# Molecular Diagnostic Approaches Responding to New Challenges in Clinical Microbiology

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Bachelor of Science (Medical Science)
Postgraduate Diploma (Biomedical Science)

*Thesis*
*Submitted to Flinders University*
*for the degree of*

**Doctor of Philosophy**

College of Medicine and Public Health
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8 September 2025

## Dedication

When I was a little boy, probably too young, my mother bought me a microscope. It was a little better than a toy as it had its own light supply, three objective lenses, a collection of pre-made slides, and materials to make your own. Prior to the microscope, a thick magnifying glass was all I had to see the smallest things known to me at that time. A whole new world appeared with the microscope. The first images were from the slides provided, which included stained fibres of flax and hemp, pollen, fly wings, and a ferocious-looking flea. Worlds-within-worlds appeared as I started to examine pond water, midges, scabs picked off my knees, and my own spit. The need to look at my blood was intense, so I pricked my finger with a pin and then fainted on my bedroom floor. Later, with my mother's encouragement, I managed to squeeze a drop on a slide and add a coverslip. The red blood cells, all perfect in shape, moving and swaying with a strange motion, had a profound effect on me. My mother taught me compassion with compassion and took great care of me, biologically in terms of medical issues, and mentally in terms of cognitive development, as I was not an easy child in either category. My mother has dedicated her life to me, unconditionally, and so I dedicate this thesis to her.

Shirley A. Ward

05/09/1951 -

## Acknowledgments

This research is supported by an Australian Government Research Training Program (RTP) Scholarship.

This thesis is an outgrowth of the influences of many people. My parents Shirley and Colin, let me run free as a child and always told me I could be anyone I wanted to be. My father showed me how to use my hands to fix anything with the right knowledge and tools. My high school biology teacher, Terry Hopkins, was very influential. Terry described biological systems very well and introduced me to phylogeny and taxonomy, as well as cultivating my mind with the method of scientific enquiry.

David Budge, a much older friend, taught me about the energy locked up in chemistry and showed me how to make gunpowder and build rockets. I remember experimenting with varying gunpowder composition and improving rocket designs to examine the effects, which is probably the first experimental science I recall. David became very successful chemical engineer and inventor. It is no surprise to me now that biology and chemistry were my leading interests. As such, microbiology, biochemistry and molecular biology were my favourite subjects as an undergraduate. I thank lecturers Martin Finn (Microbiology), Ian Lee (Biochemistry), and John Warmington (Molecular Biology) for their instruction and guidance.

My employment commenced in 1993 with a Scientific Officer role in research. I am grateful to Roger Wilson and Jerzy (Yurek) Kulski for enterococci project, which led to my first publication as a corresponding author. Roger was an excellent Clinical Microbiologist, very clever, and taught me to choose my words well. Yurek's research allowed me to learn and hone my molecular skills.

My eventual move to bacteriology was facilitated by Geoffrey Coombs. Geoff's method of scientific enquiry has had a great influence on me, and I thank Geoff for his guidance over the years. Geoff is now a Professor and has created an impressive field of intellectual integrity around his research. I also thank John Pearman who was especially kind and supportive of my ideas throughout his time as Head of Department.

The Diagnostic Molecular Laboratory took off sharply in 1996, established by Silvano Palladino and Ian Kay. Silvano and Ian played crucial roles in my development. Together, they were pioneers in molecular test validation, implementation, and reporting. I learned a great deal from being a part this process. The support of the fungal sequencing project was particularly important, due to its novel nature, as were many other molecular projects along the way. Silvano retired as the Executive Director of PathWest, leaving a lasting legacy. Ian has been the Principal Scientist for many years and continues supporting my ideas and innovations, providing a strong scientific sounding board, mentorship and leadership. Together, Ian and I have faced many challenges—this thesis examines some of the hardest.

David Whiley's postgraduate influence on me is not easily summarised. We are similar in age and were both forged in the PCR era. David established himself as an early developer and implementer of molecular assays and investigated the complexities of non-specificity, not just with *Neisseria*, but in virology as well. David convinced me to stop sitting on data and publish, and his writing style—a balance of science and conversation—has greatly influenced me. David is non-territorial about scientific problems and describes them in a way that encourages everyone to contribute. These are the most valuable traits you can find in an advisor, and I thank David for being an extremely helpful collaborator.

Although there are too many to mention by name, I also extend my sincere gratitude to all collaborators and coauthors who have contributed to my research, including those that have worked with me in the Department of Clinical Microbiology. Please know that I deeply appreciate and thank you for your dedication, hard work, and unwavering commitment to PathWest and public health.

I am also deeply grateful to Peter Boan, who is now Head of Department. Peter has involved me in several important clinical projects, awakening my interest in new areas. More importantly, Peter shares his time with me without reservation, allowing us to examine clinical problems, interpret results, and workshop laboratory solutions together.

I sincerely thank my Adjunct Supervisor, James Flexman. I have had more than 25 years of experience working with Jim in his roles as a Registrar, Clinical Microbiologist, Head of Department and Director of Pathology. Jim is always helpful, distinctly knowledgeable, and has been a tremendous source of knowledge and support over the many years.

I would like to express my deepest appreciation to my Principal Supervisor, Jillian Carr, who has been instrumental in helping me collate my publications for this thesis. Jill has kept me on track and, importantly, on topic, which has been a challenge due to the broad nature of microbiology and the diverse challenges examined in my publications. Thank you, Jill, for your guidance and persistence.

Finally, this thesis is devoted to Angela Carvosso, my partner in life and thought. The majority of the publications presented in this thesis coincide with her presence in my life—a presence marked by love, insight, and unwavering support. Angela's love and intellectual companionship have guided me through moments of uncertainty and shaped the way I approach science and life. Angela's rare blend of scientific insight and business acumen has not only enriched my professional journey but has also deepened my understanding of the interplay between knowledge and application. It is Angela's wisdom, clarity, and unwavering support that I honour here—this devotion stands as a quiet tribute to my love for her.

## Abstract

Over the past few decades, advances in microscopy instrumentation, laboratory automation for culture and susceptibility testing, and rapid organism identification using matrix-assisted laser desorption ionization-time of flight mass spectrometry have dramatically changed clinical microbiology. Molecular methods, which have rapidly evolved alongside these technologies, continue to address many longstanding challenges in microbiology—most notably the time delays for culture and subsequent pathogen identification. The development of the Polymerase Chain Reaction (PCR) technique transformed clinical microbiology, and I was fortunate to be among its early practitioners. As such, rapid pathogen detection and subsequent identification using PCR have been at the core of diagnostic molecular microbiology since its inception. Over time, a wide array of PCR assays have been applied to detect, identify and quantify many microbial pathogens. As exampled by my work in this thesis, PCR has been applied to many sub-disciplines in microbiology due to the speed of the application its high sensitivity and specificity. Today, PCR methodology continues to assist clinicians in identifying infections, promptly administering tailored therapies, and measuring the efficacy of treatment. Consequently, PCR methodologies have reduced morbidity and mortality, length of hospitalisation, inform appropriate administration of antimicrobial agents, which in turn minimises the development of antimicrobial resistance and promotes antimicrobial stewardship.

This thesis details my body of work through twelve prior publications, identifying the problems and challenges with traditional methods of diagnosis and pathogen identification in a clinical laboratory setting, and addressing these issues with contemporary molecular methodology. The publications presented in the thesis contributes to the scientific knowledgebase of molecular test development and applications in this field. The methods and concepts presented in this thesis have been developed over 30 years of experience, alongside continuous advancements in molecular techniques. Together, they contribute to a comprehensive understanding and establish a precedent for how PCR can be applied to overcome complex challenges and anticipate future problems. Here, I address three major on-going laboratory and clinical challenges: (a) the detection and identification of fungal pathogens in the context of rapid identification, cross reactivity with human DNA, and challenging biological specimens, b) improving the sensitivity and specificity of *Neisseria gonorrhoeae* testing, particularly for pharyngeal infections, whilst progressively addressing the growing rate and threat of antimicrobial resistance (AMR), via early AMR detection and individualised therapy, and c) COVID-19 pandemic, through developing novel approaches to sample preparation, testing throughput and standardisation in the context of a new pathogen, limited resources and time critical responses.

## Thesis outline

Infectious disease nucleic acid amplification tests (NAATs) have developed substantially over the last two decades, with significant advancements in nucleic acid extraction, real-time PCR chemistry, quantitative PCR, and automation. Today, NAAT's exhibit a high degree of sensitivity, specificity, reproducibility, and standardisation across a broad range of infectious diseases. This field's development has challenged many traditional methods, with numerous NAATs now considered the gold standard for infectious disease diagnosis. The need to develop, improve and implement new diagnostic assays has largely been driven by the goal of enhancing patient care and improving clinical outcomes.

The factors driving assay development include improved sensitivity, specificity, test turnaround time, throughput, ease of use, standardization, cost-effectiveness, safety, and clinical utility. However, despite individual advantages, trade-offs exist between these factors, and they are prioritised based on the laboratory and clinical challenges presented. This thesis describes three distinct microbes that have posed several challenges to diagnostic molecular microbiology. In the subsequent chapters, 11 senior author publications and one co-authored publication are presented—proposing, testing, and reporting solutions to these challenges.

**Chapter 2** focuses on the development of a rapid NAAT method for fungal identification based on broad-range PCR and sequencing, to complement or serve as an alternative to the traditional, time-consuming culture-based identification methods. This work includes the identification of fungal isolates recovered from culture and fungi growing in blood culture or other enrichment media. Four first-author publications are presented. Two publications are presented, demonstrating rapid fungal identification with a higher degree of specificity than traditional methods. These methods are also summarised in a book chapter. **Chapter 2** concludes with a publication that focuses on the rapid detection of *Aspergillus fumigatus* and *Candida albicans* directly from whole blood using a real-time PCR probe-based assay. This targeted PCR approach resolves non-specificity issues associated with broad-range PCR and highlights the challenges of achieving the analytical sensitivity required for the clinical detection of these pathogens in blood.

**Chapter 3** addresses the persistent issue of non-specificity in *Neisseria gonorrhoeae* screening assays. Both commercial and in-house screening assays for *N. gonorrhoeae* have been plagued by specificity problems associated with cross-reaction with commensal *Neisseria* species. This issue is particularly problematic for pharyngeal swabs, which are heavily colonised with commensal *Neisseria* species and are a common site of infection for *N. gonorrhoeae*. Three first-author publications are presented that

address these specificity issues. The first investigates the ongoing false-positive results of second- and third-generation commercial assays, using two supplemental confirmatory tests. The second examines the improved specificity claims of a third-generation *N. gonorrhoeae* screening assay compared to the second-generation version of the same test. The third evaluates the sensitivity and specificity of a new supplemental test that co-detects an antimicrobial resistance (AMR) marker, enhancing the clinical utility of this diagnostic approach.

**Chapter 4** presents four first-author publications and one co-authored publication that address several challenges faced throughout the SARS-CoV-2 pandemic. The publications are presented in a timeline of the challenges encountered. The first two publications assess the impact on assay sensitivity following thermal pre-treatment of clinical samples, a time-efficient process to improve safety and resource management. The third publication validates a high-throughput assay of local design and supply, aimed at increasing testing capacity while mitigating issues around the global supply shortages. The fourth co-authored publication examines the clinical associations of disease in relation to SARS-CoV-2 viral load, featuring the first report of SARS-CoV-2 RNA quantitation using an international calibration standard. Finally, the last publication focuses on increasing the SARS-CoV-2 testing capacity using a novel extraction-free method, while simultaneously assessing this method with quantitative PCR against the routine extraction-based method and sample pooling approaches. All the approaches described in **Chapter 4** were driven by the global and local circumstances of the pandemic and provide valuable lessons for similarly driven future responses.

This thesis demonstrates an original and significant contribution to scientific knowledge and understanding in the field of diagnostic molecular microbiology through publications that challenge traditional approaches. The work addresses laboratory safety, assay sensitivity, specificity, throughput and standardisation, using novel approaches and quantitative methods of experimental analysis. The relevance of this body of work was underscored during the COVID-19 pandemic, which required the rapid development and implementation of new diagnostic methods. The publications presented in this thesis detail the methodologies and scientific strategies used to overcome these challenges, resulting in changes in testing workflows and policy, and a deeper understanding of contemporary issues faced in clinical microbiology**. Chapters 2, 3 and 4** are linked by the need for NAATs in diagnostic clinical microbiology. Given the diverse nature of the microbes and clinical settings, this thesis begins with an introduction to nucleic acid amplification testing and its use in clinical microbiology, followed by more detailed introductions in each chapter.

## Declaration

I certify that this thesis:

1. does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university

2. and the research within will not be submitted for any other future degree or diploma without the permission of Flinders University; and

3. to the best of my knowledge and belief, does not contain any material previously published or written by another person except where due reference is made in the text.

4. was not compiled using generative artificial intelligence tools.

Signed: Todd M. Pryce

Date: 08/09/2025

## Statement of originality

This thesis presents 11 first-author publications and one second-author publication of original research. I declare that all portions of these published works are original. Additionally, I confirm that all these published works have not been published elsewhere, other than in the journals which originally accepted them for publication.

## Statement of editing

No professional editing services were performed.

## Publications included in this thesis

Chapter 2

1. **Pryce TM**, Palladino S, Kay ID, Coombs GW. 2003. Rapid identification of fungi by sequencing the ITS1 and ITS2 regions using an automated capillary electrophoresis system. Medical Mycology 41:369-81. http://dx.doi.org/10.1080/13693780310001600435
2. **Pryce TM**, Palladino S, Price DM, Gardam DJ, Campbell PB, Christiansen KJ, Murray RJ. 2006. Rapid identification of fungal pathogens in BacT/ALERT, BACTEC, and BBL MGIT media using polymerase chain reaction and DNA sequencing of the internal transcribed spacer regions. Diagnostic Microbiology and Infectious Disease 54:289-97. http://dx.doi.org/10.1016/j.diagmicrobio.2005.11.002
3. **Pryce TM**. 2010. Universal detection and identification of fungi by PCR and DNA sequencing. Chapter 70. In: Schuller M, Sloots T, James GS, Halliday CL, Carter IWJ. 2010. PCR for Clinical Microbiology: An Australian and International Perspective. https://link.springer.com/chapter/10.1007/978-90-481-9039-3_70
4. **Pryce TM**, Kay ID, Palladino S, Heath CH. 2003. Real-time automated polymerase chain reaction (PCR) to detect *Candida albicans* and *Aspergillus fumigatus* DNA in whole blood from high-risk patients. Diagnostic Microbiology and Infectious Disease 47:487-96. http://dx.doi.org/10.1016/s0732-8893(03)00139-1

Chapter 3

5. **Pryce TM**, Hiew VJ, Haygarth EJ, Whiley DM. 2021. Second- and third-generation commercial *Neisseria gonorrhoeae* screening assays and the ongoing issues of false-positive results and confirmatory testing. European Journal of Clinical Microbiology and Infectious Diseases 40:67-75. http://dx.doi.org/10.1007/s10096-020-04004-5
6. **Pryce TM**, Bromhead C, Whiley DM. 2023. A previously documented *Neisseria macacae* isolate providing a false-positive result with Roche cobas 4800 CT/NG does not cross-react with the later generation cobas 6800 CT/NG assay. European Journal of Clinical Microbiology and Infectious Diseases 42:121-123.

http://dx.doi.org/10.1007/s10096-022-04519-z

7. **Pryce TM**, Foti OR, Haygarth EJ, Whiley DM. 2023. Maximizing the *Neisseria gonorrhoeae* confirmatory rate and the genotypic detection of ciprofloxacin resistance for samples screened with cobas CT/NG. Journal of Clinical Microbiology 62(1), e0103923. http://dx.doi.org/doi:10.1128/jcm.01039-23

Chapter 4

8. **Pryce TM**, Boan PA, Kay ID, Flexman JP. 2021. Thermal treatment of nasopharyngeal samples before cobas SARS-CoV-2 testing. Clinical Microbiology and Infection 27:149-150. http://dx.doi.org/10.1016/j.cmi.2020.07.042
9. **Pryce TM**, Boan PA, Kay ID, Flexman JP. 2021. Qualitative and quantitative effects of thermal treatment of naso-oropharyngeal samples before cobas SARS-CoV-2 testing. Diagnostic Microbiology and Infectious Disease 101:115519. http://dx.doi.org/10.1016/j.diagmicrobio.2021.115519
10. **Pryce TM**, Haygarth EJ, Bordessa J, Boan PA. 2021. Evaluation of a PlexZyme-Based PCR Assay and Assessment of COVID-19 Surge Testing Throughput Compared to Cobas SARS-CoV-2. Pathogens 10(9). http://dx.doi.org/10.3390/pathogens10091088
11. Boan P, Jardine A, **Pryce TM**. 2022. Clinical associations of SARS-CoV-2 viral load using the first WHO International Standard for SARS-CoV-2 RNA. Pathology 54:344-350. http://dx.doi.org/10.1016/j.pathol.2021.11.006
12. **Pryce TM**, Haygarth EJ, Bordessa J, Jeffery CT, Kay ID, Flexman JP, Boan PA. 2022. High-Throughput COVID-19 Testing of Naso-Oropharyngeal Swabs Using a Sensitive Extraction-Free Sample Preparation Method. Microbiology Spectrum 10:e0135822. http://dx.doi.org/10.1128/spectrum.01358-22

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## Abbreviations

| | |
|---|---|
| AMR | Antimicrobial resistance |
| BSC | Biological safety cabinet |
| $C_T$ | Cycle of threshold |
| $C_q$ | Cycle of quantitation |
| CDC | U.S. Centers for Disease Control and Prevention |
| cDNA | Complimentary DNA |
| CFU | Colony forming units |
| CLSI | Clinical and Laboratory Standards Institute |
| CMV | Human cytomegalovirus |
| COVID-19 | Coronavirus disease-2019 |
| ddPCR | Droplet-based digital PCR |
| DNA | Deoxyribonucleic acid |
| dNTP | Deoxynucleotide triphosphate |
| EBV | Epstein-Barr virus |
| HBV | Hepatitis B virus |
| HCV | Hepatitis C virus |
| HSV | Herpes simplex virus |
| HIV | Human immunodeficiency virus |
| HybProbes | Hybridisation Probes |
| IFI | Invasive fungal infection |
| IS | International standard |
| IU | International units |
| IVD | In-vitro diagnostic device |
| MERS-CoV | Middle East respiratory syndrome coronavirus |
| NAAT | Nucleic acid amplification test (testing) |
| NGS | Next-generation sequencing |
| NIBSC | National Institute of Biological Standards and Controls |
| PCR | Polymerase chain reaction |
| POC | Point of care |

| | |
|---|---|
| PPE | Personal protective equipment |
| qPCR | Quantitative real-time polymerase chain reaction |
| rDNA | Ribosomal DNA (genes) |
| rRNA | Ribosomal RNA (genes) |
| RNA | Ribonucleic acid |
| RT | Reverse transcriptase enzyme |
| SARS-CoV | Severe acute respiratory syndrome coronavirus 1 |
| SARS-CoV-2 | Severe acute respiratory syndrome coronavirus 2 |
| STI | Sexually transmitted infection |
| UNG | Uracil N-glycosylase |
| VZV | Varicella zoster virus |
| WGS | Whole genome sequencing |
| WHO | World Health Organisation |

# Chapter 1: Introduction to nucleic acid amplification testing and the use in clinical microbiology

## 1.1 Introduction to PCR

Some readers of this thesis may have been studying or working in the field of molecular biology after the announcement of PCR in 1985 (1), from which Dr. Kary B. Mullis from Cetus Corporation, was awarded the Nobel Prize in Chemistry in October 1993. A familiar text at the time, *Molecular Cloning – A Laboratory Manual* (Cold Spring Harbor Press), began in 1980 as a collection of laboratory protocols that were used during the Cold Spring Harbor course on Molecular Cloning of Eukaryotic Genes, with the second edition published in 1989 (2). This comprehensive three-volume series served as the primary knowledge base for molecular biology, which I read cover-to-cover in 1992. The PCR process was described in one small chapter, along with some methods for molecular biology applications, such as cloning and analysis of mutations in genes.

As it was first described, PCR enables the 5-prime to 3-prime (5' → 3') in vitro synthesis of nucleic acids, allowing a DNA fragment to be replicated (amplified) in a semi-conservative manner. The segment of DNA to be amplified lies between two regions of a known sequence. Two small synthetic DNA fragments called oligonucleotides (or primers), one complementary to one strand and one complementary downstream to the opposing strand, are used to prime the PCR reaction. This is achieved by separation of the target strands by heating (denaturation) and cooling to allow primer binding to complementary sequences (annealing). A key component was the addition of the Klenow fragment of *E. coli* DNA polymerase I, which catalyses the elongation (extension) of the annealed primers (polymerisation). The Klenow fragment is produced by cleaving a portion of this DNA polymerase using the protease subtilisin (3). It retains the 5' → 3' polymerase activity and the 3’ → 5’ exonuclease activity (proofreading) but loses the 5' → 3' exonuclease activity, which would otherwise remove annealed primers. DNA polymerisation occurs as nucleoside triphosphates (dNTPs; adenine, guanine, cytosine and thymine nitrogenous bases) present in the PCR solution are incorporated into the newly formed complementary strand. This entire process is called a “cycle” which generates many copies of PCR fragments (amplicons) exponentially, one cycle at a time. In 1985, the procedure involved incorporating a new aliquot of DNA polymerase after each cycle because the high temperature (>90°C) required for denaturation, also denatured (inactivated) the DNA polymerase. These cycles were performed in water baths at various temperatures, making the process very hands-on. A machine called “Mr. Cycle” was built by Cetus to add fresh DNA polymerase to each test tube

on the completion of each cycle. However, despite this semi-automation, the yield was often poor. Amplicons were heterogenous in size due to mis-priming (non-specificity), and amplifying target sequences greater than 200 base pairs was problematic.

The first problem with DNA polymerase inactivation was resolved with the introduction of a thermally stable DNA polymerase, purified from the thermophilic bacterium *Thermus aquaticus* (*Taq* DNA Polymerase, or *Taq*) discovered in 1976 (4). This enzyme was introduced into the Mullis PCR reaction by Randy K. Saiki (5). *Taq* could withstand the high temperatures used for denaturation and did not need to be replaced after every cycle. Primer specificity problems were also resolved as annealing and extension could also be performed at higher temperatures, thereby improving the efficacy (stringency) of primer-template complementary and nucleotide paring. As such, *Taq* improved PCR specificity and target amplicon yield. Cetus joined Perkin-Elmer to develop a new PCR machine for the improved chemistry. In 1987, the group announced the commercial availability of the first thermal cycler (or thermocycler) for *Taq*, the Perkin-Elmer/Cetus "PCR-1000" and the first commercially labelled polymerase "AmpliTaq DNA Polymerase". The PCR-1000 was followed by the Perkin-Elmer/Cetus DNA 480, a refrigerant-based machine that cycled programmable temperatures via an aluminium block and held a total of 48 PCR tubes. This instrument contributed to reducing the cost and hours spent performing the PCR which allowed many new applications in research and diagnostics.

## 1.2 Early developments

The first published account of PCR used for diagnosis in clinical microbiology was for the detection of integrated human immunodeficiency virus (HIV) in the DNA of cell lines cultured from infected individuals (6), followed by the detection of HIV in the DNA of peripheral blood mononuclear cells (7). Here, the capability of PCR to revolutionise clinical microbiology became apparent; "This method of DNA amplification made it possible to obtain results within 3 days, whereas virus isolation takes up to 3 to 4 weeks…..the method may therefore be used to complement or replace virus isolation as a routine means of determining HIV-1 infection" (7). The method of HIV DNA-specific PCR product detection was cumbersome involving a radiolabelled probe, polyacrylamide gel electrophoresis, Southern blotting, and exposure to radioisotope sensitive film. It is important to note that at the time, PCR was amplifying DNA templates until a new form of PCR was developed for RNA templates. This new form used a reverse transcriptase enzyme (RT) to covert RNA to cDNA (RT-PCR), which was subsequently amplified by *Taq* (8).

With the advent of RT-PCR, the power of this new technology extended to all nucleic acid types. Further hardware improvements to thermal cycling machines were developed in the 1990s to support this version of the technique. For example, the DNA 480 instrument did not have a heated lid, so PCR reactions were overlayed with mineral oil to prevent evaporation and condensation in the top of the tube. Additionally, the speed at which temperatures could transition (ramp rates) in the refrigerant-based machines were slow, leading to many mis-priming reactions occurring at temperatures below the melting temperature ($T_M$) of the primers (the temperature at which the primer anneals at 50% efficiency). These issues were overcome with the introduction of heated lids for PCR tubes to prevent condensation and thermoelectric Peltier-based blocks with faster ramp rates. Due to this simplicity and compact size of Peltier devices, new instruments such as the MJ Research PTC-100 and PTC-200 became popular. These instruments were much smaller, more affordable, and had a larger tube capacity (up to 96 tubes).

PCR process development in the 1990s focussed on more rapid and convenient methods of PCR product detection. The standard method of PCR product detection was agarose gel electrophoresis with ethidium bromide staining, followed by visualising the DNA bands using an ultraviolet transilluminator (2). This process, termed conventional PCR throughout this thesis, was widely used during my early years in clinical microbiology. Conventional PCR (highlighted in **Chapter 2**) was used as the basis for PCR product detection for my first two publications as a first and second author (9, 10). Gel electrophoresis and Southern blotting methods were very labour intensive. New end-point PCR product detection methods were being developed into a semi-automated process using enzyme-linked immunoassay systems for detecting HIV and hepatitis B virus (HBV) as early as 1990 (11, 12). With the PCR patent rights sold to Hoffman-La Roche in 1990, which eventually became Roche Molecular Systems, the first commercial assays using this type of PCR product detection were introduced in 1992, namely Roche AMPLICOR HIV-1 MONITOR test and AMPLICOR *Chlamydia trachomatis* test. However, one of the most significant developments of PCR product detection has been the ability to visualise amplicon formation in real time during the PCR process (real-time PCR).

## 1.3 Real-time PCR

The development of quantitative real-time PCR, abbreviated to qPCR based on the current convention (13), has revolutionised both the qualitative detection (positive or negative) and the quantification (measurement of concentration) of DNA and RNA. Insights into the PCR kinetics of the reaction were also discovered along the qPCR development journey, including a deeper understanding of the efficiency of nucleic acid preparation methods and the assessment of interfering substances (14, 15).

The main benefits of qPCR included the elimination of labour-intensive gel electrophoresis, reducing post-PCR procedural handling steps as potential sources of contamination, and achieve the benefit of quantitation. Although quantification of PCR products was possible with conventional PCR, qPCR was more rapid, accurate and reproducible. As part of my earlier published work, I published a first-author chapter on quantitative real-time PCR, describing PCR kinetics, quantification using external or internal standards, detection formats and clinical uses (15).

Two common methods for the detection of PCR products in qPCR are: (a) nonspecific fluorescent dyes that intercalate with any double-stranded DNA (as for ethidium bromide), and (b) sequence-specific DNA probes consisting of oligonucleotides that are labelled with a fluorescent reporter, which allows detection only after hybridisation of the probe with its complementary sequence. Intercalating dyes can be described as sequence-independent, intercalating with any PCR product formed or native nucleic acid. Conversely, DNA probes are sequence-dependent, in theory, only bind to an internal specific sequence. As these measurements are taken directly from the PCR tube, the PCR instrumentation requires a light source (excitation) and a detector to capture the fluorescence (emission), along with an optical system to measure fluorescence and software to interpret the data and analyse the results. The fluorescence emission generates a signal that increases in direct proportion to the amplicon concentration, cycle after cycle.

The basic principle of qPCR is that as PCR products are synthesised, amplification begins with the initial flat phase, followed by an exponential phase, which decreases in the transitional phase and flattens into a plateau. The baseline, exponential, transitional, and plateau phase of the amplification curve are generated based on the quantitative relationship between the fluorescence signal accumulation and the number of cycles. Software applications provided with the instrumentation allow the user to set a baseline threshold to exclude the background fluorescence during the initial flat phase. A signal above this baseline, which can be set by the user as a threshold ($_T$), reflects a significant signal increase over the baseline, thereby distinguishing a relevant amplification signal from the background. The cycle where the fluorescence crosses the threshold is called the cycle threshold ($C_T$), which now has a standardised nomenclature of cycle of quantitation ($C_q$) (13). The $C_q$ threshold can be adjusted for each experiment to be in the region of exponential amplification across all plots. However, the placement of the threshold defined by the exponential phase can easily lead to different results for different samples. Hence, algorithms to assess individual amplification curves have been developed to determine the best point along the curve to estimate initial target quantities. The maximum value of the second derivative (of the logarithmic curve) as the $C_q$ is commonly used (16, 17). Using this approach, the $C_q$ can be calculated for each curve based on its characteristics, rather than simply

crossing a user-defined threshold. Irrespective of the method of PCR product detection, PCR amplification curve and $C_q$ analysis is the most important role of the interpretive software, either solely or in combination with the reviewing scientist. However, different detection formats can also provide more information to enhance specificity, such as the $T_M$ of double stranded DNA containing intercalating dyes, or the $T_M$ of sequence-specific probes bound to the template.

## 1.4 Sequence-specific detection formats

PCR product detection formats have progressed over time from the sequence-independent methods of PCR product detection, such as ethidium bromide used in gel electrophoresis and SYBR Green I for qPCR (18-20). Although SYBR Green provides the simplest and most economical method of PCR product detection, it detects all double-stranded DNA and primer dimers (primers bound to each other). Therefore, probe-based sequence-dependent methods of PCR product detection provide a higher degree of specificity. Many of the sequence-specific detection formats are named due to commercialisation. Numerous detection formats exist today with several reviews covering the subject (14, 21-23). Examples include TaqMan, Major Groove Binders, Hybridisation Probes (HybProbes), Molecular Beacons, Scorpion Primers, Sunrise Primers, and MNAzyme probes. Several alternative amplification methods have also been developed (for various advantages) and include Strand Displacement Amplification, Nucleic Acid Sequence Based Amplification, Transcription-Mediated Amplification, Ligase Chain Reaction, and Rolling Circle Amplification, most of which are isothermal nucleic acid amplification methods obviating the need for a thermal cycler. These methods offer potential advantages over PCR for speed, cost, scale or portability (24). In this introduction, the sequence-specific detection formats relevant to my prior published work are briefly described.

### 1.4.1 TaqMan probe chemistry

The first sequence-specific detection format for qPCR were TaqMan probes, developed by Cetus (25), with the AmpliTaq polymerase illustrated in their manuscript as the Pac-Man figure, from the video game of the same name (Figure 1). TaqMan PCR exploits the 5 → 3' exonuclease activity of *Taq*, to hydrolyse an oligonucleotide that is hybridised to the amplicon. This oligonucleotide probe, non-extendable at the 3' end, has a fluorescent reporter dye attached to the 5' end and a fluorescent quencher attached to the 3' end, hybridises to the complementary sequence. In the non-cleaved state, Fluorescent Resonance Energy Transfer (FRET) occurs, and the fluorescence from the fluorophore is absorbed by the quencher, and no signal is generated. When *Taq* encounters the probe bound to the

complementary sequence, the probe is cleaved into smaller fragments via the 5 → 3' exonuclease activity. This decouples the fluorescent and quenching dyes, resulting in fluorescence that increases in a linear relationship with the amount of probe cleavage. TaqMan probes are single oligonucleotide probes that can be combined with other probes with different fluorophores (multiplexed) to enable simultaneous detection and discrimination of multiple targets in a single PCR reaction. Although sequence-dependent, TaqMan probes only fluoresce in the cleaved state and cannot be used for $T_M$ analysis of the target. Due to their simplicity, TaqMan probes are widely used in many diagnostic microbiology assays. For example, TaqMan is the preferred chemistry of Roche Molecular Systems with the LightMix and LightMix Modular diagnostic assays, manufactured by Tib-Molbiol (Berlin, Germany). The TaqMan method of PCR product detection and the multiplexing capabilities are highlighted in **Chapter 3** and **Chapter 4**.

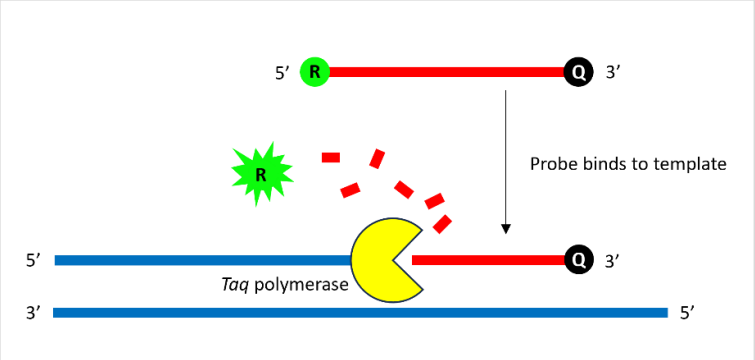



Figure 1. TaqMan probe detection. *Taq* hydrolyses an oligonucleotide probe bound to the complementary DNA strand, via the 5 → 3' exonuclease activity of *Taq* polymerase. The probe is cleaved into smaller fragments, separating the fluorescent reporter dye (R) from the quenching dye (Q), allowing emission of fluorescence. Additional probes with sequences complementary to other targets can be added that have different fluorophores attached. These additional probes are excited at different wavelengths allowing for PCR multiplexing. Figure created with PowerPoint. Pryce, TM (2024).

### 1.4.2 HybProbe chemistry

In contrast to TaqMan probes, HybProbes are oligonucleotide pairs capable of both detection and $T_M$ analysis of the target (Figure 2). One probe is labelled with a donor fluorophore at the 3' end and another probe is labelled with an acceptor fluorophore at the 5' end. The bound probes are typically separated by a few nucleotides. During the primer annealing phase of the PCR reaction, probes are

bound adjacent to each other, and excitation energy of the donor passes to the acceptor (FRET), resulting in light emission. The amount of fluorescence is proportional to the amount of PCR product. Moreover, melt curve analysis can be performed at the end of the PCR. The PCR reaction is cooled, allowing probe hybridisation, then heated incrementally while obtaining continuous fluorescence acquisitions. Upon reaching the temperature of which at least one of the probes dissociates from the template, the fluorescence drops sharply. The temperature at which this occurs is the $T_M$ of the target of interest. This measurement is used to confirm specificity of the target, matching this $T_M$ to the control $T_M$, or a matching $T_M$ to a mutation of interest. HybProbes can also be multiplexed like TaqMan probes, with both chemistries compatible with the LightCycler range of instruments and specific fluorophores to match. The HybProbe method of PCR product detection and its specificity advantages are highlighted in **Chapter 2**.

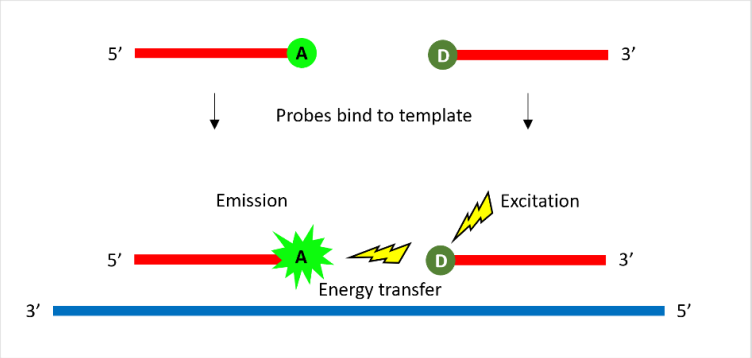



Figure 2. Hybridisation probe detection. When both probes are bound to the complementary DNA strand during the primer annealing phase of the PCR reaction, excitation and emission occurs and excitation energy of the donor (D) passes to the acceptor (A). Additional probes with sequences complementary to other targets can be added that have different fluorophores attached. These additional probes emit at different wavelengths allowing for PCR multiplexing. Figure created with PowerPoint. Pryce, TM (2024).

### 1.4.3 MNAzyme probe chemistry

In 2010, the Australian biotechnology company SpeeDx (Eveleigh, New South Wales) developed a new class of nucleic acid enzymes that function as molecular probes (26). These probes only form catalytic complexes in the presence of a target nucleic acid. Similar to Hybprobes and TaqMan probes, MNAzymes are useful for measuring the accumulation of amplicons during qPCR. Partial enzymes, “part-zyme” A and B, bind to complementary adjacent regions of the amplicon target sequence,

allowing their partial catalytic core domains to form a complete active MNAzyme core (Figure 3). The signalling probe binds across part A and B, and once assembled, the MNAzyme cleaves the bound probe, causing the fluorophore and quencher labels to separate and produce a fluorescent signal, indicating the presence of the target (similar to TaqMan). The probe-binding domain of the part-zymes can be complementary to any one of a series of well-characterized universal probes. Since there is no need to synthesize or optimize a new target-specific labelled probe for each different target, MNAzyme qPCR is more flexible and cost-effective than TaqMan or HybProbes, allowing target-specific detection with a generic fluorophore readout. This enables faster development and large-scale production of multiplex assays, with high-efficiency multiplexing and mutation detection capabilities. Like Hybprobes, the MNAzyme offers additional specificity compared to alternative detection technologies, as two partial enzymes are required to bind for detection. MNAzymes are also multiple turnover enzymes, meaning multiple probes can be cleaved during each PCR cycle, resulting in a strong and sensitive signal. However, similar to TaqMan probes, the probe bound the MNAzyme is cleaved, and $T_M$ determination is not possible. The advantages of MNAzyme signal and complex multiplexing are highlighted in **Chapter 3** (**Publication 7**) and **Chapter 4** (**Publication 10 and 12**). In addition, the rapid development and high-volume production of MNAzyme probes are also highlighted in **Chapter 4** (SARS-CoV-2 pandemic**; Publication 10**).

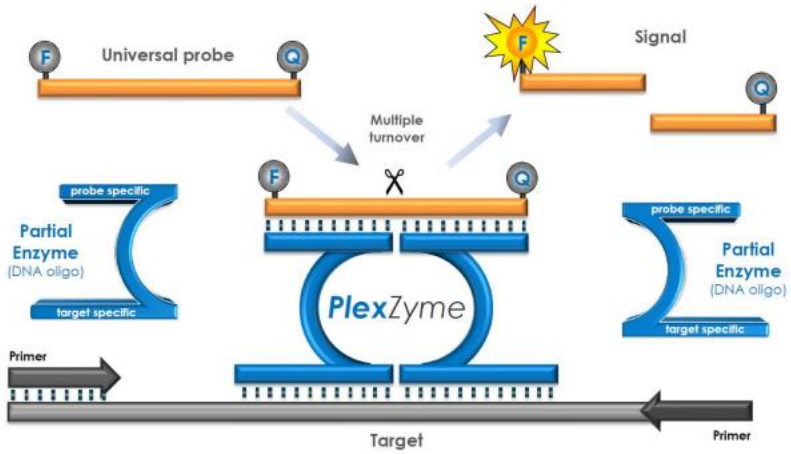



Figure 3. MNAZyme probe detection. MNAZymes (commercialised as PlexZyme) are catalytic DNA complexes composed of two DNA oligonucleotides referred to as “Partial Enzymes”. Each Partial Enzyme has a target-specific region, a catalytic core and a universal probe binding region. When the target product is present, the two Partial Enzymes bind adjacently to form the active PlexZyme which has catalytic activity to cleave a labelled probe. Cleavage separates the fluorophore (F) and quencher (Q) dyes, producing a fluorescent signal that can be monitored in real time. Additional PlexZyme

probes with sequences complementary to other targets can be added. The universal probes can have different fluorophores which excited at different wavelengths, allowing for PCR multiplexing. Image reproduced with permission from A/Professor Alison Todd, Chief Scientific Officer & Founder, SpeeDx Pty. Ltd.

## 1.5 Quantitative PCR

With the capacity to measure and detect small concentrations of nucleic acids from a wide range of samples, qPCR has greatly improved the utility of molecular diagnostics in clinical microbiology. The combination of simplicity, speed and accuracy, has made qPCR the gold standard approach for the quantitation of microbial load. In terms of history, the concept of quantitative PCR emerged in the 1990's. One of the earliest examples of target quantification in our laboratory was measurement of human cytomegalovirus (CMV) viral load in leukocytes using conventional PCR and quantitation using a gel-based end-point titration (27). However, this method was time consuming, did not allow for real-time data collection, and had challenges with reproducibility. Recognising the need for rapid and accurate quantitative results, qPCR assays were being developed with a variety of quantitative approaches. For example, qPCR for HIV-1, HBV, hepatitis C virus (HCV), and CMV using the COBAS AMPLICOR instrument revolutionised viral quantitation in the mid-1990s, with the use of probe-capture of biotinylated amplicons (28-31). The progressive development of automated systems like COBAS AmpliPrep and COBAS TaqMan, improved speed and accuracy of quantitation, with a liquid magnetic glass particle (MGP) extraction process and real-time PCR chemistry. With a focus on time to diagnosis, qPCR development further improved the detection of other viruses, such the herpes viruses (32). The biggest impact was reducing the mortality rate of central nervous system (CNS) disease with prompt antiviral therapy; a diagnosis could be made within a few hours after receiving the sample in the laboratory, rather than 2-5 days using viral culture techniques (21). As a precursor to multiplexing in a single tube, multiple assays were being designed to operate using the same thermal cycling conditions. Hence, panels of targets were being developed to screen cerebrospinal fluid samples for herpes viruses. Here, the first CNS "syndromic panels" were developed for CMV, Epstein-Barr virus (EBV), herpes simplex virus (HSV-1 and HSV-2) and varicella zoster virus (VZV) (33, 34). Early qPCR platforms such as the LightCycler, offered rapid PCR (< 1 hour), which was well-suited for this purpose (35). Our laboratory implemented such an assay in 2003, multiplexing HSV-1, HSV-2 and VZV in a single assay using this instrument. At the same time, detection and quantitation of EBV and BKV were also implemented (36). Today, the popularity of qPCR is reflected in the prodigious number of publications reporting its use, with many commercial assays available, particularly for the

blood-borne viruses. As a result of the enormous quantitative range PCR offers, coupled with the ability to detect one target in the presence of vast amounts of other targets or nucleic acids, ensured the future of qPCR for molecular diagnostics in microbiology.

There are many aspects to qPCR, such as PCR kinetics, choice of quantitation standard and detection formats, some of which have been described above. I have previously summarised the methods of quantitative PCR in one of my earlier publications in a first-author book chapter (15). For brevity, the principles of qPCR kinetics will not be described here, but in summary, a figure illustrating the qPCR relationship between $C_q$ and $\log_{10}$ concentration of the target is shown in Figure 4. Quantitation is achieved using external or internal standards and exploit the predictable kinetics of the PCR reaction. All that is required is a standard of known concentration, either a nucleic acid, or a known concentration of standardised suspension of the target (bacteria, virus etc), which is subjected to the entire nucleic acid and PCR amplification process, as for the patient samples. In general, the calculated $C_q$ at each concentration is plotted against the $\log_{10}$ concentration of the target, to generate a standard curve that is used to convert the $C_q$ of a patient sample into a concentration.

**Publications 6, 7, 9, 10-12** highlight my skills in developing quantitative assays and the importance of target quantitation for clinical interpretation, as a measure of method comparison, to understand the kinetics or efficiency of a PCR reaction, or as a tool for assay development.

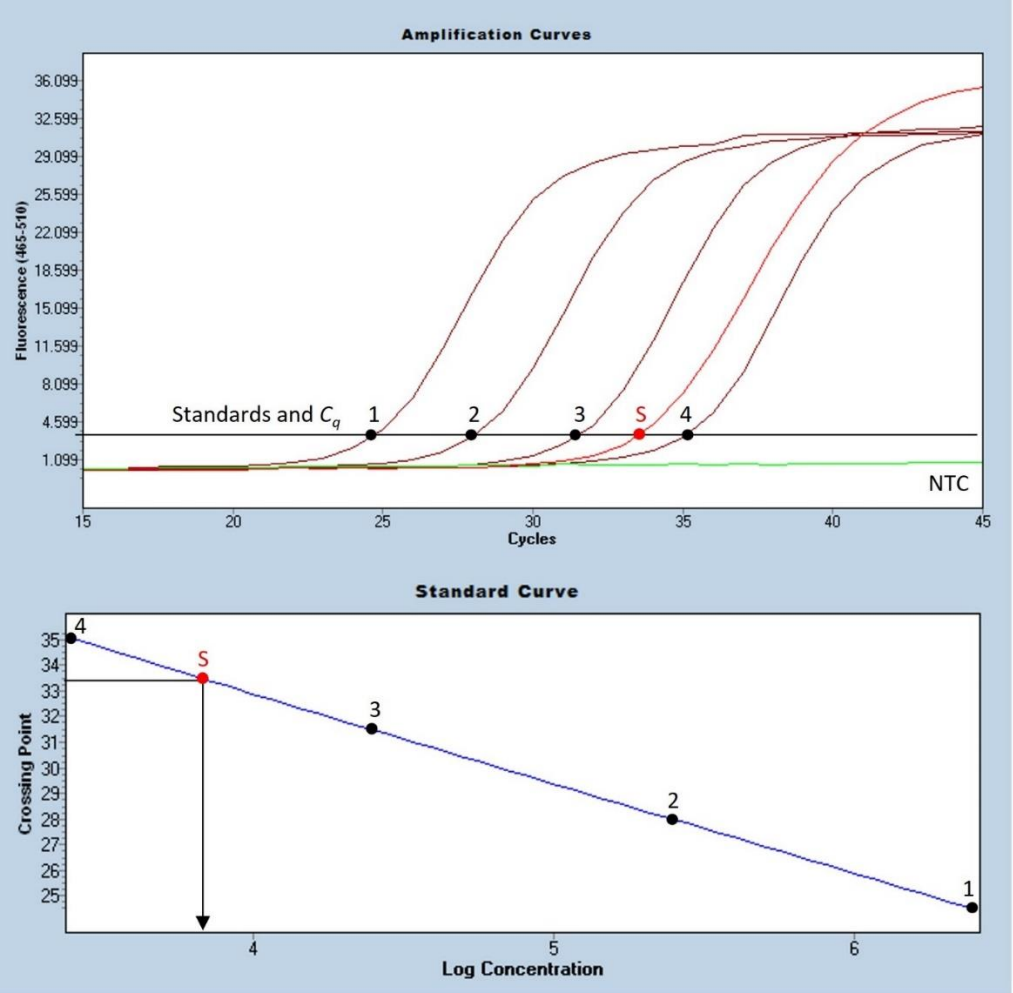



Figure 4. Quantitative PCR and standard curve generation. The amplification curve plot above shows the real-time fluorescence signal for external DNA standards 1 to 4, including a patient sample S and non-template control (NTC). The $C_q$ for each standard is calculated by the user or interpretive software. The $C_q$ (y-axis) is then plotted against $\log_{10}$ concentration (x-axis) of each standard to generate a standard curve. The $C_q$ for a patient sample S is plotted against the standard curve to calculate the concentration. Figure created with PowerPoint using standard curves calculated with LightCycler 480 result analysis software. Pryce, TM (2024).

## 1.6 Limitations of qPCR

Despite the many advantages of qPCR, there are several technical limitations. The broader limitations of analytical sensitivity, limit of detection, limit of quantification, amplification efficiency, accuracy and precision, are well-covered elsewhere (21, 37, 38). However, there are some limitations that are relevant to this thesis worth noting. The first include the limitations that stem from initial design of a qPCR assay. Firstly, some prior sequence data is needed to design a qPCR assay, with target-specific

primers and probes designed from complementary sequences of the target, using a wide range of oligonucleotide design tools. Before searchable nucleotide databases, such as GenBank, and sequence alignment tools, such as the basic local alignment search tool (known as BLAST), the assay developer tested their PCR target in the presence of co-extracted human DNA. Next, gel electrophoresis was performed to verify the correct size of the amplicon and specificity was verified using Southern blot analysis. With the advent of qPCR and the emergence of searchable databases, the investigator could perform most of the design in silico and check for nonspecific complementarity to other microorganisms or human DNA. However, throughout my career, I have encountered several examples of nonspecific interactions whilst reproducing the work of others. Although qPCR utilises a probe-based detection of amplicon, thereby mitigating the detection of nonspecific amplicons, it is still necessary to check the specificity of the PCR reaction using an alternative method, such as gel electrophoresis. Nonspecific amplicons formed during the PCR process can competitively inhibit detection of the target, thereby reducing the efficiency of the qPCR assay. An example of nonspecific human DNA amplification is highlighted in **Chapter 2** and mitigated with an improved DNA extraction technique and alternative primers. This improved extraction technique also mitigated against false-negative results caused by inadequate removal of PCR inhibitors or poor recovery of nucleic acid from the sample.

Other sources of false positive results include contamination of the PCR reaction with the target present in the environment or reagents. An example in this thesis is highlighted in **Chapter 2** (**Publication 4**), with fungal DNA present in some enzymatic reagents. PCR product carry-over presents a significant risk to the molecular laboratory and integrity of results. The ability to exponentially amplify a single copy of target to over 1 x $10^{10}$ copies of amplicon means the risk of contamination is high. Environmental and amplicon contamination can be mitigated with good pre- and post-PCR laboratory practices (which will not be discussed here). Amplicon contamination can be controlled enzymatically. For example, uracil N-glycosylase (UNG) is commonly used in PCR reactions to remove carryover amplicons. This technique is only effective for PCR assays that utilise deoxyuridine triphosphate (uracil; dUTP) as a substitute nucleotide base for deoxythymidine triphosphate (thymidine; dTTP). As such, any new PCR product contains dUTP instead of dTTP. The heat-labile UNG enzyme selectively degrades uracil-containing amplicons and does not affect native DNA containing thymidine (39). Two examples relevant to this thesis include the use of dUTP in a highly sensitive PCR assay used for the detection of *A. fumigatus* and *C. albicans* in whole blood (**Chapter 2**). Here, dUTP was introduced as a safeguard to amplicon carry-over, given the high sensitivity of this assay. It is noteworthy that the use of dUTP is a mainstay in all commercial screening assays developed by Roche (**Chapter 3 and 4**).

Other important limitations include nucleotide polymorphisms in the primer or probe binding domains, which may result in reduced amplification efficiency or false-negative results. These polymorphisms may not have been characterised during the initial primer and probe design due to a lack of broader sequence information of similar species or strains. These polymorphisms are often a feature of the biology of the organism. A classic historical example is the detection of 2009 H1N1 influenza, with the virus consisting of a reassortment of 6 gene segments from the triple reassortment of swine-origin virus and 2 gene segments from the Eurasian influenza A (H1N1) swine virus lineage, requiring new H1N1 assays to be developed due to subtype differences in nucleic acid sequences (40). A more recent example in a co-authored publication, we describe several single-point mutations in the N gene of SARS-CoV-2 which adversely impacted SARS-CoV-2 detection in a commercial assay (41). In this case, this assay was multiplexed with the SARS-CoV-2 E gene, which was positive, thereby mitigating a false-negative result. This dual-target approach is highlighted in **Chapter 4** with SARS-CoV-2 testing. Other examples of false-positive and false negative results related to the biology of the organism are highlighted in this thesis. For example, the frequency of genetic exchange within the *Neisseria* species causing specificity issues with *N. gonorrhoeae* screening assays are examined **Chapter 3** with my prior publications in this field.

In summary, these short-listed limitations relevant to this thesis highlight some of the more important problems that can lead to erroneous results. In the following chapters, I present several publications that highlight some of these limitations and develop effective diagnostic strategies to mitigate them.

## 1.7 Applications of PCR in Clinical Microbiology

Due to the extensive published literature of the subject and the vast array of PCR applications in clinical microbiology, a review of the many applications of PCR will not be covered in this thesis. Instead, this this section focusses on PCR applications relevant to my personal experience in clinical microbiology and the development of this thesis via prior publications. For a broader context of the applications, texts such as *Molecular Microbiology: Diagnostic Principles and Practice*, Second Edition (42), and Third Edition (37), cover the principles, applications and emerging technologies over the past decade. To introduce these chapters in this thesis, I present a summary of the background and challenges faced, including illustrations, highlighting the publications that addressed some of the specific issues encountered.

### 1.7.1 Molecular detection and identification of fungal pathogens

Fungi are eukaryotes that represent a diverse group of microorganisms, most of which are saprophytic and soil-dwelling, with decomposition and recycling of organic material as their main role in the ecosystem (43). Consequently, fungi are in constant contact with humans, leading to colonisation and establishment as normal human flora. Passive exposure occurs though skin and mucous membranes, ingestion and inhalation. Fungi can be described in terms of the relationship to the human host, some mostly harmless to the host (saprobes), compared to facultative or obligate pathogens. Fungi are mostly opportunistic pathogens that cause a broad spectrum of diseases, from superficial skin and nail infections to life-threatening invasive infection of the bodily tissues and fluids. While the majority of fungal infections are superficial and relatively treatable, invasive fungal infections, commonly caused by *Candida albicans*, *Aspergillus fumigatus*, and *Cryptococcus neoformans* are more difficult to diagnose and treat, resulting in high mortality rates that can reach 90% in immunocompromised individuals (44). Furthermore, the increase in antifungal resistance further challenges our ability to treat these diseases, further increasing the mortality rate for some infections (44). A summary of the most common human fungal pathogens and their distribution is shown in Table 1.

Table 1. Common human fungal pathogens and their distribution. Commonly encountered fungi showing genus and phylum association, in association with common human diseases and geographical distribution. Table prepared from mycology texts (45-47).

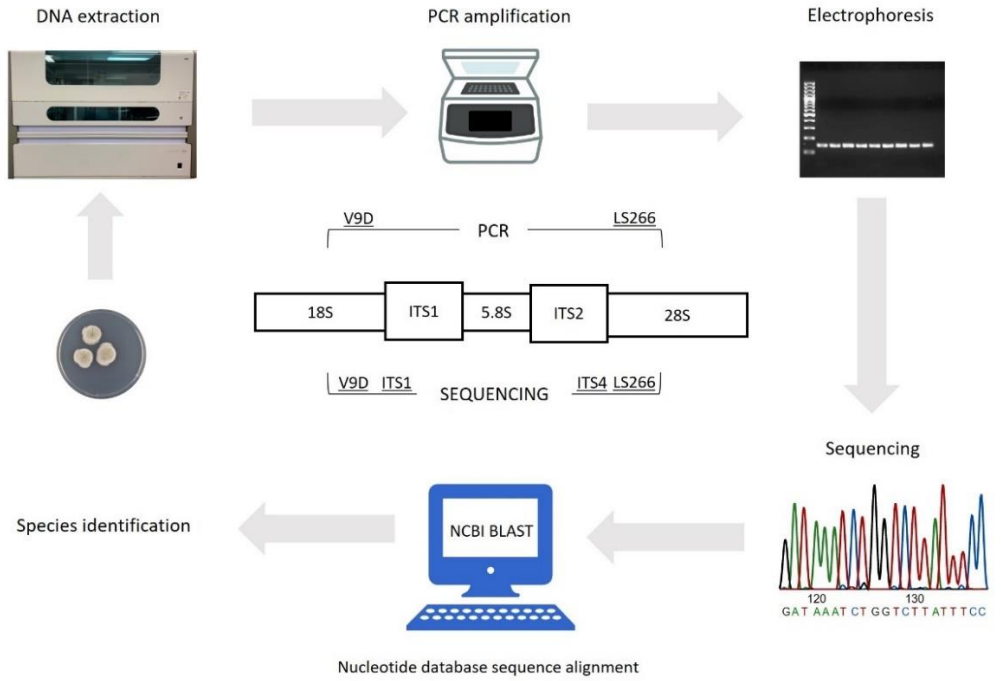
| **Genus** | **Phylum** | **Human Disease Caused** | **Example** | **Distribution** |
|---|---|---|---|---|
| *Aspergillus* | Ascomycota | Aspergillosis<br>Allergic bronchopulmonary aspergillosis<br>Allergic *Aspergillus* sinusitis<br>Aspergilloma<br>Chronic pulmonary aspergillosis Invasive aspergillosis<br>Cutaneous aspergillosis | *Aspergillus fumigatus* | Global |
| *Cryptococcus* | Basidiomycota | Cryptococcal meningitis<br>Cryptococcosis | *Cryptococcus neoformans* | Primarily Sub-Saharan Africa Asia-Pacific |
| *Candida* | Ascomycota | Candidiasis<br>Vaginal candidiasis (thrush)<br>Invasive candidiasis<br>Oropharyngeal candidiasis<br>Candidemia | *Candida albicans* | Global |
| *Histoplasma* | Ascomycota | Histoplasmosis | *Histoplasma capsulatum* | Central and Eastern USA<br>Central and South America Africa, Asia, Australia |
| *Blastomyces* | Ascomycota | Blastomycosis | *Blastomyces dermatitidis* | Eastern USA and Candida<br>Africa, Middle East, India, North America |
| *Coccidioides* | Ascomycota | Coccidioidomycosis | *Coccidioides immitis* | Southwestern USA, Mexico, Central South America |
| *Paracoccidioides* | Ascomycota | Paracoccidioidomycosis | *Paracoccidioides brasiliensis* | Mexico and Central and South America |
| *Sporothrix* | Ascomycota | Sporotrichosis | *Sporothrix schenckii* | Global<br>Endemic in Latin America |
| *Scedosporium* | Ascomycota | Scedosporiosis | *Lomentospora prolificans* | Global<br>Australia (*S. aurantiacum*) |
| *Trichophyton* | Ascomycota | Tinea (ringworm)<br>Onychomycoses (nail) | *Trichophyton interdigitale* | Global |

Clinical diagnosis of fungal infection is challenging, particularly in the immunocompromised host, due to the variable and nonspecific clinical signs of fungal infection. It is often difficult to distinguish colonisation from invasive disease (48). Laboratory diagnosis is also challenging. This is due to lack of

sensitivity and specificity of microscopy and culture-based techniques for their detection and identification (48). Morphological approaches have major limitations, including the slow growth rate of fungi, the need for specialised culture, and difficulty in identifying morphologically similar fungi. These limitations lead to delayed species identification, potential misidentification and challenges in disease diagnosis. Furthermore, identification is complicated due to the diversity of fungi, requiring high-level expertise in phenotypic identification methods, which are associated with high labour and consumable costs. Newly described species of fungi that do not exhibit distinguishing features in a timely fashion, also represent difficult challenges for the mycologist. Other challenges include the species-level identification required for the reliable interpretation of some antifungal susceptibility results, difficulties in recovering and identifying emerging pathogens (such as *Candida auris*), and common issues affecting all laboratories, such as increasing workload, insufficient staffing levels, and declining expertise in mycology. Due to these problems, molecular methods and other newer methods of identification, such as matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF), provide clear advantages over traditional methods. Most well-equipped bacteriology laboratories now utilise MALDI-TOF for rapid fungal identification of many common fungal pathogens. Briefly, MALDI-TOF is used in microbiology as a rapid, accurate, and cost-effective method for identifying microorganisms. A typical experiment consists of the growth of the organism, colony selection, placement on a target slide and the addition of matrix, which is a small organic molecule to facilitate the ionisation process by absorption of UV light. The sample is subsequently ionised by a laser and the particles are separated by their mass-to-charge ratio and their time of flight to the detector measured. MALDI-TOF generates characteristic mass spectral fingerprints which are compared with large library of mass spectra. As the spectral fingerprints are unique signatures for each microorganism, accurate microbial identification at the genus and species levels is performed using bioinformatics pattern profiling.

Molecular methods of fungal identification focus on the identification of pathogens cultured from clinical samples and/or the detection and identification of pathogens directly in clinical samples. Earlier molecular methods commonly targeted the ribosomal RNA (rRNA) genes used for taxonomic classification of fungi (46). During this early development, the internal transcribed spacer (ITS) regions of the rRNA gene complex, were favoured as they demonstrated a high degree of specificity for a broad range of fungi encountered in the clinical microbiology laboratory (49). The ITS regions were chosen by taxonomists for molecular phylogeny because of their favourable properties in terms of biology: a) small in size, b) highly conserved flanking sequences for universal primers, c) ease of detection due to the high copy number of the rRNA gene clusters, and d) suitable sequence variation between closely related species caused by the relatively low evolutionary pressure acting on such non-

coding ITS sequences (46, 49). The main advantage of the ITS regions for diagnostic purposes is the ease of amplification using universal primers and the number of internal primers available for Sanger sequencing, enabling good coverage of the entire ITS1 and ITS 2 regions (Figure 5). Combining these favourable properties, the ITS regions became the primary fungal DNA target for species identification.




| Primers | Direction (5'-3') | Position (*S. cerevisiae*) | Region | Useful for |
|---|---|---|---|---|
| **V9D** | Forward | 18S: 1609-1627 | 18S = 1800 bp | Genus and some species |
| **ITS1** | Forward | 18S: 1769-1787 | 5.8S = 160 bp | Universal primer sites |
| ITS5 | Forward | 18S: 1745-1767 | | (highly conserved) |
| ITS2 | Reverse | 5.8S: 50-31 | 28S = 3400 bp | Genus |
| ITS3 | Forward | 5.8S: 34-51 | ITS = variable | Species and strain identification |
| **ITS4** | Reverse | 28S: 41-60 | | |
| **LS266** | Reverse | 28S: 287-266 | | |

Figure 5. ITS PCR and sequencing for fungal identification. Diagrammatic representation of the ITS PCR and sequence-based identification method. The rDNA complex (centre) is shown to illustrate the location of the of the rRNA genes (18S, 5.8S, and 28S) and the ITS regions. PCR amplification primers (V9D and LS266) and sequencing primers (V9D, ITS1, ITS4 and LS266) are shown. The locations of primers and region lengths correspond to genomic positions of *Saccharomyces cerevisiae* (46). Figure created with PowerPoint using several copyright-free images as detailed in the Appendix. Pryce, TM (2024).

A comprehensive review in *Medical Mycology* of the ITS regions inspired an investigation of the ITS sequencing approach, with the ITS regions demonstrating the greatest potential for the universal identification of fungi (49). However, the authors concluded that although the ITS regions are more useful than the other rRNA gene targets, they may lack the necessary species-level discriminatory power, or that the morphological-based taxonomic schemes for the identification of some fungi may need to be reviewed. At the time, the laboratory was routinely performing ITS PCR and sequencing to identify fungi from cultures and clinical samples. The concluding statement of Iwen et al. (2002); "A simple, rapid and sensitive test…..that can be routinely used in the clinical mycology laboratory is an important clinical goal for the proper management of patients with an invasive fungal disease", served as a key motivator for the completion and subsequent publication of this work in the same journal (**Publication 1**) (50).

An important advantage of the broad-range PCR and sequencing approach is the improved time to detection and identification compared to microscopy and culture-based techniques (49). For example, ITS PCR amplification can detect a fungus on day one and sequenced the next day (Figure 6). In comparison, yeasts were traditionally identified using germ-tube, Dalmau plate slide culture, VITEK YBC card identification and API ID32C sugar assimilation tests. Filamentous fungi were identified using a plethora of culture media, temperature studies, microscopy and dichotomous keys (46, 51). Several days could be saved for yeast identification and a week or more for filamentous fungi. In contrast, the ITS sequencing method is universal and standardised in approach; identification can be achieved with one method in a few days for any fungus, irrespective of genus. More importantly, ITS sequencing can more accurately speciate most clinically important fungi than traditional methods, which were hampered by paucity and variability of microscopic features. Additionally, ITS sequencing can identify fungi that are slow to develop distinguishing features or do not sporulate (49). The result was an earlier and more accurate identification to assist disease diagnosis and treatment. Finally, earlier identification may also result in an earlier change from broad-spectrum antifungal agents to more tailored or efficacious treatment regimen, or cessation in therapy altogether for a non-pathogenic fungus (Figure 6). The application of this approach is highlighted in Chapter 2 for fungi isolated from culture (**Publication 1**) and blood cultures (**Publication 2**), with the entire approach summarised in **Publication 3.**

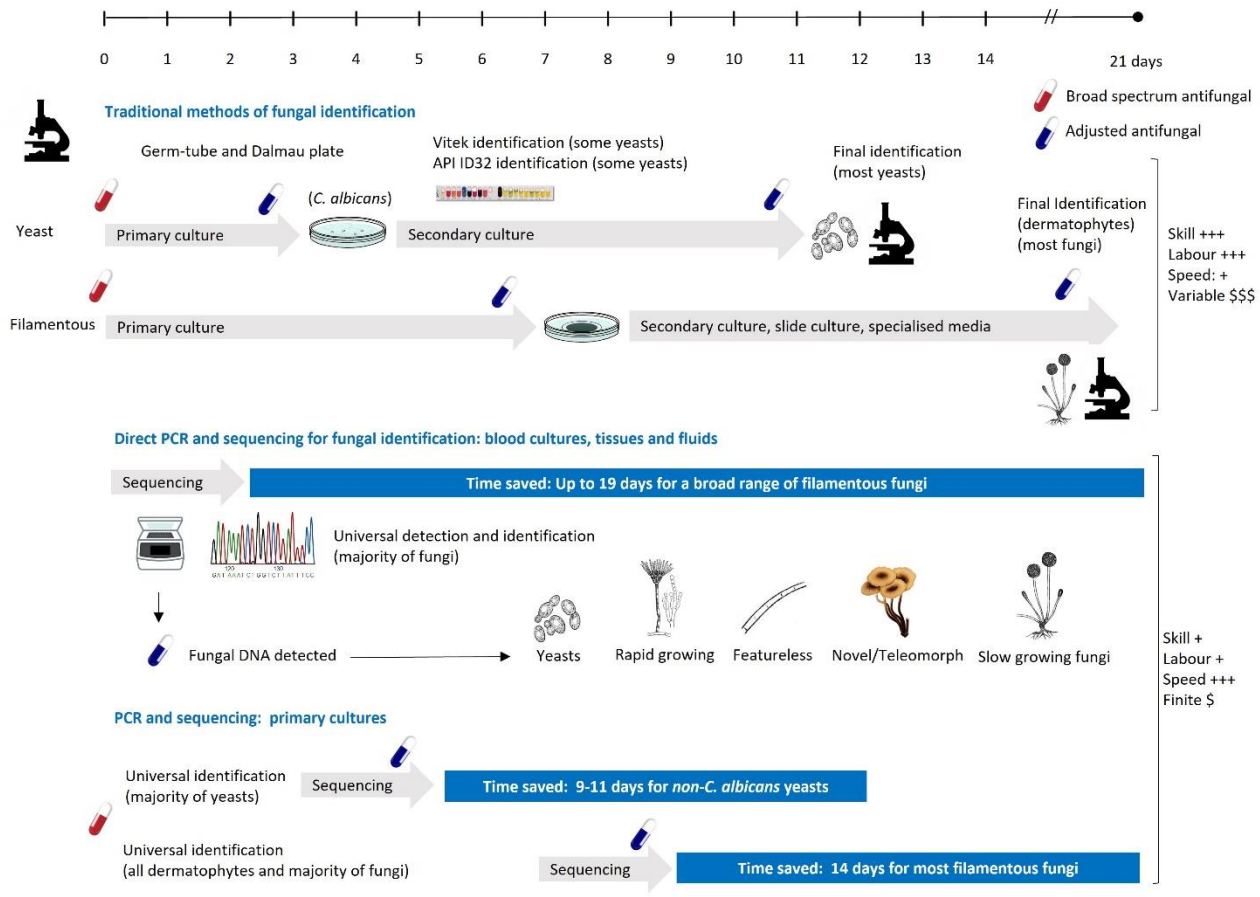



Figure 6. ITS sequencing compared to traditional methods. Schematic representation of the impact of ITS sequencing to detect and identify fungi in a timelier and cost-efficient manner compared to the older traditional methods of microscopy and culture. The time saved for PCR and sequencing direct from clinical samples and primary culture is compared to traditional methods of culture and identification. The potential change from empirical broad-spectrum therapy to a more efficacious therapy is also shown. Figure created with PowerPoint using several copyright-free images as detailed in the Appendix. Pryce, TM (2024).

In terms of diagnostic progression, the traditional germ-tube, Dalmau plate slide culture, VITEK YBC card identification and API ID32C sugar assimilation tests, were slowly being supplemented or superseded with new chromogenic medium to differentiate common yeasts (51). MALDI-TOF (introduced in 2010 in our bacteriology laboratory) was also proving useful to identify common yeasts (52). The combination of chromogenic agar and MALDI-TOF reduced the time to identify yeasts to a few hours (Figure 7). The method could also be applied directly to blood culture fluid with a moderate degree of success, thereby saving a few days normally required for subculture identification.

MALDI-TOF was also proving to be useful for the identification of common dermatophytes and other common species in the genus *Aspergillus Penicillium*, *Fusarium*, and some Zygomycetes. Speed and cost was the main driver MALDI-TOF implementation with same-day identification at a unit cost measured in cents. Today, the fungal species represented in MALDI-TOF databases are much-improved, however, MALDI-TOF is still limited in resolving to the species level for some fungi and has unresolved challenges direct from clinical samples.

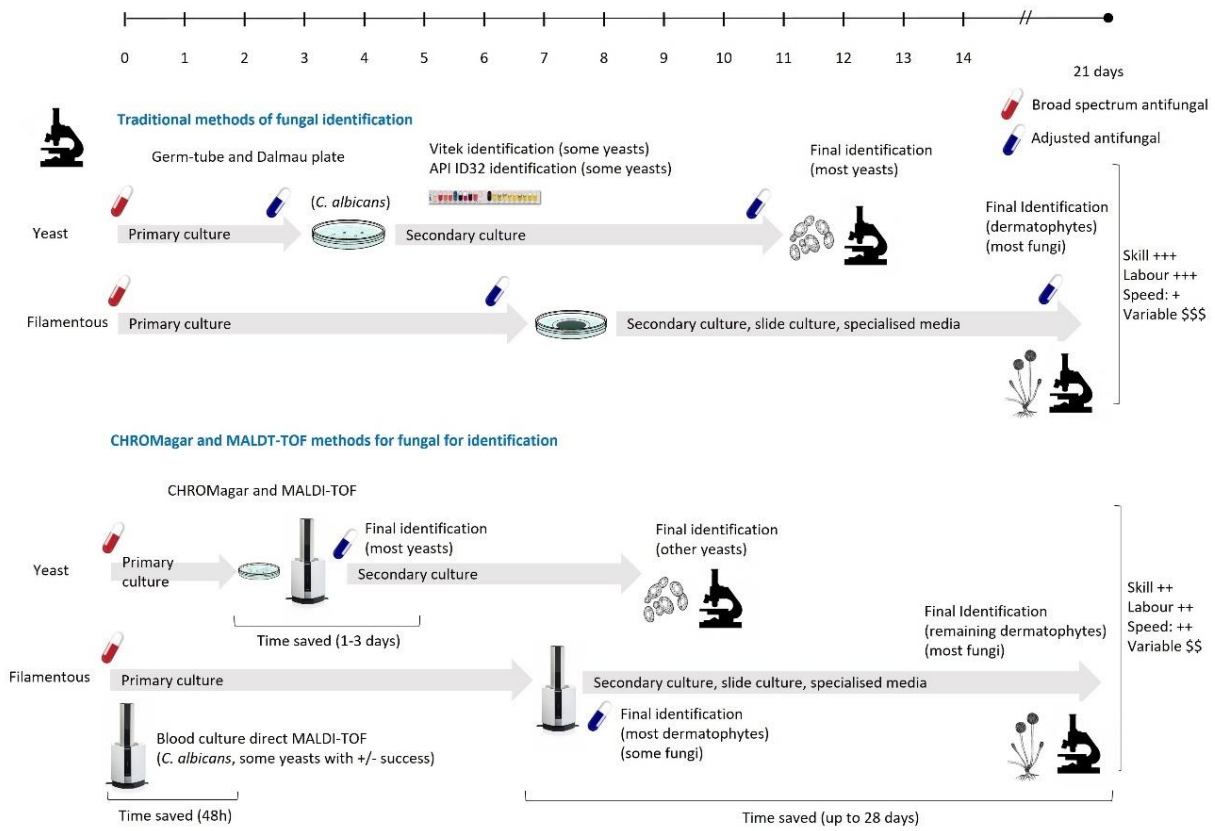



Figure 7. Impact of chromogenic medium and MALDI-TOF. Schematic representation of the impact of chromogenic medium and MALDI-TOF to detect and identify fungi in a timelier manner compared to traditional methods of microscopy and culture. The time saved using chromogenic medium and MALDI-TOF is compared to traditional methods of culture and identification. The potential change to from empirical broad-spectrum therapy to a more efficacious therapy is also shown. Figure created with PowerPoint using several copyright-free images as detailed in the Appendix. Pryce, TM (2024).

In the early 2010s, MALDI-TOF and molecular approaches were converging together as synergistic tools to further improve the time to identification, reduce costs and resolve issues around workflow

and hands-on time (Figure 8). While chromogenic agar and MALDI-TOF are frontline identification methods for positive blood cultures and cultured isolates, ITS PCR and sequencing in combination with some secondary phenotypic tests, are used to resolve identification to the species level with a high degree of accuracy (52). However, the identification of fungi using chromogenic agar and MALDI-TOF still relied on primary culture. Selective PCR, targeting specific fungal pathogens, or broad-range PCR, targeting several pathogens, were being applied directly to nucleic acids recovered from clinical samples, leading to a much earlier diagnosis. An application of this technique using a targeted PCR approach as described in **Chapter 2** (**Publication 4**). To conclude, **Chapter 2** presents four publications highlighting several molecular methods that have evolved over time to advance the laboratory workflows in mycology to provide greater diagnostic value to clinicians and patients.

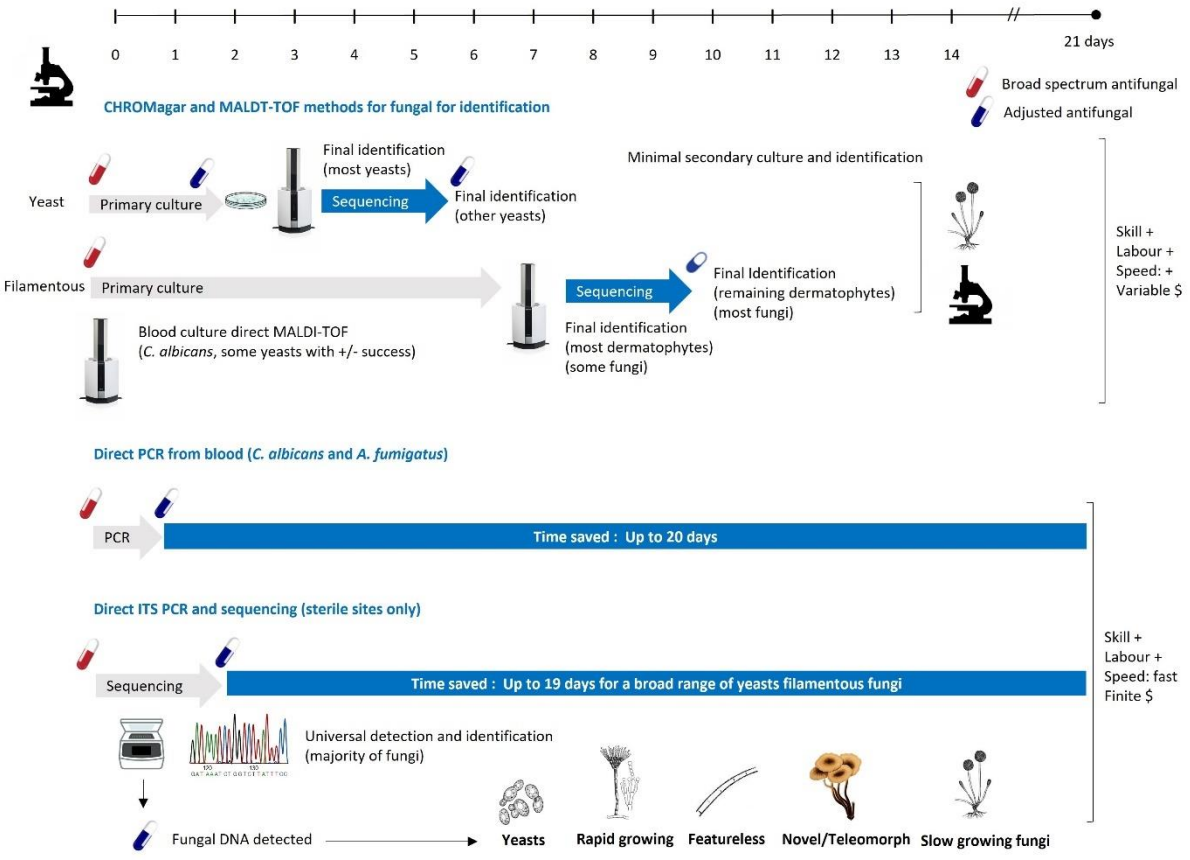



Figure 8. Impact of MALDI-TOF combined with PCR methods. Schematic representation of the impact of PCR applied directly to nucleic acids from clinical samples, compared to, and working synergistically with culture and MALDI-TOF. The potential change to from empirical broad-spectrum therapy to a more efficacious therapy is also shown. Figure created with PowerPoint using several copyright-free images as detailed in the Appendix. Pryce, TM (2024).

### 1.7.2 *Neisseria gonorrhoeae* NAATs and specificity issues

Sexually transmitted infections (STIs) have a profound impact on sexual and reproductive health worldwide and are a significant public health issue. According to World Health Organisation (WHO) reports, more than 1 million curable STIs are acquired every day by people 15–49-years, the majority of which are asymptomatic. Annually, an estimated 374 million people are infected with one of four curable STIs: *Treponema pallidum* (syphilis), *Chlamydia trachomatis* (CT), *Neisseria gonorrhoeae* (NG) and *Trichomonas vaginalis* (53). *N. gonorrhoeae* infections (gonococcal, gonorrhoea) account for approximately 87 million of these STIs per year (53). The focus in this thesis is on *N. gonorrhoeae.*

All persons that have unprotected vaginal, oral, or anal sex with infected individuals are at risk. Sites of infection are categorised as urogenital (urethra, urine, vaginal, endocervical), extragenital (oropharynx, anorectal, ocular), or invasive (blood, synovial fluid, cerebrospinal fluid) (54, 55). Infections range from symptomatic urogenital infections to asymptomatic cases, particularly in women. Extragenital infections, such as those in the oropharynx and rectum, are often asymptomatic and more common in some populations. For example, men who have sex with men are a group disproportionately affected by gonococcal infection (53). Rectal gonorrhoea is also associated with increased transmission and susceptibility to HIV infection due to local inflammation, increasing the number of CD4+ T-cells and dendritic cells in the rectal tissue and compromising protective mucosal barriers (56). Early diagnosis and effective treatment is important as untreated gonorrhoea infections can lead to sequalae such as urethritis, epididymo-orchitis, proctitis, pelvic inflammatory disease, infertility and neonatal health issues (53). Although STI testing services are widespread, prevention services are often underutilised due to the social stigma of STI testing. STI testing is also associated with ethical issues, such as partner notification, contact tracing, cases involving sexual assault and infections in minors (57, 58). Consequently, there are significant medical and legal implications that relate to STI testing, with significant pressure on the laboratory to deliver results with a high degree of accuracy.

Prior to molecular methods, the laboratory diagnosis for *N. gonorrhoeae* was limited to Gram's stain and culture. For symptomatic male urethral swabs, microscopy is suitably sensitive when compared to culture However, microscopy is less sensitive and specific especially with specimens collected from the vagina, rectum and oropharynx. For the latter, this is due to low load of *N. gonorrhoeae* and the presence of other commensal *Neisseria* species. The first commercial molecular methods for *N. gonorrhoeae* became available in the early 1990s, with the development of hybridisation capture assays. However, these assays reported sensitivity and specificity below that of culture as they were target capture with no nucleic acid amplification (59, 60). The first automated NAAT for

*N. gonorrhoeae* was the Roche Amplicor CT/NG test, which was launched in the USA in 1999 and first evaluated in a multicentre evaluation in 2000 (61). Other first-generation tests from different manufacturers were soon to follow, with the consensus that amplification technologies greatly improved sensitivity and specificity of *N. gonorrhoeae* detection. However, over the next decade, many reports emerged regarding false positive and false negative results for a few of the commercial assays and some in-house tests (Table 2).

Table 2. Reports of specificity issues with commercial and in-house NAATs. Published reports of *N. gonorrhoeae* specificity issues with commercial and in-house NAATs from 1999 to 2022. The target for each assay is shown according to the specificity issue and the *Neisseria* species associated.

| Year | Assay/Platform | Target | Specificity issue | Cross-reactivity with other *Neisseria* species or nature of error | References |
|---|---|---|---|---|---|
| 1999-2003 | Roche cobas Amplicor | *Cytosine DNA methyltransferase gene* | False positive | *N. flavescens*<br>*N. lactamica*<br>*N. sicca*<br>*N. subflava*<br>*N. cinerea* | (62-65) |
| 1999-2003 | BD ProbeTec | *Piv*NG | False positive | *N. flavescens*<br>*N. lactamica*<br>*N. subflava*<br>*N. cinerea*<br>*N. sicca* | (62, 64-67) |
| 2003 | In-house (68) | *cppB* | False positive | *N. cinerea* | (62) |
| 2011-2012 | In-house | *porA* | False negative | *N. gonorrhoeae* (*N. meningitidis porA* sequence) | (69, 70) |
| 2013 | Gen-Probe Aptima | 16S rRNA | False positive | *N. meningitidis* (*N. gonorrhoeae* 16S sequence) | (71) |
| 2013 | Roche cobas 4800 | DR9 | False positive | *N. macacae* | (72) |
| 2013-2018 | Cepheid GeneXpert | NG2 and NG4 (chromosomal targets) | False positive | *N. mucosa* (NG4)<br>*N. subflava* (NG4)<br>*N. oralis* (NG2)<br>*N. bergeri* (NG2) | (73, 74) |
| 2022 | In-house | *Opa* | False positive | *N. meningitidis* | (75) |

These early reports of non-specificity mostly occurred in urogenital samples. However, the problem was exacerbated when these assays were used for testing extragenital sites, driven by clinical need to test pharyngeal and rectal sites, particularly for high-risk patient groups. The occurrence and abundance of commensal *Neisseria* species, especially in the oropharynx, combined with the frequent horizontal genetic exchange occurring within the *Neisseria* genus, created ideal conditions for assay failure. Hence, further reports followed of issues with assay specificity, leading to false positive identification of gonococcal infection, or false negative outcomes, particularly for pharyngeal testing. Most false positive results occurred via genetic exchange whereby commensal *Neisseria* species acquired *N. gonorrhoeae* genes or shared related genes of a similar sequence. False negative results

occur though loss of the target sequence in *N. gonorrhoeae* or have acquired a different gene from another non-gonococcal *Neisseria* species. Two classic examples from each category include false positive reports of commensal *Neisseria* species cross reacting with the cytosine DNA methyltransferase gene targets designed for *N. gonorrhoeae* (62, 63) and false negative reports caused by a *N. gonorrhoeae* harbouring a *N. meningitidis porA* sequence (69). It should be noted, however, that the assays presented in Table 2 did not have testing claims for testing extragenital sites.

To address these issues of non-specificity and the lack of extragenital testing claims, supplementary testing (whereby samples testing positive in a screening NG-NAAT are confirmed by a second NAAT) has been widely implemented (76-79). Many commercial assays have improved assay specificity and include extragenital testing claims (Table 3). For example, Roche changed targets and developed a dual target assay for the second-generation cobas CT/NG test on the cobas 4800 system. The assay utility was further improved with the third-generation cobas CT/NG test on the cobas 6800 system, including an extragenital claim. Dual target assays offer some redundancy for target drop out, polymorphisms in primer/probe binding sites, or horizontal gene transfer, especially if the dual target assay amplifies two different genes. Throughout successive generations of commercial screening assays, analytical sensitivity has improved, with most screening assays offering dual target tests and the ability to detect 1 colony forming unit (CFU) per millilitre of sample tested. These assays are summarised in Table 3, as part of a recently authored review of the current challenges of molecular testing for *N. gonorrhoeae* (80). These ongoing challenges with *N. gonorrhoeae* NAAT assay non-specificity are highlighted in **Chapter 3**, as I present a series of linked publications demonstrating the issue and supplementary test methods used to minimise false positive results.

Table 3. High throughput sample-to-result *N. gonorrhoeae* testing assays. High throughput sample-to-result *N. gonorrhoeae* testing assays available in Australia as of June 2024[1]. Table reproduced from Pryce (2024) with permission from the publisher (80).

| Assay | Systems | *N. gonorrhoeae* targets | Analytical Sensitivity | Sample collection device storage temperature (days stability) | Extragenital site claim (date) | On-board NG supplemental testing (manufacturer-supplied, third party or in-house) | Reportable results | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | 8 hours | 24 hours |
| Alinity m STI[2] | Alinity m | *opa* gene DNA | 1.5 CFU[7]/assay | 2-30°C (14 days) | Oropharyngeal and anorectal (5/11/2021) | LDT available: (Alinity m You-Create) | 300 | 1080 |
| BD CTGCTV2[3] | BD COR™ | *opc*A & *var* genes (dual target) | Urine: 20-30 CFU/mL<br>Urogenital swab: 30-40 CFU/mL<br>Rectal swab: 20-25 CFU/mL<br>Oropharyngeal swab: 10-20 CFU/mL | 2-30°C (21 days) | Oropharyngeal and anorectal (01/06/2023) | LDT not currently available | 580 | 1008 |
| Xpert® CT/NG[4] | GeneXpert® Instrument Systems (Infinity 80) | Chromosomal NG2 and NG4 (dual target) | Vaginal swab: 1.5-1.6 CFU/mL<br>Male urine: 1.2-2.7 CFU/mL<br>Pharyngeal swab: 6.4-7.1 CFU/mL<br>Rectal swab: 4.9-5.3 CFU/mL | Female urine 2-30°C (3 days)<br>Male urine: 2-30°C (45 days)<br>Swabs: 2-30°C (60 days) | Oropharyngeal and anorectal (01/03/2019) | LDT not currently available | 400 | 1200 |
| Aptima Combo 2®[5] | Panther® System | 16S rRNA target | 50 cells / assay<br>(0.10 CFU/mL for extragenital sites) | Urine 2-30°C (30 days)<br>Swabs: 2-30°C (60 days) | Oropharyngeal and anorectal (09/10/2017) | Aptima GC Assay (different 16S target) LDT available (Open Access) | 270 | 1220 |
| cobas® CT/NG[6] | cobas® 5800/6800/8800 Systems | DR-9 region (dual target) | 1 CFU/mL | 2-30°C (365 days) | Oropharyngeal and anorectal (7/03/2017) | LDT available (cobas® omni Utility Channel) | 380 | 1410 |

1. Capable of up to 1000 reportable results in a 24 -hour period (verified by the manufacturer)
2. Abbott Molecular Inc., Des Plaines, IL, USA
3. Becton Dickinson, Sparks, MD, USA
4. Cepheid AB, Solna, Sweden
5. Hologic, Inc., San Diego, CA, USA
6. Roche Molecular Systems, Branchburg, NJ, USA
7. CFU: colony forming units
8. LDT: laboratory-defined testing (open channel)
9. Reported results rounded to nearest 10 samples

### 1.7.3 *Neisseria gonorrhoeae* and antimicrobial resistance detection

The testing of samples from extragenital sites is not just restricted to specificity concerns. Pharyngeal infections also play an important role in the epidemiology of *Neisseria* as a site for genetic exchange and reservoirs for antimicrobial resistance (AMR) in *N. gonorrhoeae* (81-83). Commensal *Neisseria* species can develop and carry genetic AMR elements from prior exposure to antibiotics (82). Furthermore, antibiotic concentrations in the oropharynx are suboptimal, hindering effective treatment, promoting antibiotic overuse that increases AMR in the biome (84). Globally, ceftriaxone, an extended-spectrum cephalosporin, is the first front-line treatment for gonococcal infections. However, resistance to ceftriaxone is emerging, particularly in pockets within the Asia-Pacific region (85, 86). To monitor and report on gonococcal AMR, global, regional and local surveillance systems have been established through an extended laboratory network to report data to the WHO. In Australia, the National *Neisseria* Network (NNN) of collaborating laboratories, including our organisation (PathWest), fulfils this role (87). The NNN also reviews diagnostic tests and promotes further test development, especially for AMR. The extraordinary capacity to acquire and retain AMR in *N. gonorrhoeae* requires rapid laboratory testing to include molecular predictors of AMR (88). Until recently, detecting AMR markers was limited to in-house tests, which require extensive validation, quality control and more work effort, limiting the uptake in laboratories. While a small number of commercial assays are available, they are limited, targeting only a few antimicrobials such as ciprofloxacin, azithromycin and ceftriaxone (Table 4).

Table 4. Commercial assays capable of detecting gonococcal AMR markers. The targets, mutations and the test turnaround time for each assay is shown.

| Assay | Assay type | Antimicrobial | Target | Mutation | Test turnaround |
|---|---|---|---|---|---|
| Seegene Allplex NG & DR Assay | Supplemental | Azithromycin | 23S rRNA | A2059G and C2611T | 2.5 hours |
| | | Ciprofloxacin | Gyrase A | S91F | |
| SpeeDx ResistancePlus GC | Supplemental | Ciprofloxacin | Gyrase A | S91F | 2.5 hours |
| AusDiagnostics Urinogenital and Resistance 12-well | Screening and supplemental | Ceftriaxone | *penA-60.001* | A311V | 2.5 hours |

**Chapter 3** describes the SpeeDx ResistancePlus GC supplementary test for *N. gonorrhoeae* detection, which also includes a molecular predictor for ciprofloxacin (**Publication 7**). In summary, the *N. gonorrhoeae* species presents some considerable challenges to molecular diagnostics, both in terms of specificity and the growing state of AMR. **Chapter 3** presents a series of linked publications that demonstrate non-specificity issues and the supplementary test methods used to minimise false positive results, includes the use of new contemporary methods of *N. gonorrhoeae* AMR detection.

### 1.7.4 SARS-CoV-2 pandemic NAAT implementation

The outbreak of coronavirus disease-2019 (COVID-19) was first reported in Wuhan city, Hubei province, China in December 2019 (89, 90). The epidemic commenced on 12 December 2019 and caused 2,794 laboratory-confirmed infections including 80 deaths by 26 January 2020 (90). The virus shared 80% sequence identity to severe acute respiratory syndrome coronavirus (SARS-CoV) and 96% sequence identity at the whole genome level to a bat coronavirus of probable origin (90). The virus was subsequently named SARS-CoV-2 by an International Committee on Taxonomy of Viruses (ICTV) Study Group (91). Research is ongoing to determine the origin, directly from bats or indirectly through an intermediate host, with a spillover event to humans (92, 93). The growing list of investigations, scientific theories, conspiracy theories as to the spillover event are overwhelming and beyond the scope of this thesis. Regardless of the nature of the spillover event, SARS-CoV-2 quickly first spread through China, Thailand, Japan and the Republic of Korea. The World Health Organisation (WHO) issued daily situation reports from 20 January 2020. At the end of April 2020, the total number of cases reported to the World Health Organisation (WHO) was 3.1 million with a total of 218,000 deaths (94). At the end of July 2020, the number of cases had risen to 17.1 million cases and 669,000 deaths (95). The number of cases peaked globally at week 25 (December 2022), with 42.2 million cases reported in one week. Overall, the number of cases and deaths cumulatively reported to WHO over six-monthly intervals from December 2020 to December 2024 are shown in Table 5 (96). As of December 2024, the total number of cases and deaths was 777 million and 7.1 million, respectively.

Table 5. COVID-19 cases and deaths reported to WHO. The number of global COVID-19 cases and deaths over six-monthly intervals from December 2022 to December 2025 (96).

| End of month | Year | Cumulative cases reported to WHO (million) | Deaths (million) |
|---|---|---|---|
| December | 2020 | 79.2 | 1.7 |
| June | 2021 | 180.5 | 3.9 |
| December | 2021 | 278.7 | 5.4 |
| June | 2022 | 541.3 | 6.3 |
| December | 2022 | 649.2 | 6.6 |
| June | 2023 | 767.4 | 6.9 |
| December | 2023 | 772.8 | 7.0 |
| July | 2024 | 775.7 | 7.1 |
| December | 2024 | 776.7 | 7.1 |

The SARS-CoV-2 pandemic brings into sharp focus the importance of early laboratory diagnosis coordinated with public health responses to identify infected individuals and contain the spread of the virus. Many laboratories already performing respiratory PCR were, for the most part, adequately equipped to quickly implement a new in-house or published method or a commercial assay for SARS-CoV-2 testing, with existing processes for safe laboratory testing. However, these laboratories were not prepared for the unforeseen challenges; the global shortage of reagents and equipment, disruptive supply chains, shortage of labour and the scale of testing required. RT-PCR was already a globally recognised approach for other RNA viruses, including SARS-CoV and Middle East respiratory syndrome coronavirus (MERS-CoV) (97, 98). Although other detection tools were possible, such as human antibody detection and serological methods for viral antigens, nucleic acid detection using amplification techniques has historically demonstrated earlier detection and greater reliability for respiratory viruses than serological-based methods (Figure 9) (99). At the time, there were two immediate challenges for our laboratory: a suitable RT-PCR assay compatible with our instrumentation and the safe handling of samples. Given assay design and selection drive sensitivity and specificity, RT-PCR assay selection was the priority. Safety can be managed in accordance with the Australian/New Zealand Standard for microbiological safety and containment (AS/NZS 2243.3:2010), as applied to other SARS coronavirus testing involving Risk Group 2 viruses. Our laboratory is a NATA ISO 15189-accredited Category GX physical containment level 2 (PC2) pathology facility. Accordingly, specific work practices for handling Risk Group 2 microorganisms transmissible via the respiratory route—or procedures that generate significant aerosol risk—include the use of class II biological safety cabinets (BSC), gloves, eye protection, and laboratory gowns. Additional personal protective equipment (PPE), such as face shields, N95 masks, and disposable gowns, can also be used to further enhance protection if required.

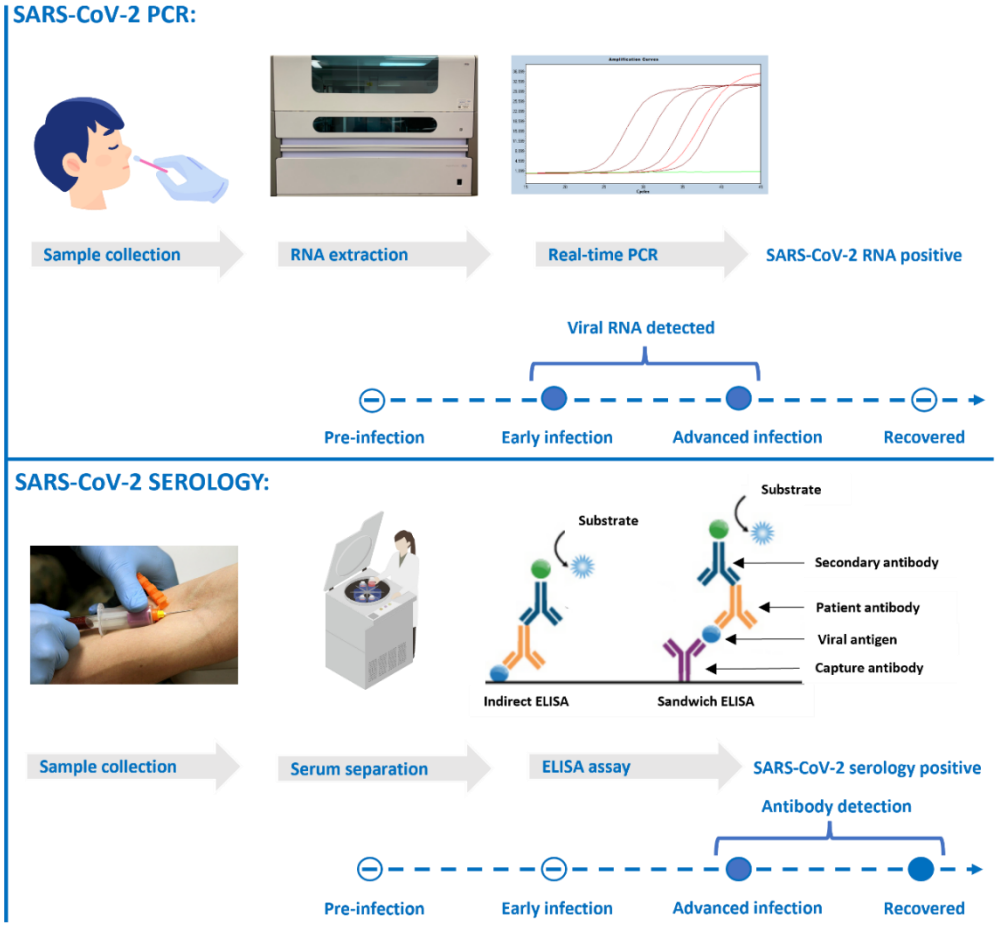



Figure 9. SARS-CoV-2 PCR testing strategy compared to SARS-CoV-2 serology. Schematic representation comparing PCR to serology for the diagnosis and monitoring of SARS-CoV-2 infections. The key point of difference is that PCR can detect a genomic sequence earlier than antibody tests. Specific antibody tests detect infection usually at a later stage, including past exposure. Figure adapted from El-Daly (2024) (99) and created with PowerPoint. Pryce, TM (2024).

All PCR assay development begins with a target sequence. Whole genome sequencing (WGS) methods were used in the initial identification of SARS-CoV-2 (89) and, according to reports, were uploaded into Genbank on 5 January 2020, but were not made immediately available to the public. A collaboration by Dr. Zhang Yong-Zhen, a virologist at Fudan University, along with Dr. Edward Holmes, an evolutionary biologist and virologist at the University of Sydney, made the genome available to the public on January 11, 2020 (accession MN908947). The European collaborative group, which developed the MERS-CoV RT-PCR assay targeting the envelope protein gene (E gene) and open reading frame (ORF) (98), rapidly developed and published SARS-CoV-2 RT-PCR assays targeting E gene, nucleocapsid protein gene (N) and RNA-dependent RNA polymerase gene (RdRp) on January 23, by Corman et al. (2020) (Figure 10) (100). The second assay for E gene was published on January 24 by Huang et al. (2020) (101).

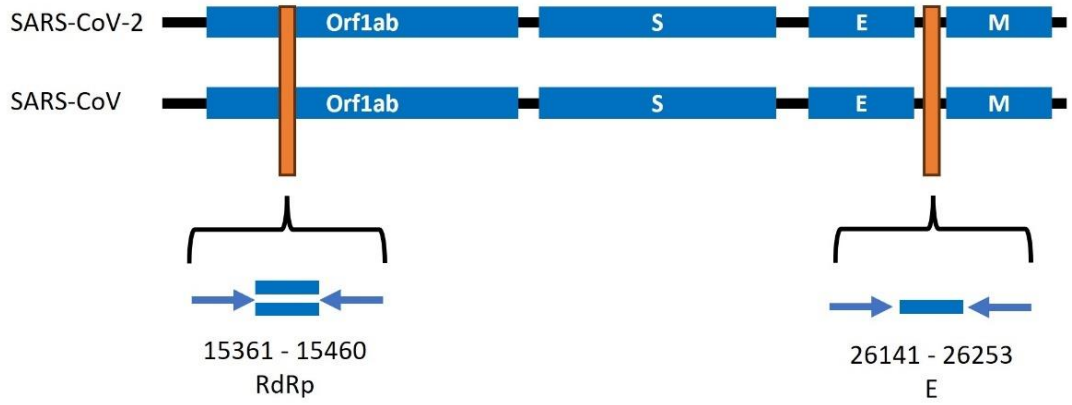



Figure 10. SARS-CoV-2 genomic PCR targets. Relative positions of amplicon targets comparing SARS-CoV to the 2019 novel coronavirus genome (SARS-CoV-2), with the amplicon genome positions according to SARS-CoV, GenBank NC_004718. E: envelope protein gene; M: membrane protein gene; ORF: open reading frame; RdRp: RNA-dependent RNA polymerase gene. Figure adapted from Corman et al. (2020) (100). Figure created with PowerPoint. Pryce, TM (2024).

The biggest challenge faced by the European collaborative group was developing an assay and workflow for SARS-CoV-2 screening and specific confirmation without available virus isolates or original patient samples. The design and validation were enabled by the close genetic relatedness to the 2003 SARS-CoV, aided by synthetic nucleic acid technology. This effort was facilitated by coauthor Dr. Olfert Landt, founder and Chief Executive Officer (CEO) of Tib-Molbiol. The authors developed an E gene assay by aligning primer binding domains from the published sequences from SARS-CoV-2, SARS-CoV, and selected bat-associated SARS-related coronavirus., (102). To discriminate SARS-CoV-2 from SARS-CoV, the authors added an RdRp assay so that it contains two probes; a broad-range probe reacting with SARS-CoV and SARS-CoV-2, and an additional probe that reacts only with SARS-CoV-2. The authors confirmed the specificity of the E and RdRp assays for SARS-CoV-2, yielding no false positive outcomes for seasonal human coronaviruses (HKU1, OC43, NL63, 229E), MERS-CoV, and a broad range of influenza A strains and other viral and bacterial respiratory pathogens. Consequently, the E gene and RdRp assays became quickly available from Tib-Molbiol, which was rapidly deployed, as highlighted in **Chapter 4.**

Following these initial publications, several in-house assays targeting various genomic targets were published (103-108). Recognising the seriousness of the infection and potential commercial

opportunities, the well-established molecular diagnostic industry progressed to commercial assay development, with many becoming available throughout 2020. For many laboratories, the decision to implement a SARS-CoV-2 RT-PCR assay was based on the existing respiratory PCR platforms or in-house tests already established in the laboratory. At the time, diagnostic testing at Fiona Stanley Hospital for other respiratory viruses was restricted to the rapid molecular tests, such as BioFire Film Array (bioMérieux) and GeneXpert (Cepheid) which were low in throughput and high in cost. This new molecular testing for SARS-CoV-2 needed to leverage our existing throughput for nucleic acid extraction equipment, or our sample-to-result high-throughput Roche cobas 6800 system, which handled all the high-throughput virology. Consequently, our SARS-CoV-2 testing journey commenced in early March 2020 and continues to this day.

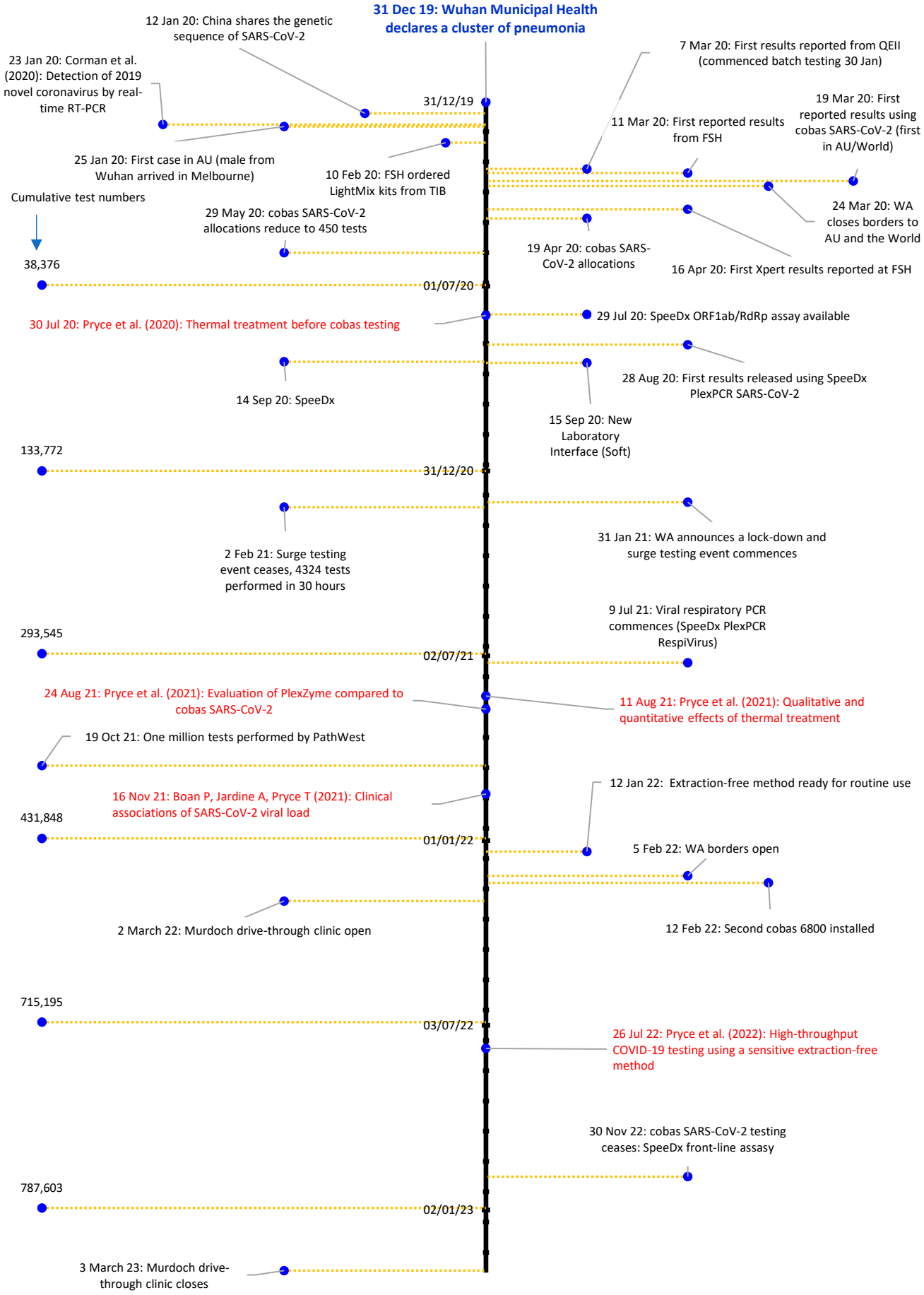
The timeline of the SARS-CoV-2 pandemic in Western Australia (WA) in relation to our laboratory based at Fiona Stanley Hospital (FSH) (Murdoch, WA), is shown in Figure 11. Some of this detail is further described in **Chapter 4** in a series of linked publications using molecular methods to resolve several challenges faced during the pandemic:

a) Safer laboratory handling of samples.
b) Improving the workflow in a time of unprecedented reagent and PPE shortage.
c) Improving the laboratory output by combining several testing strategies.
d) Improving the efficiency and throughput using alternative nucleic acid preparation methods.
e) Quantifying these assays for assay development and for clinical correlation of disease and for estimating potential infectivity.

The publications in **Chapter 4** are presented in a timeline narrative, highlighting the problems faced and investigative techniques to resolve these challenges, concluding this thesis with SARS-CoV-2, the worst pandemic the world has seen in 100 years.

Figure 11 (overleaf). SARS-CoV-2 pandemic timeline and our laboratory response. A timeline of significant events highlighting the response to the SARS-CoV-2 pandemic from the Department of Clinical Microbiology, PathWest Laboratory Medicine WA, FSH. Significant events such as the first reported results, border control events in WA, surge testing events, opening of the PathWest Murdoch drive-through collection facility are shown. The cumulative testing numbers are also shown, including publications presented in this thesis (highlighted in red). Figure created with Excel. Pryce, TM (2024).

## SARS-CoV-2 Pandemic Timeline: Department of Clinical Microbiology Fiona Stanley Hospital



# Chapter 2: Rapid detection and identification of fungi

## 2.1 Introduction to prior publications

Mycological identification is primarily relies on a complex system of microscopic features, culture characteristics and dichotomous keys, covering an exceptional degree of biological complexity (109, 110). A major challenge for diagnostic mycology in the early 2000s was the timely identification of yeasts, dermatophytes and pathogenic dimorphic fungi (111). It is important to recognise that laboratories servicing large tertiary hospitals performing bone marrow, hematopoietic stem cell, and solid organ transplantation face the task of identifying any opportunistic fungus which can grow at 37°C and gain access to the bloodstream to cause disseminated infection. The increased incidence of opportunistic fungal infections and the diversity of fungi causing them occurred in parallel to aggressive post-transplant immunosuppression, lowering resistance of the host. In the 1990s, candidiasis accounted for 44-80% of fungal infections in cancer, organ transplant recipients and patients diagnosed with AIDS (111). Fortunately, yeasts are easily detected in clinical samples by either Gram's stain or potassium hydroxide preparations and *C. albicans* could be rapidly identified by the germ tube test—a 3-hour screening test where the production of germ tubes by the cells were diagnostic for *C. albicans* (51). Demonstration of the germ tube was often sufficient for the identification of *C. albicans* for non-invasive infections. However, in the late 1990s a related yeast, *Candida dubliniens*is emerged in HIV-positive patients. It was also germ tube positive and largely morphologically indistinguishable from *C. albicans* (112). Azole resistant *C. dubliniensis* quickly emerged, and consequently, all germ tube positive yeasts from immunocompromised patients and isolates recovered from invasive infections required further identification, such as temperature growth studies and chlamydospore differentiation (50, 113).

The identification of fungi worldwide has been guided by several definitive mycological texts, while comprehensive, are often overwhelming in terms of size and complexity (110, 114, 115). The first Australian handbook for the identification of fungi was authored by David H. Ellis (Mycology Unit, Adelaide Women's and Children's Hospital) and published in 1994, summarising these complex methods of identification (111). This work played a key role providing the mycological component of the Microbiology Quality Assurance Program organised by the Royal College of Pathologists of Australasia, with the publication of the *Descriptions of Medical Fungi* (51). Following these guidelines, *C. dubliniensis* and all germ tube negative yeasts required microscopy from Dalmau plate culture and carbohydrate assimilation tests for accurate identification (51). These carbohydrate assimilation tests were performed manually with traditional in-house prepared sugars or a variety of commercial tests available at the time, such as Vitek YBC, API 32C or API Candida (116). However, misidentification of

yeasts with these tests was well known due to variable results and convergent carbohydrate assimilation pathways among the yeasts; mycological features from slide culture were still required for definitive identification (117, 118). The introduction of *Candida* CHROMagar in the early 2000s assisted with the presumptive identification of *C. albicans*, *Candida tropicalis, Candida krusei* and *Candida glabrata*, but could not differentiate *C. dubliniensis* from *C. albicans*, nor identify *Candida parapsilosis* or other members of the genus *Candida* (119, 120). Despite these advances in phenotypic, biochemical, and chromogenic methods of identification, the overall process of yeast identification often took 1-3 days for a final result, with some non-*C. albicans* yeasts taking up to 7 days (51, 110, 120).

The identification of dermatophytes was also slow and challenging. Identification relies solely on microscopy from tedious slide culture preparation and macroscopic culture-based techniques (51, 111). Correct identification is necessary, particularly for clinical and epidemiological investigations of zoonotic acquired human infections (51). *Trichophyton* species were identified using the Kaminski identification scheme, examining macroscopic and microscopic features using six different media types, including Littman Oxgall agar, Lactritmel agar, Sabouraud's Dextrose agar with 5% NaCl, 1% Peptone agar, hydrolysis of urea, combined with the hair perforation test (51, 121, 122). These plates were prepared individually or nested together in a singular rectangular plate called a "Trich tray". Distinguishing features were used for identification such as surface colour, reverse colour, texture, height, microconidia shape and the presence of macroconidia. This identification scheme for common dermatophytes required a high-level of expertise and was very time consuming. Given the increasing workload, staffing challenges, and operational costs of the mycology laboratory, a new approach was needed to maintain diagnostic accuracy. Rapid and simple methodologies that did not rely on expert mycologists were required to speciate common yeasts and dermatophytes in a timely manner.

Additionally, several highly pathogenic dimorphic fungi and antifungal resistant fungi, often associated with a high degree of morbidity and mortality, require more rapid identification methods. For example, *Histoplasma capsulatum* causing disseminated histoplasmosis, *Sporothrix schenckii* causing serious subcutaneous infections (sporotrichosis), and *Scedosporium* spp., causing serious disseminated infections (mycetomas, eye, ear, central nervous system, and internal organs) that were often un-treatable with antifungal agents and often require aggressive surgery (111). In addition, the culture-based identification of some dimorphic fungi, namely *H. capsulatum* and *Coccidioides immitis*, posed a danger to laboratory workers as biosafety level 3 organisms (46). Therefore, timely and alternative methods of identification were needed for this select group of pathogens.

Prior experience with the limitations of RFLP and PCR-based diagnostic methodologies for bacterial detection and identification were useful to resolve these challenges (9, 10). The second edition of the Atlas of Clinical Fungi published rRNA gene endonuclease restriction maps for many type strains of fungal species (46). These maps included restriction sites for one or more of 18S, ITS1, ITS2 and 28S rRNA genes, along with associated primers, covering the majority of clinical fungi (46). These RFLP and probe-based methods were commonly used but had limited applicability for routine use due to tedious DNA extractions and labour-intensive blotting techniques, using species-specific digoxigenin or radiolabelled probes (49). The major disadvantage of RFLP was the labour-intensive nature of gel electrophoresis, which was not well-suited for routine diagnostic laboratories. Fundamentally, RFLP analysis became redundant if the whole sequence could be derived (10). On the other hand, direct sequence analysis methods of the ITS regions were showing promise for the identification of *Candida* spp. (123, 124), dermatophytes (125), *Scedesporium* spp. (126) and *H. capsulatum* (127). These regions were shorter (<800 bp) and more compatible with the sequencing technology. However, most of these reports identified a limited range of fungi within a genus, used incomplete GenBank ITS1 or ITS1 sequences, or relied on GenBank records that have been derived from non-curated strains (49). In addition, the traditional Sanger sequencing methods were slow and not practical for routine use.

**Chapter 2** includes four publications where molecular methods are used to address the difficulties with traditional methods of identifying fungi grown from clinical samples, and detecting and identifying fungi directly from clinical samples, ultimately leading to a timelier identification and diagnosis. The key aims were:

a) Investigate the utility of a new universal PCR and a new capillary-based sequencing approach to rapidly detect and identify fungi grown from clinical samples.
b) to improve the time to identification and the accuracy of identification, cost-effectively and more efficiently.
c) Investigate the utility of a new qPCR method for the diagnosis of fungaemia, to improve the time to diagnosis and provide better clinical outcomes for patients.

The objectives were:

a) Develop a universal extraction method for fungi.
b) Develop a universal ITS PCR and capillary-based Sanger sequencing approach to rapidly identify a wide range of fungi.
c) Investigate the utility of ITS sequencing to rapidly identify fungi commonly encountered in the clinical laboratory.

d) Compare the ITS sequencing identification with the identification by traditional methods and investigate discrepancies.
e) Determine if there were any specimen types where traditional mycology could be rationalised, to improve laboratory efficiency and costs.

For the preparation of the first publication, an in silico sequence-based identification of a wide range of yeasts and dermatophytes reported from our laboratory was performed, including other highly pathogenic fungi, by mining representative ITS1-5.8S-ITS2 sequences from GenBank. Secondly, I defined missing fungal sequences, incomplete or partial ITS sequences from GenBank, then procured a collection of type strain representatives for ITS sequencing. The sequences derived from these were deposited in GenBank, thereby compiling our first curated ITS sequencing database. Thirdly, I developed a universal, simple and robust nucleic acid extraction technique, standardised PCR amplification conditions, PCR product purification methods, and optimised a rapid capillary-based Sanger sequencing method. Finally, the ITS PCR and sequencing method was used to identify a collection of clinical isolates and controls that were previously identified using traditional methods. Here, the correlation between phenotypic and molecular identification is presented, along with an investigation of discrepancies and an assessment of cost and time comparisons in **Publication 1**, titled "Rapid identification of fungi by sequencing the ITS1 and ITS2 regions using an automated capillary electrophoresis system" (50).

The second publication was inspired by success of the ITS sequencing method for cultured isolates, which was routinely implemented in the laboratory for identifying yeasts, dermatophytes and other pathogenic fungi. Continuously monitored blood culture systems are widely deployed in clinical laboratories for the detection and the isolation of bacteria, mycobacteria and fungi from patients with blood stream infections (128). Furthermore, inoculation of blood culture media with other body fluids or tissues is a widely accepted method to improve recovery of microorganisms (118). Following confirmation of positive microscopy (yeasts or fungal hyphae seen), subcultures are performed for the identification of fungi using traditional methods. At the time, it was conceivable that the same ITS sequencing method described in **Publication 1** could be used to identify fungi directly from enrichment media, thereby dramatically improving the time for a definitive identification compared to traditional techniques. However, based on prior experience for the detection of mycobacteria in blood culture media, interfering substances present in the media such as sodium polyanetholsulfonate can cause a loss in sensitivity due to PCR inhibition (9). Additionally, pilot experiments revealed contamination of the ITS sequence chromatogram with nonspecific co-

amplified human DNA from the ITS PCR reaction, albeit in small amounts (Pryce, 2003, unpublished data). For the second publication our objectives were:

a) Develop and refine a rapid universal fungal extraction method which:
   - could be applied to a variety of enrichment media where fungi were likely to be cultured.
   - removed inhibitors and reduce the concentration of human DNA.
   - successfully lyse fungal cells and extract nucleic acid.
b) Prospectively assess the utility of the method combined with ITS sequencing for the rapid identification of fungi in enrichment media.
c) Compare the ITS sequencing identification to that of traditional methods.
d) Assess cost and time comparisons.

For the development of the second publication, a novel sodium hydroxide (NaOH) pretreatment step was developed based on prior work isolating mycobacterial DNA from blood cultures (9). I tested the NaOH pretreatment step, in combination with the extraction method and ITS sequencing technique described in **Publication 1,** using enrichment media commonly used to isolate fungi from blood, tissues and fluids. This approach simultaneously addressed two challenges: the removal of SPS and the hydrolysis of contaminating human DNA present in the blood culture fluid. The fungal DNA, protected by the alkali resistant fungal cell wall, would not be affected by the NaOH. The method was tested prospectively on microscopy-positive enrichment media, and the ITS sequencing identification was compared to the identification using traditional methods. Time and cost comparisons were also made. This study is presented in **Publication 2** titled "Rapid identification of fungal pathogens in BacT/ALERT, BACTEC, and BBL MGIT media using polymerase chain reaction and DNA sequencing of the internal transcribed spacer regions" (129).

The third publication was initiated by invitation to publish a chapter in the book titled "*PCR for Clinical Microbiology: An Australian and International Perspective*" (130). Chapter 70 summarises the molecular methods, applications and limitations of **Publication 1** and **Publication 2**, highlighting the contribution of this methodology to the advancement of this field of molecular diagnostics. This work is presented as **Publication 3** titled "Universal detection and identification of fungi by PCR and DNA sequencing " (131).

Whilst the work from these publications focused on improving the speed and specificity of identification from mycological cultures (**Publication 1**) and further improving the speed of identification by applying this method directly to blood cultures (**Publication 2**), the ultimate tool for the clinician is the detection and identification directly from clinical samples (49, 132). During the

implementation of the ITS sequencing method for the identification of fungal cultures in 2003, there was significant a significant requirement to develop an ITS method that can be applied directly to blood, fluids and tissues for the identification of invasive fungal infections, with the potential for rapid, same-day results. This would allow for earlier clinical intervention to improve patient outcomes, particularly for the critically ill. However, this posed a challenge to the ITS sequencing method in terms of both sensitivity and specificity. The NaOH wash step may result in the loss of fungal cells, reducing assay sensitivity, and the ITS primers might still cross-react with human DNA given the high number of PCR cycles required for optimal sensitivity.

The 18S rRNA regions had previously been used for whole blood which did not show cross-reactivity with human DNA (132-134). As such, a highly sensitive RT-PCR assay targeting the 18S region may generate earlier results than blood culture. For the fourth publication our objectives were:

a) Design novel 18S primers and probes to detect *C. albicans* and *A. fumigatus* DNA.
b) Assess the analytical sensitivity, specificity and reproducibility of the novel 18S primers and probes, including published primers and probes.
c) Based on these results, develop an optimised extraction and RT-PCR method for the detection of *C. albicans* and *A. fumigatus* DNA in whole blood.
d) Evaluate the RT-PCR on whole blood, from patients with proven invasive fungal disease, using standardised consensus criteria for invasive fungal infection.

As an alternative to ITS presented in **Publication 1** and **Publication 2**, I designed new primers and added probes targeting the 18S, based on sequence alignments for *C. albicans* and *A. fumigatus* DNA, which are still common pathogens of fungaemia (135). These new primers and probes were compared to other primers and probes from previously published work (132, 133), and were tested for analytical sensitivity, specificity and reproducibility. A refined RT-PCR method to detect *C. albicans* and *A. fumigatus* DNA in whole blood was developed and tested on whole blood samples from patients with proven invasive fungal infection. This study is presented as **Publication 4** titled "Real-time automated polymerase chain reaction (PCR) to detect *Candida albicans* and *A. fumigatus* DNA in whole blood from high-risk patients" (136). Overall, the ITS sequencing method (**Publication 1 and 2**) is most applicable to broad fungal identification of isolates recovered from culture in the diagnostic setting and is summarised in a book chapter (**Publication 3**), while the 18S rRNA PCR methodology (**Publication 4**) is a more specialised and rapid approach for identifying fungi such as *C. albicans* or *A. fumigatus* that can be used for urgent diagnostic testing.

## 2.2 **Publication 1**. Pryce et al., 2003. Rapid identification of fungi by sequencing the ITS1 and ITS2 regions using an automated capillary electrophoresis system.

**Pryce TM**, Palladino S, Kay ID, Coombs GW. 2003. Rapid identification of fungi by sequencing the ITS1 and ITS2 regions using an automated capillary electrophoresis system. Medical Mycology 41:369-81.

DOI link 10.1080/13693780310001600435

As corresponding first author, I conceptualised the study, methods and procedures. I collated all the samples for the investigation and performed all the laboratory testing and experiments. I wrote the protocol, collected and analysed the results and was the main person drafting the manuscript. All other authors either performed supervisory roles and/or contributed to the writing and editing. All authors reviewed the manuscript prior to publication.

2024 Clarivate journal impact factor from Journal Citation Reports: 1.4

*Medical Mycology* October 2003, **41**, 369–381

Taylor & Francis
healthsciences

# Rapid identification of fungi by sequencing the ITS1 and ITS2 regions using an automated capillary electrophoresis system

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We developed a standardized DNA sequence-based approach for the accurate and timely identification of medically important fungi by sequencing polymerase chain reaction (PCR) products with a rapid automated capillary electrophoresis system. A simple DNA extraction method and PCR amplification using universal fungal primers was used to amplify ribosomal DNA from a range of clinical isolates and reference strains. The entire internal transcribed spacer (ITS) 1-5.8S-ITS2 ribosomal DNA region was sequenced using automated dye termination sequencing for 89 clinical isolates. These had previously been identified by traditional methods and included 12 ascomycetous yeast species, three basidiomycetous yeast species, eight dermatophyte species and two thermally dimorphic fungi, *Scedosporium prolificans* and *S. apiospermum*. Furthermore, 21 reference strains representing 19 different *Candida* species, *Geotrichum candidum* and *Malassezia furfur* were also sequenced as part of this study and were used either as standards for sequence-based comparisons, or as assay controls. Sequence-based identification was compared to traditional identification in a blinded manner. Of the clinical isolates tested, 88/89 had DNA sequences that were highly homologous to those of reference strains accessioned in GenBank, and 87/89 gave a sequence-based identification result that correlated with the traditional identification. In contrast to relatively slow conventional methods of identification, a sequence-based identification from a pure culture can be obtained within 24 h of a DNA extraction carried out after a minimal period of culture growth. We conclude that this approach is rapid, and may be a more accurate cost-effective alternative than most phenotypic methods for identification of many medically important fungi frequently encountered in a routine diagnostic microbiology laboratory.

**Keywords** DNA sequencing, identification, ITS regions

## Introduction

Fungi, all but a few species of which were once considered to be microbiological curiosities, have increasingly emerged as human pathogens capable of causing life-threatening disease, particularly in immunocompromised and other high-risk patient groups [1]. Rapid and accurate identification of fungi is essential for guiding early appropriate therapy. Many clinically important fungi, however, may take weeks to grow in the laboratory [2]. Identification of many fungi can be time consuming and complex, and can require the use of a wide range of specialized laboratory culture media [3,4]. Traditional identification methods for dermatophytes may take many weeks, are labour intensive, may lack specificity, and require experienced personnel to identify less commonly encountered pathogens or

Received 14 January 2003; Accepted 27 January 2003
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DOI: 10.1080/13693780310001600435

variant strains [5]. Traditional methods of reference yeast identification, based on carbohydrate assimilation and fermentation tests, are cumbersome and not suitable for the non-specialized clinical microbiology laboratories [6]. Rapid kits and automated identification systems have been developed but are often unreliable and may take up to 7 days for a final result [7,8].

With the emergence of fungi that are resistant to many of the antifungal drugs available, rapid species-level identification of significant clinical isolates is important, as delays in the initiation of appropriate therapy often correlate with poor outcomes [2,6,9]. It has therefore become essential to have rapid and accurate methods for identification of fungi that can easily be implemented in a routine diagnostic microbiology laboratory. Current diagnostic methods to identify many clinically important fungi combine morphological criteria using identification keys and physiological tests with molecular diagnostics [10]. Molecular methods are increasingly being utilized to aid traditional identification and to study the phylogeny of many clinically important fungi [11–20]. These studies have identified ribosomal DNA (rDNA) sequence information for a variety of fungal species and analysis of this region has been used as the basis for the organization of fungi into taxonomic groups. The intervening internal transcribed spacer (ITS) regions have become important molecular targets for taxonomy and identification [21]. Due to greater sequence variation, the ITS1/ITS2 domains are more suited for species and strain identification than the 18S region (small subunit), the 5.8S region and the 28S region (large subunit) [21]. Several groups have developed methods utilizing the ITS regions to identify species and strains of a range dermatophytes, yeasts and moulds [17,22–26]. Despite the variety of methods described, most have limited applicability for routine use in a clinical laboratory due to the use of tedious DNA extractions and labour-intensive blotting techniques using species-specific digoxigenin or radiolabelled probes. Useful sequence-based approaches have been reported to identify fungi rapidly [27,28] and show great promise. However, most reports identify a limited range of fungi within a genus, use GenBank ITS sequences that are incomplete or use GenBank records that have been derived from a non-referenced culture.

In this report, we describe the development of a fungal identification strategy based on direct sequence analysis of amplified rDNA using an automated capillary electrophoresis system. The ITS1-5.8S-ITS2 region from reference strains and clinical isolates from members of the genus *Candida*, members of the family Arthrodermataceae (dermatophytes) and other medically important fungi were amplified, sequenced and compared with reference strain sequences in GenBank. Sequence-based identification of 89 clinical isolates previously identified by traditional methods was evaluated in a blinded study. The validity of this approach for rapid and accurate identification of a variety of clinically important fungi in routine diagnostic microbiology is reported.

## Materials and methods

### *Fungal strains*

Referenced fungal organisms used as controls for this study were obtained from the American Type Culture Collection (ATCC), Manassas, VA, USA and the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands. Clinical isolates used in this study were obtained from the Royal Perth Hospital Mycology Culture Collection (RPMCC), Department of Microbiology and Infectious Diseases, Royal Perth Hospital, Perth, Australia. All clinical isolates and controls were previously identified by colony characteristics, microscopic morphology and physiological testing, using identification keys [10] and specialized handbooks [29–31]. Where possible, commercially available systems to identify yeasts were also used. All clinical isolates and controls are shown in Table 1. Furthermore, a variety of CBS type strains were sequenced for this study and used as standards for DNA sequence comparisons (Table 2).

### *Identification of ascomycetous and basidiomycetous yeasts*

The germ tube broth was prepared and performed from direct culture for all presumptive yeast isolates. Typical *Candida albicans* isolates, identified by a positive germ tube test, were not evaluated in this study, because presumptive identification of *C. albicans* (in a manner not excluding the uncommonly encountered *C. dubliniensis*) using the germ-tube test is simple and cost-effective. However, germ-tube-positive yeasts isolated from the oral cavity of HIV-positive patients, from bloodstream infections of neutropenic patients and from patients with other immune disorders were further identified to exclude *C. dubliniensis*. A dark-green appearance on CHROMagar Candida (CHROMagar Company, Paris, France), inhibited growth at 45 °C, and the morphological characteristics seen on corn-meal/Tween 80 (CMAT) were used to identify *C. dubliniensis*. Common clinical ascomycetous yeasts (*Saccharomyces cerevisiae*, *Geotrichum candidum*, *C. glabrata*, *C. parapsilosis*, *C. krusei*, *C. tropicalis*, *C. guilliermondii*, *C. kefyr*, *C. famata*) that were germ

**Table 1** Clinical isolates and controls analysed in the study

| Organism and conventional identification* | Strain no. | Source of isolate | rDNA sequence-based identification results† | | |
|---|---|---|---|---|---|
| | | | Sequence-based identification | GenBank accession no. | Reference source |
| Ascomycetous yeasts | | | | | |
| *Candida* species | | | | | |
| *C. albicans* | Control 14053 | ATCC 14053 | *C. albicans* | AF217609 | ATCC 28516 |
| *C. albicans* (germ tube negative) | RPMCC 2031 | Clinical isolate | *C. albicans* | AF217609 | ATCC 28516 |
| *C. dubliniensis* | RPMCC 8357 | Clinical isolate | *C. dubliniensis* | AB049123 | CBS 7987 |
| *C. dubliniensis* | RPMCC 1075 | Clinical isolate | *C. dubliniensis* | AB049123 | CBS 7987 |
| *C. famata* (*Debaryomyces hansenii* var. *hansenii*) | Control 1962 | CBS 1962 | *D. hansenii* | AF210327 | CBS 767 |
| *C. famata* (*D. hansenii*) | RPMCC 8165 | Clinical isolate | *D. hansenii* | AF210326 | CBS 789 |
| *C. glabrata* | Control 2238 | ATCC 2238 | *C. glabrata* | AF167993 | ATCC15545 |
| *C. glabrata* | RPMCC 9700 | Clinical isolate | *C. glabrata* | AF167993 | ATCC15545 |
| *C. glabrata* | RPMCC 9703 | Clinical isolate | *C. glabrata* | AF167993 | ATCC15545 |
| *C. glabrata* | RPMCC 9162 | Clinical isolate | *C. glabrata* | AF167993 | ATCC15545 |
| *C. glabrata* | RPMCC 4635 | Clinical isolate | *C. glabrata* | AF167993 | ATCC15545 |
| *C. glabrata* | RPMCC 6240 | Clinical isolate | *C. glabrata* | AF167993 | ATCC15545 |
| *C. glabrata* | RPMCC 5779 | Clinical isolate | *C. glabrata* | AF167993 | ATCC15545 |
| *C. glabrata* | RPMCC 4759 | Clinical isolate | *C. glabrata* | AF167993 | ATCC15545 |
| *C. glabrata* | RPMCC 0935 | Clinical isolate | *C. glabrata* | AF167993 | ATCC15545 |
| *C. glabrata* | RPMCC 9223 | Clinical isolate | *C. glabrata* | AF167993 | ATCC15545 |
| *C. glabrata* | RPMCC 3217 | Clinical isolate | *C. glabrata* | AF167993 | ATCC15545 |
| *C. glabrata* | RPMCC 1041 | Clinical isolate | *C. glabrata* | AF167993 | ATCC15545 |
| *C. glabrata* | RPMCC 0130 | Clinical isolate | *C. glabrata* | AF167993 | ATCC15545 |
| *C. glabrata* | RPMCC 8378 | Clinical isolate | *C. glabrata* | AF167993 | ATCC15545 |
| *C. glabrata* | RPMCC 5435 | Clinical isolate | *C. glabrata* | AF167993 | ATCC15545 |
| *C. guilliermondii* (*Pichia guilliermondii*) | RPMCC 5150 | Clinical isolate | *P. guilliermondii* | AB054109 | JCM 10735 |
| *C. guilliermondii* (*P. guilliermondii*) | RPMCC 9646 | Clinical isolate | *P. guilliermondii* | AB054109 | JCM 10735 |
| *C. intermedia* | RPMCC 2222 | Clinical isolate | *C. intermedia* | Authors' data | CBS 572 |
| *C. kefyr* (*Kluyveromyces marxianus*) | RPMCC 2951 | Clinical isolate | *K. marxianus* | AJ401699 | CBS 4857 |
| *C. kefyr* (*K. marxianus*) | RPMCC 0901 | Clinical isolate | *K. marxianus* | AJ401699 | CBS 4857 |
| *C. krusei* (*Issatchenkia orientalis*) | Control 6528 | ATCC 6528 | *I. orientalis* | AF246989 | ATCC 6258 |
| *C. krusei* (*I. orientalis*) | RPMCC 1726 | Clinical isolate | *I. orientalis* | AF246989 | ATCC 6258 |
| *C. krusei* (*I. orientalis*) | RPMCC 6136 | Clinical isolate | *I. orientalis* | AF246989 | ATCC 6258 |
| *C. krusei* (*I. orientalis*) | RPMCC 2106 | Clinical isolate | *I. orientalis* | AF246989 | ATCC 6258 |
| *C. krusei* (*I. orientalis*) | RPMCC 1609 | Clinical isolate | *I. orientalis* | AF246989 | ATCC 6258 |
| *C. krusei* (*I. orientalis*) | RPMCC 8980 | Clinical isolate | *I. orientalis* | AF246989 | ATCC 6258 |
| *C. krusei* (*I. orientalis*) | RPMCC 2409 | Clinical isolate | *I. orientalis* | AF246989 | ATCC 6258 |
| *C. parapsilosis* | Control 22019 | ATCC 22019 | *C. parapsilosis* | AF287909 | ATCC 22019 |
| *C. parapsilosis* | RPMCC 3185 | Clinical isolate | *C. parapsilosis* | AF287909 | ATCC 22019 |
| *C. parapsilosis* | RPMCC 2897 | Clinical isolate | *C. parapsilosis* | AF287909 | ATCC 22019 |
| *C. parapsilosis* | RPMCC 8359 | Clinical isolate | *C. parapsilosis* | AF287909 | ATCC 22019 |
| *C. parapsilosis* | RPMCC 7653 | Clinical isolate | *C. parapsilosis* | AF287909 | ATCC 22019 |
| *C. parapsilosis* | RPMCC 5108 | Clinical isolate | *C. parapsilosis* | AF287909 | ATCC 22019 |
| *C. parapsilosis* | RPMCC 8632 | Clinical isolate | *C. parapsilosis* | AF287909 | ATCC 22019 |
| *C. parapsilosis* | RPMCC 2150 | Clinical isolate | *C. parapsilosis* | AF287909 | ATCC 22019 |
| *C. parapsilosis* | RPMCC 9373 | Clinical isolate | *C. parapsilosis* | AF287909 | ATCC 22019 |
| *C. parapsilosis* | RPMCC 0770 | Clinical isolate | *C. parapsilosis* | AF287909 | ATCC 22019 |
| *C. parapsilosis* | RPMCC 0979 | Clinical isolate | *C. parapsilosis* | AF287909 | ATCC 22019 |
| *C. parapsilosis* | RPMCC 1812 | Clinical isolate | *C. parapsilosis* | AF287909 | ATCC 22019 |
| *C. tropicalis* | Control 750 | ATCC 750 | *C. tropicalis* | AF287910 | ATCC 750 |
| *C. tropicalis* | RPMCC 6961 | Clinical isolate | *C. tropicalis* | AF287910 | ATCC 750 |
| *C. tropicalis* | RPMCC 1670 | Clinical isolate | *C. tropicalis* | AF287910 | ATCC 750 |
| *C. tropicalis* | RPMCC 0998 | Clinical isolate | *C. tropicalis* | AF287910 | ATCC 750 |
| *C. tropicalis* | RPMCC 4936 | Clinical isolate | *C. tropicalis* | AF287910 | ATCC 750 |
| *C. tropicalis* | RPMCC 3412 | Clinical isolate | *C. tropicalis* | AF287910 | ATCC 750 |
| *Geotrichum* species | | | | | |
| *G. candidum* (*Galactomyces geotrichum*) | Control 772.71 | CBS 772.71 | *G. geotrichum* | AJ279451 | CBS 121.22 |
| *G. candidum* (*G. geotrichum*) | RPMCC 5416 | Clinical isolate | *G. geotrichum* | AJ279451 | CBS 121.22 |

**Table 1** (*Continued*)

| Organism and conventional identification* | Strain no. | Source of isolate | rDNA sequence-based identification results† | | |
|---|---|---|---|---|---|
| | | | Sequence-based identification | GenBank accession no. | Reference source |
| *Saccharomyces* species | | | | | |
| *S. cerevisiae* | Control 9763 | RPMCC 9763 | *S. cerevisiae* | Z95940 | CBS 4903 |
| *S. cerevisiae* | RPMCC 1490 | Clinical isolate | *S. cerevisiae* | Z95940 | CBS 4903 |
| *S. cerevisiae* | RPMCC 5845 | Clinical isolate | *S. cerevisiae* | Z95940 | CBS 4903 |
| Basidiomycetous yeasts | | | | | |
| *Malassezia* species | | | | | |
| *M. furfur* | Control 1878 | CBS 1878 | *M. furfur* | AF246896 | ATCC 44344 |
| *Malassezia* sp. | RPMCC 9603 | Clinical isolate | *M. furfur* | AF246896 | ATCC 44344 |
| *Malassezia* sp. | RPMCC 4701 | Clinical isolate | *M. furfur* | AF246896 | ATCC 44344 |
| *Malassezia* sp. | RPMCC 5576 | Clinical isolate | *M. furfur* | AF246896 | ATCC 44344 |
| *Malassezia* sp. | RPMCC 5621 | Clinical isolate | *M. furfur* | AF246896 | ATCC 44344 |
| *Trichosporon* species | | | | | |
| *T. asahii* | RPMCC 0065 | Clinical isolate | *T. asahii* | AF444457 | CBS 8520 |
| *T. asahii* | RPMCC 2911 | Clinical isolate | *T. asahii* | AF444457 | CBS 8520 |
| *T. asahii* | RPMCC 8399 | Clinical isolate | *T. asahii* | AF444457 | CBS 8520 |
| *T. inkin* | RPMCC 3804 | Clinical isolate | *T. inkin* | AF444420 | CBS 5585 |
| Dermatophytes | | | | | |
| *Epidermophyton* species | | | | | |
| *E. floccosum* | RPMCC 3409 | Clinical isolate | *E. floccosum* | AJ000629 | CBS 358.93 |
| *E. floccosum* | RPMCC 6580 | Clinical isolate | *E. floccosum* | AJ000629 | CBS 358.93 |
| *E. floccosum* | RPMCC 1320 | Clinical isolate | *E. floccosum* | AJ000629 | CBS 358.93 |
| *Microsporum* species | | | | | |
| *M. canis* (*A. otae*) | RPMCC 3893 | Clinical isolate | *A. otae* | AJ252339 | CBS 495.86 |
| *M. gypseum* (*A. gypseum*) | RPMCC 2631 | Clinical isolate | *A. gypseum* | AF168128 | CBS 170.64 |
| *Trichophyton* species | | | | | |
| *T. rubrum* | RPMCC 5907 | Clinical isolate | *T. rubrum* | AF170472 | ATCC 28188 |
| *T. rubrum* | RPMCC 0263 | Clinical isolate | *T. rubrum* | AF170472 | ATCC 28188 |
| *T. rubrum* | RPMCC 6206 | Clinical isolate | *T. rubrum* | AF170472 | ATCC 28188 |
| *T. rubrum* | RPMCC 6691 | Clinical isolate | *T. rubrum* | AF170472 | ATCC 28188 |
| *T. rubrum* | RPMCC 6205 | Clinical isolate | *T. rubrum* | AF170472 | ATCC 28188 |
| *T. rubrum* | RPMCC 7086 | Clinical isolate | *T. rubrum* | AF170472 | ATCC 28188 |
| *T. rubrum* | RPMCC 4735 | Clinical isolate | *T. rubrum* | AF170472 | ATCC 28188 |
| *T. rubrum* | RPMCC 5918 | Clinical isolate | *T. rubrum* | AF170472 | ATCC 28188 |
| *T. rubrum* | RPMCC 3361 | Clinical isolate | *T. rubrum* | AF170472 | ATCC 28188 |
| *T. rubrum* | RPMCC 1133 | Clinical isolate | *T. rubrum* | AF170472 | ATCC 28188 |
| *T. interdigitale* (*A. vanbreuseghemii*) | RPMCC 4677.1 | Clinical isolate | *A. vanbreuseghemii* | AF170466 | UAMH 8544 |
| *T. interdigitale* (*A. vanbreuseghemii*) | RPMCC 7122 | Clinical isolate | *A. vanbreuseghemii* | AF170466 | UAMH 8544 |
| *T. interdigitale* (*A. vanbreuseghemii*) | RPMCC 6772 | Clinical isolate | *A. vanbreuseghemii* | AF170466 | UAMH 8544 |
| *T. interdigitale* (*A. vanbreuseghemii*) | RPMCC 2939 | Clinical isolate | *C. indicum*‡ | AJ005369 | CBS 117.63 |
| *T. interdigitale* (*A. vanbreuseghemii*) | RPMCC 8529 | Clinical isolate | *A. vanbreuseghemii* | AF170466 | UAMH 8544 |
| *T. interdigitale* (*A. vanbreuseghemii*) | RPMCC 5294 | Clinical isolate | *A. vanbreuseghemii* | AF170466 | UAMH 8544 |
| *T. mentagrophytes* | RPMCC 4677.2 | Clinical isolate | *A. vanbreuseghemii* | AF170466 | UAMH 8544 |
| *T. tonsurans* | RPMCC 0167 | Clinical isolate | *T. tonsurans* | AF170479 | UAMH 8552 |
| *T. violaceum* | RPMCC 5519 | Clinical isolate | *T. violaceum* | AJ270811 | CBS 319.31 |
| Dimorphic fungi | | | | | |
| *Sporothrix* species | | | | | |
| *S. schenckii* (*Ophiostoma*) | RPMCC 1254 | Clinical isolate | *S. schenckii* | AF364061 | ATCC 14284 |
| *S. schenckii* (*Ophiostoma*) | RPMCC 0485 | Clinical isolate | *S. schenckii* | AF364061 | ATCC 14284 |
| *Histoplasma* species | | | | | |
| *H. capsulatum* (*Ajellomyces capsulatus*) | RPMCC 2889 | Clinical isolate | *A. capsulatus* | AF038353 | UAMH 7141 |
| *H. capsulatum* (*A. capsulatus*) | RPMCC 5400 | Clinical isolate | *A. capsulatus* | AF038353 | UAMH 7141 |
| Other fungi | | | | | |
| *Scedosporium* species | | | | | |
| *S. apiospermum* (*Pseudallescheria boydii*) | RPMCC 3039 | Clinical isolate | *P. boydii* | AF022486 | CBS 101.22 |

**Table 1** (*Continued*)

| Organism and conventional identification* | Strain no. | Source of isolate | rDNA sequence-based identification results† | | |
|---|---|---|---|---|---|
| | | | Sequence-based identification | GenBank accession no. | Reference source |
| *S. apiospermum* (*P. boydii*) | RPMCC 3293 | Clinical isolate | *P. boydii* | AF022486 | CBS 101.22 |
| *S. apiospermum* (*P. boydii*) | RPMCC 6397 | Clinical isolate | *P. boydii* | AF022486 | CBS 101.22 |
| *S. prolificans* | RPMCC 2811 | Clinical isolate | *S. prolificans* | AF022484 | CBS 114.90 |
| *S. prolificans* | RPMCC 4297 | Clinical isolate | *S. prolificans* | AF022484 | CBS 114.90 |

ATCC, American Type Culture Collection, Manassas, VA, USA; CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; JCM, Japan Collection of Microorganisms, Institute of Physical and Chemical Research, Wako, Japan; RPMCC, Royal Perth Hospital Mycology Culture Collection, Perth, Australia; UAMH, University of Alberta Microfungus Collection and Herbarium, Edmonton, AB, Canada.
*Teleomorph in parentheses if known.
†Based on the highest bit score (Materials and methods).
‡Confirmed as *Chrysosporium* species by traditional mycological techniques.

tube negative were identified by the Yeast Biochemical Card (YBC) system (BioMérieux Vitek, Inc., Hazelwood, MO, USA). The identification was confirmed by examination of conventional morphological characteristics using CMAT plate culture and CHROMagar. For common clinical yeasts, identification was achieved within 3–4 days of initial isolation. Yeasts not identified using the YBC system were identified within 48 h using the ID 32 C system (BioMérieux, Marcy l'Etoile, France). Clinical basidiomycetous yeasts such as *Trichosporon* species were also identified with the ID 32 C system. Primary isolation and subculture of *Malassezia* spp. was performed using Dixon's agar. These isolates were not identified to the species level due to the lack of reliable methods available in our laboratory.

### *Identification of dermatophytes*

Microscopic morphology was studied on primary isolation media [Sabouraud's glucose agar with chloramphenicol, Casamino acids erythritol albumin agar, Mycosel agar (Difco, Detroit, MI, USA)]. Physiological testing included the following for suspected dermatophytes: growth at 27 °C and 37 °C on Sabouraud's glucose agar; dermatophyte test medium (DTM) was used according to Taplin *et al*. [32]; hydrolysis of urea by production of urease; growth characteristics on Littman oxgall agar, lactritmel agar, Sabouraud's glucose agar with 5% NaCl, 1% peptone agar, bromcresol purple–milk solids–glucose agar (BCP-MS-G) and *Trichophyton* agars (Difco); vitamin-free casamino acids agar (T1); vitamin-free casamino acid agar+inositol (T2); vitamin-free casamino acid agar+inositol+thiamine (T3); vitamin-free casamino acid agar+thiamine (T4); vitamin-free casamino acid agar+nicotinic acid (T5); vitamin-free ammonium nitrate agar (T6); vitamin-free ammonium nitrate agar+L-histidine (T7). Other tests that assisted in the identification of the dermatophytes tested included growth on boiled polished rice grains and examination of reverse pigment on potato glucose agar. Tests were incubated at 27 °C and incubated up to 4 weeks in an air incubator. For common clinical dermatophytes, identification was achieved within 2 weeks of the initial isolation.

### *Identification of other fungi*

Other filamentous fungi in this study were identified using traditional mycological methods using specialized mycological techniques, handbooks and identification keys based on colony characteristics, microscopic morphology and physiological testing [10,29]. In most cases, identification was achieved within 2 weeks of the initial isolation.

### *Culture preparation and DNA isolation*

A simple and universal DNA isolation method was used for all fungi tested. Fungi used in this study were subcultured onto routine culture media (Sabouraud's glucose agar or Dixon's agar) and incubated at 30 °C until the earliest visible signs of growth were noted. A small amount (approximately 1–2 $mm^2$) of fungal mycelial mass or yeast colony was removed and emulsified in 100 µl of extraction buffer (10 mm Tris–HCl [pH 8.0], 10 U of partially purified lyticase [Sigma, Castle Hill, NSW, Australia]) in a 1.5-ml microfuge tube. The tubes were incubated at 37 °C for 45 min then vortexed for 30 s. Chelex 100 (Bio-Rad Laboratories, Hercules, CA, USA) was added to each tube to a final concentration of 10% (w/v). All tubes were heated to 95 °C in a heating block for 10 min then allowed to cool before centrifugation for 2 min at $13000 \times g$. Samples were used immediately for polymerase chain reaction (PCR) or stored at −70 °C until use. *S.*

**Table 2** Medically important fungi used as reference strains in this study

| Organism | GenBank accession no.(s)* | Strain(s)† |
|---|---|---|
| Ascomycetous yeasts | | |
| *C. albicans* | AF217609 | ATCC 28516 |
| *C. catenulata* | Authors' data | CBS 565 |
| *C. chiropterorum* | Authors' data | CBS 6064 |
| *C. ciferrii* (*Stephanoascus ciferrii*) | Authors' data | CBS 5295 |
| *C. dubliniensis* | AB049123, AB035590 | CBS 7987, CBS 7988 |
| *C. famata* (*D. hansenii*) | AF210326, AF210327 | CBS 789, CBS 767 |
| *C. glabrata* | AF167993 | ATCC 15545 |
| *C. guilliermondii* (*P. guilliermondii*) | AB054109 | JCM 10735 |
| *C. haemulonii* | Authors' data | CBS 5149 |
| *C. intermedia* | Authors' data | CBS 572 |
| *C. kefyr* (*K. marxianus*) | AJ401699 | CBS 4857 |
| *C. krusei* (*I. orientalis*) | AF246989 | ATCC 6258 |
| *C. lipolytica* (*Yarrowia lipolytica*) | Authors' data | CBS 6124 |
| *C. lusitaniae* (*Clavispora lusitaniae*) | Authors' data | CBS 1944 |
| *C. norvegensis* (*Pichia norvegensis*) | Authors' data | CBS 6564 |
| *C. parapsilosis* | AF287909 | ATCC 22019 |
| *C. pelliculosa* (*Pichia anomala*) | Authors' data | CBS 110 |
| *C. rugosa* | Authors' data | CBS 613 |
| *C. tropicalis* | AF287910 | ATCC 750 |
| *C. utilis* (*Pichia jadinii*) | Authors' data | CBS 621 |
| *C. viswanathii* | Authors' data | CBS 4024 |
| *C. zeylanoides* | Authors' data | CBS 619 |
| *G. candidum* (*G. geotrichum*) | AJ279451 | CBS 121.22 |
| *S. cerevisiae* | Z95940 | CBS 4903 |
| Basidiomycetous yeasts | | |
| *M. furfur* | AF246896 | ATCC 44344 |
| *T. asahii* | AF444457 | CBS 8520 |
| *T. inkin* | AF444420 | CBS 5585 |
| Dermatophytes | | |
| *E. floccosum* | AJ000629 | CBS 358.93 |
| *M. audouinii* | AJ252333, AJ252332 | CBS 344.50, CBS 317.51 |
| *M. canis* (*A. otae*) | AJ252339 | CBS 495.86 |
| *M. ferrugineum* | AJ252335 | CBS 457.80 |
| *M. gypseum* (*A. gypseum*) | AF168128 | CBS 170.64 |
| *T. concentricum* | Z98012 | CBS 196.26 |
| *T. erinacei* (*Arthroderma benhamiae*) | Z97996 | CBS 344.79 |
| *T. interdigitale* (*A. vanbreuseghemii*) | Z98001, AF170466 | CBS 558.66, UAMH 8544 |
| *T. mentagrophytes* | Z97995 | CBS 318.56 |
| *T. rubrum* | Z97993, AF170472 | CBS 392.58, ATCC 28188 |
| *T. schoenleinii* | Z98011 | CBS 855.71 |
| *T. simii* (*Arthroderma simii*) | Z98017 | CBS 417.65 |
| *T. tonsurans* | Z98009 | CBS 292.81 |
| *T. vanbreuseghemii* (*Arthroderma gertleri*) | Z98013 | CBS 598.66 |
| *T. verrucosum* | Z98003 | CBS 134.66 |
| *T. violaceum* | AJ270811 | CBS 319.31 |
| Other fungi | | |
| *S. schenckii* (*Ophiostoma*) | AF364061 | ATCC 14284 |
| *H. capsulatum* (*A. capsulatus*) | AF038353, AF038354 | UAMH 7141, UAMH 3536 |
| *S. apiospermum* (*P. boydii*) | AF022486 | CBS 101.22 |
| *S. prolificans* | AF022484 | CBS 114.90 |
| *C. indicum* | AJ005369 | CBS 117.63 |

ATCC, American Type Culture Collection, Manassas, VA, USA; JCM, Japan Collection of Microorganisms, Institute of Physical and Chemical Research, Wako, Japan; CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; WB, Laboratory for Mycology and Molecular Biology, ENT-University Hospital, Graz, Austria; UAMH, University of Alberta Microfungus Collection and Herbarium, Edmonton, AB, Canada.

Teleomorph in parentheses.

*GenBank accession numbers for rDNA sequences used as references.

†Culture collections and strain numbers of sequences reported in GenBank, respectively.

*cerevisiae* (RPMCC 9763) was used as a positive control for DNA isolation, PCR and DNA sequencing for each experiment.

### *Primers*

The primers used for universal fungal DNA amplification from all isolates were V9D, 5′-TTA AGT CCC TGC CCT TTG TA-3′ [33], and LS266, 5′-GCA TTC CCA AAC AAC TCG ACT C-3′ [34]. These primers bind to conserved regions, with corresponding positions to *S. serevisiae* 18S (1609–1627) and 26S (287–266) rRNA genes, and amplify a product that encompasses a portion of the 18S and 26S rRNA gene and the entire intervening ITS1, 5.8S and ITS2 rRNA gene regions. The size of the product generated varies according to the organism tested. Primers used for direct sequencing were ITS1; 5′-TCC GTA GGT GAA CCT GCG G-3′ (position corresponding to *S. serevisiae* small subunit 1769–1787), or both ITS1 and ITS4; 5′-TCC TCC GCT TAT TGA TAT GC-3′ (position corresponding to *S. cerevisiae* large subunit 41–60) [20]. All primers were synthesized by Gibco BRL, Life Technologies, Melbourne, Australia.

### *PCR amplification*

The PCR assay was performed with 5 μl of DNA template in a total reaction volume of 50 μl. The PCR reaction mixture contained 5 μl of 10 × reaction buffer (Applied Biosystems, Foster City, CA, USA); 3 μl of 25 mm $MgCl_2$; 1.5 μl of 20 μm of each oligonucleotide; 200 μm of each deoxynucleoside triphosphate; dATP, dGTP, dCTP, dTTP; 2.25 U of AmpliTaq Gold (Applied Biosystems); and 33 μl of sterile distilled $H_2O$. The PCR was performed in a DNA Engine, PTC-200 Peltier Thermal Cycler (MJ Research Inc., Watertown, MA, USA) with the following program; 95 °C for 9 min initial inactivation step followed by 95 °C for 30 s, 62 °C for 60 s, 72 for 2 min for 33 cycles, and then the mixture was incubated at 72 °C for 5 min for final extension.

### *Agarose gel electrophoresis*

Detection of PCR-amplified product was performed by electrophoresis on a 2% (w/v) agarose gel stained with ethidium bromide. A volume of 5 μl of PCR-amplified product and 1 μl of Gel Loading Solution (Sigma) was loaded into each lane. A volume of 2 μl of a 50–2000-bp molecular weight marker (AmpliSize Molecular Ruler, Bio-Rad) was run in parallel to approximate PCR-amplified product size. Amplified DNA from *S. cerevisiae* (RPMCC 9763) was used as a control.

### *DNA sequencing and editing*

All PCR-amplified products were sequenced at the West Australian Genome Resource Centre at Royal Perth Hospital by automated dye termination sequencing. Each PCR-amplified product was purified with UltraClean PCR Clean-up Kit (MO BIO Laboratories, Carlsbad, CA, USA) and sequenced using a 16-capillary 3100 Genetic Analyzer (Applied Biosystems). The ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit Version 3 (Applied Biosystems) was used with protocols supplied by the manufacturer. PCR-amplified products from clinical isolates were directly sequenced using the ITS1 primer. PCR-amplified products from referenced cultures were sequenced in a forward and reverse direction using ITS1 primer and the ITS4 primer respectively. Sequences were visualized and edited using Chromas Version 1.45; Technelysium Pty. Ltd. [http://www.technelysium.com.au/chromas.html]) or SEQSCAPE Version 1.1 (Applied Biosystems).

### *GenBank search*

Sequence search was performed using the BLAST standard nucleotide-nucleotide basic local alignment search tool [National Center for Biotechnology Information (NCBI), Library of Medicine, Bethesda, MD, USA (http://www.ncbi.nlm.nih.gov/BLAST/)]. All GenBank, EMBL, DDBJ, PDB sequences (but no EST, STS, GSS, or phase 0, 1 or 2 HTGS sequences) were searched with the expectation frequency minimized at 0.0001. Sequences were filtered for low complexity.

### *Clinical isolate identification study*

Eighty-nine clinical isolates previously identified by traditional mycological techniques were assigned unique laboratory identification numbers prior to DNA extraction (Table 1). The universal DNA extraction was performed as described earlier, and the PCR products were sequenced by personnel from a remote laboratory in a blinded manner. Sequence editing and analysis to determine the sequence-based identification was performed by a second person blinded to the traditional identification results. Where possible, sequence-based identification was determined from the entry with the highest bit score listed in the BLAST search with an expect threshold closest to zero that fulfilled the following additional criteria: (i) the sequence included the entire ITS1-5.8S-ITS2 region; (ii) the sequence was derived from a referenced culture; (ii) the nomenclature ascribed to the referenced culture was valid. The

GenBank records of referenced cultures obtained in this manner are shown in Table 2. A few clinical isolates could not be identified using this approach because the organism identification obtained from a BLAST search did not meet all the criteria described. These identifications were considered a preliminary result until confirmed by the following process. A well characterized reference culture of current nomenclature representing the preliminary species identification was sequenced using ITS1 and ITS4 primers and an accurate consensus sequence was generated using SEQSCAPE. Alignment of this sequence with the test sequence was performed using the BLAST 2 alignment tool (http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html) using identical parameters as for the initial BLAST search. Greater than 98% homology with the referenced culture was required to confirm the preliminary identification of the test sequence. A more detailed alignment to confirm this result was performed using the GeneDoc Multiple Sequence Alignment Editor and Shading Utility Version 2.6.001, Pittsburgh Supercomputing Center (PSC), Carnegie Mellon University, University of Pittsburgh, Pittsburgh, PA, USA [35]. Sequences from other fungi were also included to cover a range of medically important fungi that may be encountered in a clinical microbiology laboratory (Table 2). Furthermore, entire target sequences from certain medically important fungal reference strains could not be found in GenBank prior to this study. Hence, to improve the range of fungi able to be identified using this approach, sequences from 13 reference strains of medically important fungi not reported in GenBank at the time of this study were sequenced (Table 2).

## Results

### *Sequence-based identification and correlation with phenotypic identification*

Among the 89 clinical isolates, 87 had a sequence-based identification result that correlated with conventional phenotypic identification (Table 1). Furthermore, nine control strains sequenced in this study also had a sequence-based identification that correlated with their known identification (Table 1). Forty-eight clinical ascomycetous yeasts identified phenotypically as germ-tube-negative *C. albicans* ($n = 1$), *C. dubliniensis* ($n = 2$), *C. famata* ($n = 1$), *C. glabrata* ($n = 14$), *C. guilliermondii* ($n = 2$), *C. intermedia* ($n = 1$), *C. kefyr* ($n = 2$), *C. krusei* ($n = 6$), *C. parapsilosis* ($n = 11$), *C. tropicalis* ($n = 5$), *S. cerevisiae* ($n = 2$), and *G. candidum* ($n = 1$) were reliably identified to the species level using sequence-based identification. The sequence-based identification also correlated with routine identification for the basidiomycetous yeasts identified as *Trichosporon asahii* ($n = 3$) and *T. inkin* ($n = 1$). *Malassezia* spp. ($n = 4$) were not identified to the species level using recently developed physiological methods; all had sequenced-based identifications of *M. furfur*. Twenty-two clinical dermatophyte isolates previously identified as *Epidermophyton floccosum* ($n = 3$), *Microsporum canis* ($n = 1$), *M. gypseum* ($n = 1$), *Trichophyton rubrum* ($n = 10$), *T. interdigitale* ($n = 5$), *T. tonsurans* ($n = 1$) and *T. violaceum* ($n = 1$) were also reliably identified to the species level using sequence-based identification, as were dimorphic fungi identified by routine methods as *Sporothrix schenckii* ($n = 2$), *Histoplasma capsulatum* ($n = 2$). *Scedosporium apiospermum* ($n = 3$) and *S. prolificans* ($n = 2$) were also recognized by sequencing.

Two discordant identification results were observed between the phenotypic identification and the sequence-based identification. One isolate identified phenotypically as *T. interdigitale* had a 99% homology (591/592 bp) with a GenBank sequence from a referenced *Chrysosporium indicum* culture, CBS 117.63. In contrast, no significant alignments were possible ($< 50\%$ homology) when compared to the reference sequence from *T. interdigitale* strain CBS 558.66 using BLAST 2. The isolate produced a pH change on DTM, demonstrated a moderate alkaline change on BCP-MS-G, and grew well at 37 °C. Small, slender, clavate, smooth-walled conidia were seen; however, the presence of small numbers of cymbiform (boat-shaped) conidia were initially overlooked, and the absence of small numbers of macroconidia after 7 days of incubation was not considered unusual for some strains of *T. interdigitale*. Repeat microscopy and culture for 14 days revealed moderate numbers of cymbiform conidia and the absence of macroconidia, as well as a stronger alkaline reaction on BCP-MS-G agar. This isolate was subsequently confirmed as belonging to the genus *Chrysosporium* and the initial identification of *T. interdigitale* was amended to the sequence-based identification of *C. indicum*. The second discordant result was an isolate identified phenotypically as *T. mentagrophytes* (RPMCC 4677.2). This isolate shared a 99% sequence homology (669/676 bp) with the reference sequence from the teleomorph *Arthroderma vanbreuseghemii* UAMH 8544 (University of Alberta Microfungus Collection, Edmonton, AB, Canada), and a 99% sequence homology (657/664 bp) with the reference sequence from the anamorph *T. interdigitale* CBS 558.66. In both cases, a 7-bp insertion in the test sequence was responsible for the difference. In contrast, the same clinical isolate

shared a 96% sequence homology (641/667 bp) with the reference sequence from *T. mentagrophytes sensu stricto*, CBS 318.56. This included a 6-bp insertion and an additional 20 base changes comprising of 14 substitutions, three insertions and three deletions (sequence data not shown). The original laboratory phenotypic identification was reviewed and the organism was found to be granular in appearance and the microscopy was consistent with the recently refined *sensu stricto* concept of *T. mentagrophytes* except for the absence of favic chandelier-like structures. However, these structures are only found in some strains. There were no additional definitive phenotypic characteristics to aid identification and the initial identification of *T. mentagrophytes* had been made on the basis of colony morphology and microscopy. However, this isolate was co-isolated with a downy *T. interdigitale* also identified in this study (RPMCC 4677.1) from a chronic low-grade tinea pedis infection from a city-dweller. Sequence alignment and comparison of ITS sequences from the two isolates showed that they were identical. Furthermore, there was no supporting clinical evidence of animal contact. Based on the clinical evidence, co-isolation with a typical *T. interdigitale* and the sequence-based result, we conclude that the isolate is most likely to be a granular *T. interdigitale*.

### *Cost and time comparisons*

For yeasts other than *C. albicans*, the average total cost in Australian dollars of performing conventional identification was $20.07 per isolate. This comprised reagents ($13.75) and labour ($6.32). Time taken for a final identification was 3–4 days for common yeasts and up to 11 days for less commonly encountered yeasts. The average total cost of identification of dermatophytes, in Australian dollars was $25.01 per isolate. This comprised reagents ($10.25) and labour ($14.76). Time taken for a final identification of these organisms ranged from 14 to 21 days. Cost and time comparisons for the identification of other filamentous fungi have not been reported, as labour and consumable costs vary significantly depending on the organism.

Based on identifying a batch of six isolates, the total cost in Australian dollars of sequence-based identification, which includes the initial amplification step plus the sequencing reaction, averaged $16.66 per isolate. This comprised reagents ($10.12) and labour ($6.54). A final identification was usually obtained within 48 h of obtaining a pure isolate, but could be available within 24 h if required.

## Discussion

This present work is based on the premise that more rapid methods of identifying fungi are required, particularly with the increase in morbidity and mortality associated with fungal infections caused by resistant fungi [9]. Many traditional methods of fungal identification are slow, based on colony characteristics, microscopic morphology and physiological tests. In most cases, confident identification of fungi to the species level requires specialized staff trained in the use of mycological identification keys and culture techniques.

Commercial yeast identification systems have provided laboratories with a reasonably accurate and cost-effective means to identify clinical isolates. However, some systems have proven to be unreliable and they may be restricted to common clinical isolates [7,8]. Some may lack the ability to identify a number of species altogether due to incomplete biochemical databases [36]. The performance of commercial systems may also vary according to inoculum and geographic isolate source [6].

Classical parameters for the identification of dermatophytes and other filamentous fungi are broad, and include conidial morphology, cultural characteristics, physiological tests and clinical features. Identification of dermatophytes is time-consuming and requires extensive familiarity with the microscopic and cultural characteristics of these taxa [5]. Furthermore, dermatophyte identification can be difficult due the variety of species names ascribed for the same organism. In contrast to the rapid commercial systems available for yeasts, identification of dermatophytes and other filamentous fungi are often based solely on phenotypic methods. Reliable identification to the species level may often take many weeks. As a strategy to overcome these limitations, we have developed a sequenced-based approach for the identification of slow-growing fungi from the early stages of growth.

Common molecular targets for rapid methods identification include the ribosomal small subunit (SSU) and large subunit (LSU) rDNA regions. Traditionally, these regions have been used to study phylogenetic relationships, because they evolve slowly and are relatively conserved among fungi [20]. However, these regions subtend relatively large DNA sequences and may lack sufficient heterogeneity for species identification. This has led to the increased use of the ITS regions as targets for identifying fungi to the species level. Recent molecular methods using the ITS regions as targets to separate and identify fungal species have been reviewed, demonstrating that the ITS regions offer

a powerful tool for the identification and typing of fungi [21]. Most studies have shown that sufficient variation exists within the ITS regions to allow for species identification [21]. The ITS regions have been used as targets to investigate the validity of dermatophyte taxonomy [14,15,18], and to determine the phylogenetic relationships among other fungal species [11,16,19,22]. Some of the cited studies (and others) have provided ITS sequence information for a variety of fungi and are available in GenBank for analysis. However, until recently the use of direct sequence analysis for diagnostic purposes has been limited due to the lack of automated high-throughput DNA sequencing instruments and high running costs.

Current molecular methods utilizing the ITS regions for the identification of fungi include the use of genus- or species-specific primers and probes, restriction fragment length polymorphism of amplified DNA or direct sequence analysis of amplified DNA. These methods are commonly used to identify and type many fungi [21]. Molecular methods using species-specific primers have been used to rapidly identify *T. asahii* and *Paracoccidioides brasiliensis* [26,37,38]. The major disadvantage with species-specific PCR assays is that multiple assays would need to be implemented to cover a range of medically important fungi encountered in a clinical microbiology laboratory. In contrast, methods used for the rapid identification of one or more fungal pathogens often involve the use of universal primers combined with genus- or species-specific probes to identify or detect a range of fungal pathogens in cultures or clinical samples [24,25,39,40]. Although these approaches have the ability to differentiate fungal species, some probes may lack specificity [24]. These approaches are more suited for diagnostic use, particularly in clinical samples, because more than one pathogen may be detected. However, the use of probes to identify a range of fungi is problematic due to the need to develop species-specific probes that have similar properties for DNA hybridization. That is, probes used for species identification must be free of secondary structure, must have appropriate melting temperatures and must not cross-react with other species. To develop a panel of probes with these characteristics to accurately identify many different fungal species is challenging and would involve considerable development cost. Recently, a PCR-enzyme immunoassay (EIA) technique was developed that used streptavidin-coated 96-well microtitre plates, a universal capture probe and eight species-specific digoxigenin probes to identify yeast-like fungal pathogens [25]. Such methods are well suited to clinical laboratory use because of the rapid and convenient EIA detection format. However, probe design, high development costs and a small number of species identified are still the limiting factors for the development and implementation of routine PCR-EIA systems to identify a broad-range of fungal pathogens in a clinical microbiology laboratory.

Another disadvantage of some molecular approaches to identify fungi is the use of tedious methods of DNA isolation. Methods include freezing and grinding mycelium, detergent lysis, extraction with solvents and precipitation with alcohol [17]. Others use commercial kits [25] or a combination of detergent lysis with a commercial kit [27]. Overall, commercial methods of DNA extraction decrease the time required for DNA isolation and are well suited for diagnostic use. However, commercial methods have a relatively high unit cost per purification. Overall, the rapid cost-effective DNA extraction method ( $< 1$ h) described in this study performed well, with sufficient DNA present in each tube to amplify a single PCR product from all clinical isolates and controls. The band intensity of each amplification was relatively uniform and slight variations in PCR product concentrations had minimal or no effect on the DNA sequencing result. Furthermore, non-specific PCR products were not amplified from any media or the lyticase lysing enzyme (data not shown).

In the methodology described in this study, we refer to sequences from referenced cultures in GenBank as standards for identification of fungi. In a similar approach, medically important *Aspergillus* species were identified to the species level when sequences from test isolates were compared to sequences in GenBank using BLAST [27]. An evaluation of a blind clinical study was performed and 11 clinical isolates could be correctly identified to the species level. The evaluators concluded that both the ITS1 and ITS2, combined with the highest BLAST bit score, were required for the accurate identification of *Aspergillus* species. The method showed promise for rapid identification and earlier initiation of appropriate therapy for the treatment of invasive aspergillosis.

In our approach, a sequence with the highest bit score and with the expectation frequency value closest to zero that was derived from a referenced culture was considered a match. The selection of DNA sequences (GenBank records or a reference cultures sequenced as part of this study) to be used as standards for sequence-based identification was based on the following criteria: (i) the sequence should be complete, i.e. it should represent the whole region of interest and not just a part thereof; (ii) all sequences used should be derived from cultures obtained from reference collections,

preferably cultures nomenclaturally designated as ex-type (the fungal equivalent to bacteriological-type strains) or, failing that, cultures identified by use of the most stringent level of traditional mycological techniques based on authoritative monographs and up-to-date literature in an upper level reference laboratory and; (iii) to the best of the investigator's knowledge, each sequence should have been designated with a species name that was nomenclaturally valid and currently recognized as correct at the time of this study. Following these criteria we were able to overcome some of the major limitations concerning the use of sequences in GenBank for organism identification. These include the use of incomplete sequences or sequences derived from non-referenced [27] or even misidentified cultures. The development of a reliable database of entire ITS1-5.8S-ITS2 sequences from a collection of reference cultures may further overcome these limitations. Such databases are currently being developed for the identification of bacteria, fungi and mycobacteria [41].

Using a list of selected GenBank records and sequences derived from referenced cultures, each of the fungi tested in this study could be identified to the species level. We observed two discordant results between the phenotypic identification and the sequenced-based identification, as detailed above. Our discovery that a granular-textured dermatophyte isolate most closely resembling *T. mentagrophytes ss. str.* phenotypically had a sequence-based identification of *T. interdigitale* (*A. vanbreuseghemii*) was of particular interest. It is well known that these two species are physiologically indistinguishable and demonstrate variable cultural and morphological differences. Furthermore, granular strains corresponding to the current molecular concept of *T. interdigitale* are relatively well known, e.g. as mating type strains of *A. vanbreuseghemii* [R.C. Summerbell, personal communication]. Clinical differences do occur, and *T. mentagrophytes ss. str.* is predominantly a zoophilic species capable of causing human infection, whereas *T. interdigitale*, as seen in the clinical but not the veterinary laboratory, is predominantly anthrophilic. In this particular case, animal contact was unlikely and the clinical presentation was more supportive of a chronic low-grade tinea pedis infection. In each case, the discordant identification was resolved in favour of the sequencing result, highlighting the usefulness of this approach, particularly in cases when the phenotypic characteristics show variability and are not sufficiently conclusive to allow a definitive identification.

Our approach has a relatively high set-up cost; however, significant savings in variable costs have been achieved. Although the long-term financial advantages are yet to be assessed, the impact of an earlier and more definitive diagnosis may improve the clinical outcome for some patients. For example, a more rapid result may lead to the earlier initiation of therapy, or the sequence-based identification may alter the therapeutic management. This may occur when the clinical diagnosis remains uncertain, or when the laboratory has isolated a fungus of an unknown clinical significance. Additionally, the time taken for a final result ranged from 3–21 days for phenotypic identification, compared to 24–48 h for sequence-based identification from the first visible signs of growth. Carry-over of small amounts of growth medium does not affect the final result. Colonies of this size are usually sterile and, in microscopy, can only be used to make elementary distinctions such as aseptate mucoralean zygomycetous vs. septate hyphae. Morphological features may take a further 3–5 days to develop before a preliminary identification is possible. Final species identification may take an additional 7 days using traditional mycological techniques. Rapid sequence-based identification from single colony at the earliest signs of growth may be useful for guiding early appropriate therapy. However, several issues may effect the time required to obtain a result, including the availability of a DNA sequencer and repeat sequencing due to poor signal strengths. Repeating the DNA extraction and sequencing for a second time delays the final result only by 48 h. This differs from repeating or adding confirmatory phenotypic tests, a procedure that may require an additional 7 days. Finally, the total cost of a sequence-based identification and the time taken for a final identification remained constant and did not vary from one organism to the next, compared to significant variations in cost and time for phenotypic methods.

At the present time, direct DNA sequencing of ITS1-5.8S-ITS2 rDNA to identify fungi may only be suitable for larger reference laboratories. However, the development of compact DNA sequencers, or the use of commercial DNA sequencing services, may allow other laboratories with access to a thermal-cycler, to utilize this approach. Additional isolates representing the other fungal species not encountered in this study are needed to further evaluate this approach, as relatively few species have as yet had their ITS regions evaluated and some fungi may not show sufficient ITS sequence variation for reliable species separation. In some cases, re-evaluation of the phenotypic positioning or the proposed revision of morphological-based taxonomic schemes of some fungal species may be required. Whether or not ITS sequences will provide the ultimate sequence-based reference method needs to be estab-

lished [21]. More reference sequences are being added constantly and will eventually constitute a very large collection. We are currently evaluating an in-house sequence database for future implementation as a diagnostic tool for rapid fungal identification. A large blinded clinical evaluation ( > 1000 isolates) is currently under investigation to assess more accurately the utility of DNA sequencing to identify a broader range of medically important fungi in a diagnostic clinical microbiology laboratory.

## Acknowledgements

We thank David Sayer, Linda Smith and the staff at the West Australian Genome Resource Centre at Royal Perth Hospital for their expertise, technical assistance, and performing the sequencing reactions for this study. We also thank Erica Loke and Dianne Price for their valuable technical assistance.

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## 2.3 **Publication 2**. Pryce et al., 2006. Rapid identification of fungal pathogens in BacT/ALERT, BACTEC, and BBL MGIT media using polymerase chain reaction and DNA sequencing of the internal transcribed spacer regions.

**Pryce TM**, Palladino S, Price DM, Gardam DJ, Campbell PB, Christiansen KJ, Murray RJ. 2006. Rapid identification of fungal pathogens in BacT/ALERT, BACTEC, and BBL MGIT media using polymerase chain reaction and DNA sequencing of the internal transcribed spacer regions. Diagnostic Microbiology and Infectious Disease 54:289-97.

DOI link 10.1016/j.diagmicrobio.2005.11.002

As corresponding first author, I conceptualised the study, methods and procedures. I collated all the samples for the investigation and performed all the laboratory testing and experiments. I wrote the protocol, collected and analysed the results and was the main person drafting the manuscript. All other authors either performed supervisory roles, provided clinical information and/or contributed to the writing and editing. All authors reviewed the manuscript prior to publication.

2024 Clarivate journal impact factor from Journal Citation Reports: 2.1

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Diagnostic Microbiology and Infectious Disease 54 (2006) 289–297

DIAGNOSTIC MICROBIOLOGY AND INFECTIOUS DISEASE

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# Rapid identification of fungal pathogens in BacT/ALERT, BACTEC, and BBL MGIT media using polymerase chain reaction and DNA sequencing of the internal transcribed spacer regions

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Received 7 September 2005; revised 3 November 2005; accepted 7 November 2005

**Abstract**

We report a direct polymerase chain reaction/sequence (d-PCRS)-based method for the rapid identification of clinically significant fungi from 5 different types of commercial broth enrichment media inoculated with clinical specimens. Media including BacT/ALERT FA (BioMérieux, Marcy l'Etoile, France) ($n$ = 87), BACTEC Plus Aerobic/F (Becton Dickinson, Microbiology Systems, Sparks, MD) ($n$ = 16), BACTEC Peds Plus/F (Becton Dickinson) ($n$ = 15), BACTEC Lytic/10 Anaerobic/F (Becton Dickinson) ($n$ = 11) bottles, and BBL MGIT (Becton Dickinson) ($n$ = 11) were inoculated with specimens from 138 patients. A universal DNA extraction method was used combining a novel pretreatment step to remove PCR inhibitors with a column-based DNA extraction kit. Target sequences in the noncoding internal transcribed spacer regions of the rRNA gene were amplified by PCR and sequenced using a rapid (24 h) automated capillary electrophoresis system. Using sequence alignment software, fungi were identified by sequence similarity with sequences derived from isolates identified by upper-level reference laboratories or isolates defined as ex-type strains. We identified *Candida albicans* ($n$ = 14), *Candida parapsilosis* ($n$ = 8), *Candida glabrata* ($n$ = 7), *Candida krusei* ($n$ = 2), *Scedosporium prolificans* ($n$ = 4), and 1 each of *Candida orthopsilosis*, *Candida dubliniensis*, *Candida kefyr*, *Candida tropicalis*, *Candida guilliermondii*, *Saccharomyces cerevisiae*, *Cryptococcus neoformans*, *Aspergillus fumigatus*, *Histoplasma capsulatum*, and *Malassezia pachydermatis* by d-PCRS analysis. All d-PCRS identifications from positive broths were in agreement with the final species identification of the isolates grown from subculture. Earlier identification of fungi using d-PCRS may facilitate prompt and more appropriate antifungal therapy.


*Keywords:* Fungi; Identification; Blood cultures; PCR; DNA sequencing; Capillary electrophoresis; Internal transcribed spacer regions; ITS; *Candida*; BacT/ALERT; BACTEC; BBL MGIT

## 1. Introduction

The incidence of invasive fungal infections (IFIs) has increased significantly in the past decade as a direct consequence of increasing patient populations at risk for developing serious fungal infections (Hajjeh et al., 2004; Walsh et al., 2004). Opportunistic fungi other than *Candida albicans* and *Aspergillus fumigatus* are being reported with increased frequency from blood stream infections (BSIs) and IFIs (Pfaller and Diekema, 2004). These include non-*albicans Candida* species, such as *Candida glabrata*, *Candida parapsilosis*, *Candida tropicalis*, and *Candida krusei*, which account for more than 50% of all BSIs caused by *Candida* (Rangel-Frausto et al., 1999). Other fungi increasingly encountered include opportunistic yeast-like fungi such as *Trichosporon* spp., *Rhodotorula* spp., *Geotrichum capitatum*, and filamentous fungi such as *Scedosporium* spp., *Fusarium* spp., *Acremonium* spp., and Mucorales (Pfaller and Diekema, 2004; Walsh et al., 2004). In addition, certain species of fungi are associated with a high degree of mor-

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doi:10.1016/j.diagmicrobio.2005.11.002

tality, and some are inherently less susceptible to standard antifungal therapy (Pfaller and Diekema, 2004). The rapid and reliable detection and subsequent identification of fungi from blood and other important clinical specimens remain critical in deciding whether to initiate antifungal therapy and in the choice of agent used.

Continuously monitored automated blood culture systems have improved the detection and isolation of fungi from patients with BSIs compared with manual methods of culture (Nolte et al., 1993; O'Hara et al., 2003). Furthermore, inoculation of blood culture media with other types of fluid or tissue samples has been widely implemented in clinical microbiology laboratories to improve recovery of some fungi (Thomson and Miller, 2003). Regardless of the type of sample and system used, the laboratory handling of positive cultures is usually the same; the presence of fungi is confirmed by microscopy and the broth is subcultured onto solid mycological media for phenotypic identification. However, many fungi are slow growing, and current methods of fungal identification are labor-intensive, may lack specificity, and require a wide range of specialized laboratory media and specialized trained staff (Pfaller and Fromtling, 2003).

Numerous molecular methods have been developed to rapidly identify fungi from solid media (Luo and Mitchell, 2002; Shin et al., 1999) and directly from positive blood culture fluids (Borst et al., 2001; Chang et al., 2001; Li et al., 2003; Shin et al., 1997). Polymerase chain reaction (PCR) methods for identifying fungi directly from blood cultures usually target a few species within the genus *Candida*. Molecular targets for PCR amplification and detection are usually conserved nucleotide sequences within phylogenetically informative genetic regions such as the rRNA gene complex. Ribosomal RNA genes are also targets for sequence-based identification of fungi (Chen et al., 2001; Hall et al., 2003, 2004; Henry et al., 2000; Pryce et al., 2003). PCR amplification using universal primers targeted to conserved regions within the rRNA complex, followed by DNA sequencing of the internal transcribed spacer (ITS) regions, shows promise to identify a broad range of fungi to the species level (Chen et al., 2001; Henry et al., 2000; Iwen et al., 2002; Pryce et al., 2003).

We previously reported a rapid (24 h) sequence-based approach to identify clinically important yeasts and filamentous fungi from the first visible signs of growth on solid media (Pryce et al., 2003). The aim of this investigation was to evaluate a similar approach for the rapid and accurate identification of fungi directly from commercial broth enrichment media. To our knowledge, we report for the first time the use of DNA sequencing for the rapid identification of fungi directly from commercial broth enrichment media. In addition, we report a reliable DNA extraction method to isolate fungal nucleic acids from a range of commercial broth enrichment media commonly used in diagnostic microbiology laboratories.

## 2. Materials and methods

### 2.1. Clinical samples and controls

A total of 140 samples were collected from 138 patients from the Royal Perth Hospital and the Princess Margaret Hospital for Children, Perth, Australia. Clinical specimens included blood ($n$ = 121), vitreous fluid ($n$ = 3), synovial fluid ($n$ = 2), peritoneal fluid ($n$ = 3), skin biopsy ($n$ = 1), and a variety of other tissues and fluids ($n$ = 10). Commercial broth enrichment media including BacT/ALERT FA (BioMérieux, Marcy l'Etoile, France), BACTEC Plus Aerobic/F (Becton Dickinson, Microbiology Systems, Sparks, MD), and BACTEC Lytic/10 Anaerobic/F (Becton Dickinson) were inoculated with 5–10 mL of blood, synovial fluid, or peritoneal fluid. BACTEC Peds Plus/F media (Becton Dickinson) were inoculated with 3–5 mL of blood or peritoneal fluid, and BBL MGIT media (Becton Dickinson) were inoculated with approximately 0.5 mL of processed tissue or fluid. Overall, 45 broth cultures were microscopy-positive for fungi. Negative controls included culture-negative blood cultures ($n$ = 80) and positive blood cultures growing different bacterial species ($n$ = 15).

### 2.2. Culture and phenotypic identification of isolates

All inoculated media were incubated for a minimum of 14 days or until a signal indicating growth was detected by the automated system used. During routine processing, a small aliquot was withdrawn from each bottle when a signal indicating growth was detected or when the culture was reported as negative. From cultures that signaled positive, the aliquot was used for a Gram stain and inoculation onto routine solid media including chocolate agar, anaerobic blood agar, Sabouraud dextrose agar, and CHROMagar *Candida* (CCAN) (Becton Dickinson). Negative bottles were terminally subcultured on Sabouraud dextrose agar and incubated at 30 °C for 14 days. All isolates were initially identified in a blinded fashion (the medical scientist performing the phenotypic identification was not aware of the direct PCR/sequence [d-PCRS] result). Yeasts were identified by germ tube formation, VITEK Yeast Biochemical Card (YBC) (BioMérieux), appearance on CCAN, and microscopic characteristics such as the presence of pseudomycelium, blastoconidia, and chlamydospore production on cornmeal-Tween 80 agar (CMAT) (Hazen and Howell, 2003). A positive urease and brown pigment production on birdseed agar was used to confirm *Cryptococcus neoformans*. Yeasts not identified using YBC were identified using the ID32C system (BioMérieux). Other fungi were identified based on morphologic and physiologic characteristics using standard mycological techniques (de Hoog et al., 2000).

### 2.3. DNA extraction from commercial broth enrichment media for d-PCRS

A 0.1-mL aliquot of broth enrichment media was added to 1.0 mL of alkali wash solution (0.5 mol/L NaOH,

0.05 mol/L tri-sodium citrate dihydrate) to overcome potential PCR inhibitors (Kulski and Pryce, 1996). Samples were vortexed for 15 s, incubated at room temperature for 5 min, centrifuged at 16000 × *g* for 5 min, and the supernatant was removed. The pellet was resuspended in 1.0 mL of PBS (0.137 mol/L NaCl, 3 mmol/L KCl, 8 mmol/L $Na_2HPO_4$, 1 mmol/L $KH_2PO_4$) by gentle pipetting, vortexed for 15 s, and centrifuged at 16000 × *g* for 5 min. The supernatant was discarded, and the pellet containing fungal cells and charcoal/resin was resuspended in 0.2 mL of double-distilled $H_2O$ ($ddH_2O$) containing 50 U of lyticase (Sigma-Aldrich, Steinheim, Germany). Tubes were incubated at 37 °C for 1 h, then heated to 95 °C for 10 min. Fungal DNA was isolated using the High Pure PCR Template Preparation Kit (Roche Diagnostics, Castle Hill, Sydney, Australia) with the following modification to the manufacturer's method. Before DNA precipitation with isopropanol, tubes were centrifuged at 16000 × *g* for 2 min to deposit the activated charcoal or resin. The supernatant was removed (0.35 mL) and 0.1 mL of isopropanol was added. The samples were transferred to the glass fiber columns and processed following the protocol described by the manufacturer. An elution volume of 0.2 mL was used. Samples were used immediately for PCR or stored at −70 °C until use.

### 2.4. DNA extraction for PCR/sequence-based identification of the isolates grown from subculture

DNA extraction of isolates grown from subculture was performed as previously described (Pryce et al., 2003), with some modifications. Briefly, a small amount (~1–2 $mm^2$) of fungal mycelial mass or yeast colony was removed and emulsified in 0.1 mL of $ddH_2O$ containing 50 U of lyticase (Sigma-Aldrich) in a 2.0-mL screw-cap tube. Samples were incubated at 37 °C for 1 h then heated to 95 °C for 10 min. Proteinase K (Roche Diagnostics) was added to a final concentration of 65 μg/mL and incubated at 60 °C for 10 min. Samples were diluted with 60 μL of a solution containing 50% (wt/vol) of Chelex-100 (Bio-Rad Laboratories, Hercules, CA) with $ddH_2O$. Samples were heated to 95 °C for 10 min to further facilitate lysis. All tubes were centrifuged at 16000 × *g* for 5 min then diluted 1:10 with a solution containing 10% (wt/vol) of Chelex-100 with $ddH_2O$. Samples were used immediately for PCR or stored at −70 °C until use. A control (*Candida albicans* ATCC 14053) was used as a positive control for DNA extraction.

### 2.5. PCR amplification and detection

DNA extracts from commercial broth enrichment media and isolates grown from subculture were tested with primers V9D (5′-TTA AGT CCC TGC CCT TTG TA-3′) (de Hoog and Gerrits van den Ende, 1998) and LS266 (5′-GCA TTC CCA AAC AAC TCG ACT C-3′) (Masclaux et al., 1999) in a PCR reaction (PCR-A) as previously described (Pryce et al., 2003). These primers bind to conserved regions, with corresponding positions to *Saccharomyces cerevisiae* 18S (1609–1627) and 28S (287–266), and amplify an 800- to 1300-bp product that encompasses a portion of the 18S and 28S rRNA gene and the entire intervening ITS1, 5.8S, and ITS2 rRNA gene regions. Those samples negative by PCR-A were tested using primers ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) (White et al., 1990) in a second PCR reaction (PCR-B). These primers bind to conserved regions, with corresponding positions to *Saccharomyces cerevisiae* 18S (1769–1787) and 28S (41–60), and amplify a 400- to 900-bp product that encompasses the entire ITS1, 5.8S, and ITS2 rRNA gene regions. Both PCR-A and PCR-B were designed to use the same PCR conditions. Each PCR assay was performed with 5 μL of DNA template in a total reaction volume of 50 μL. The PCR reaction mixture contained 5 μL of 10× PCR buffer (Roche Diagnostics); 3 μL of 25 mmol/L $MgCl_2$; 1.5 μL of 20 μmol/L of each oligonucleotide; 200 μmol/L of each deoxynucleoside triphosphate; dATP, dGTP, dCTP, dTTP (Amersham Biosciences, Sydney, Australia); 2.25 U of FastStart Taq DNA Polymerase (Roche Diagnostics); and 33 μL of sterile distilled $H_2O$. The PCR was performed in a MyCycler (Bio-Rad Laboratories, Sydney, Australia) with the following program: 95 °C for 9 min, followed by 95 °C for 30 s, 62 °C for 60 s, 72 °C for 2 min for 33 cycles, followed by 72 °C for 5 min. PCR-amplified products were detected by gel electrophoresis using a 2% (wt/vol) agarose gel stained with ethidium bromide.

### 2.6. PCR amplification controls and assessment of PCR inhibition

DNA extracted from *Candida albicans* ATCC 14053 (1 pg/μL) was used as a positive PCR amplification control for each experiment. In addition, DNA extracts from all negative controls ($n$ = 95) were assessed for the presence of PCR inhibitors. A second PCR master mix was prepared containing DNA from *Candida albicans* ATCC 14053 to reach a final concentration of 1 pg per reaction. A 5-μL aliquot from each DNA extract was added to a PCR tube containing 45 μL of master mix. PCR amplification and detection were performed as previously described. The absence of PCR inhibition was determined by the presence of a PCR product the same molecular weight as the control.

### 2.7. Sequencing PCR-amplified products

All PCR-amplified products were sequenced at the West Australian Genome Resource Centre at Royal Perth Hospital. ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit Version 3 (Applied Biosystems, Foster City, CA) and the ABI Prism 3730 Genetic Analyzer were used (Applied Biosystems) following protocols supplied by the manufacturer. Primers used for sequencing PCR-A–

amplified products were V9D, LS266, ITS1, and ITS4. Primers used for sequencing PCR-B–amplified products were ITS1 and ITS4. Sequencing controls included PCR products from the DNA extraction control and PCR amplification control (*Candida albicans* ATCC 14053).

### 2.8. Sequence assembly, assessment of quality, and editing

The electropherograms were visualized and edited using SeqScape Software Version 2.0 (Applied Biosystems). DNA sequencing analysis and interpretation standards were used as a guide (Taylor, 2003).

### 2.9. Sequence similarity searching of the GenBank database using BLAST and species identification

Sequence search was performed using the BLAST standard nucleotide–nucleotide basic local alignment search tool (Altschul et al., 1997). A test sequence was assigned to a species identification by selecting the GenBank record with the highest bit score with an expect value equal to zero from the list of GenBank entries in the BLAST search, which have been identified as reliable based on previously published criteria (Pryce et al., 2003). Briefly, sequences used as standards for sequence-based identification (reference sequences) were selected based on the following criteria: (i) the sequence should be complete, that is, the sequence should represent the whole region of interest and not just a part thereof; (ii) all sequences used should be derived from cultures obtained from reference collections where possible, preferably cultures nomenclaturally designated as ex-type (the fungal equivalent to bacteriologic-type strains), or alternatively, cultures identified by the use of the most stringent level of traditional mycological techniques based on authoritative monographs and up-to-date literature in an upper-level reference laboratory; and (iii) to the best of the investigator's knowledge, each sequence should be designated with a species name that is nomenclaturally valid and currently recognized as correct at the time of the investigation.

## 3. Results

### 3.1. Assessment of the specificity of the PCR method and performance of the DNA extraction methods used

All broth enrichment media determined to be culture-positive for fungi were positive by PCR ($n$ = 45). All isolates grown from subculture were also positive by PCR ($n$ = 46). All positive samples except from patient 5 (*Cryptococcus neoformans*) were successfully amplified using PCR-A (Table 1). The positive sample from patient 5 was successfully amplified using PCR-B. A single PCR product was observed for all PCR-positive samples (data not shown). PCR products were obtained from a single DNA extraction for all broth enrichment media determined to be positive for fungi and from isolates grown from subculture. All broth enrichment media determined to be culture-negative for fungi were negative by PCR, and no PCR inhibition was detected ($n$ = 95).

### 3.2. d-PCRS and phenotypic identification of culture-positive specimens

For isolates able to be identified using phenotypic methods, d-PCRS identification for all positive specimens was 100% concordant with phenotypic identification of the isolates grown from subculture. However, 1 isolate (*Candida albicans*) was not detected from a mixed culture using d-PCRS (patient 43). This isolate was subsequently identified using phenotypic tests and PCR/sequence (PCRS) of the isolate. Subsequent investigations revealed that *Candida parapsilosis* was the predominating yeast on subculture. In all other positive specimens the d-PCRS result was 100% concordant with the PCRS identification of the isolates grown on solid media (data not shown).

Microscopic characteristics observed on CMAT correlated with the VITEK identification for the majority of isolates. However, for some isolates the VITEK identification was inconclusive and/or the microscopic features on CMAT did not correlate with the species reported by the VITEK system. In these circumstances a definitive phenotypic identification by examination of microscopic features on CMAT was difficult because of many yeasts sharing similar microscopic characteristics. The d-PCRS identification was useful to resolve ambiguous VITEK identifications and guide the selection of the most appropriate phenotypic tests to establish a final phenotypic identification (Table 2).

For 2 isolates the d-PCRS result was the only effective means of identification in our laboratory. One isolate identified by d-PCRS as *Candida orthopsilosis* (patient 3) and phenotypically as *Candida parapsilosis* was unable to be confirmed by further phenotypic tests (Table 2). The final identification of *Candida orthopsilosis* was established by PCRS identification of the isolate. Another isolate (patient 40; *H. capsulatum*) was unable to be identified in a timely fashion by phenotypic tests. The BBL MGIT tube contained clumps of septate hyphae with no distinct features. The fluid was initially subcultured onto solid media for routine identification. However, a more rapid identification was required to determine the clinical significance and selection of appropriate antifungal therapy. Following the d-PCRS result of *H. capsulatum*, subsequent growth on solid media developed microscopic features consistent with *H. capsulatum*. Because of the hazardous nature of this fungus, further phenotypic tests were not performed.

### 3.3. Time to identification from culture positivity and clinical significance

The time taken to definitively identify fungal isolates from positive fluids using conventional tests ranged from 2 to 13 days (mean, 4.8 days). The time taken to identify fungi from positive fluids using d-PCRS ranged from 24 to

Table 1
Identification of fungi from positive commercial broth enrichment media and sequence analysis

| Patient | Sample | Media | Days to positivity | Identification[a] | BLAST results[b] | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | Bit score[c] | Identity[d] | % Similarity | Accession no. | Strain no. |
| 1 | B | A | 1 | *Candida albicans* | 1046 | 535/536 | 99.8 | AF217609 | ATCC 28516 |
| 22 | B | A | 1 | *Candida albicans* | 1046 | 535/536 | 99.8 | AF217609 | ATCC 28516 |
| 20 | B | A | 1 | *Candida albicans* | 1046 | 535/538 | 99.4 | AF217609 | ATCC 28516 |
| 14 | B | A | 2 | *Candida albicans* | 1063 | 536/537 | 99.8 | AF217609 | ATCC 28516 |
| 21 | B | A | 2 | *Candida albicans* | 1063 | 536/536 | 100 | AF217609 | ATCC 28516 |
| 24 | B | A | 2 | *Candida albicans* | 1063 | 536/536 | 100 | AF217609 | ATCC 28516 |
| 8 | B | A | 2 | *Candida albicans* | 1063 | 536/536 | 100 | AF217609 | ATCC 28516 |
| 29 | B | A | 3 | *Candida albicans* | 1063 | 536/536 | 100 | AF217609 | ATCC 28516 |
| 2 | B | A | 3 | *Candida albicans* | 1063 | 536/536 | 100 | AF217609 | ATCC 28516 |
| 16 | B | A | 3 | *Candida albicans* | 1063 | 536/536 | 100 | AF217609 | ATCC 28516 |
| 39 | B | A | 3 | *Candida albicans* | 1049 | 536/537 | 99.8 | AF217609 | ATCC 28516 |
| 35 | PF | A | 1 | *Candida albicans* | 1049 | 536/537 | 99.8 | AF217609 | ATCC 28516 |
| 4 | B | A | 3 | *Candida glabrata* | 1199 | 629/634 | 99.2 | AY198398 | CBS 138 |
| 26 | B | A | 4 | *Candida glabrata* | 1203 | 622/626 | 99.4 | AY198398 | CBS 138 |
| 36 | B | A | 3 | *Candida glabrata* | 1203 | 622/626 | 99.4 | AY198398 | CBS 138 |
| 27 | B | A | 5 | *Candida glabrata* | 1199 | 627/632 | 99.2 | AY198398 | CBS 138 |
| 33 | VF | A | 3 | *Candida glabrata* | 1212 | 627/632 | 99.2 | AY198398 | CBS 138 |
| 31 | VF | A | 3 | *Candida glabrata* | 1195 | 621/626 | 99.2 | AY198398 | CBS 138 |
| 28 | B | A | 1 | *Candida parapsilosis* | 1031 | 520/520 | 100 | AY391843 | CBS 604 |
| 7 | B | A | 2 | *Candida parapsilosis* | 1031 | 520/520 | 100 | AY391843 | CBS 604 |
| 38 | B | A | 3 | *Candida parapsilosis* | 1031 | 520/520 | 100 | AY391843 | CBS 604 |
| 3 | B | A | 6 | *Candida orthopsilosis* | 914 | 461/461 | 100 | AJ698048 | ATCC 96139 |
| 32 | VF | A | 2 | *Candida dubliniensis* | 1001 | 505/505 | 100 | AB049123 | CBS 7987 |
| 23 | PF | A | 1 | *Candida kefyr* | 1255 | 633/633 | 100 | AJ401699 | CBS 4857 |
| 30 | B | A | 2 | *Candida tropicalis* | 898 | 519/523 | 99.2 | AF321539 | FEMS Yeast Res. |
| 5 | B | A | 7 | *Cryptococcus neoformans* | 952 | 489/492 | 99.4 | AF444326 | CBS 132 |
| 6 | B | A | 2 | *Saccharomyces cerevisiae* | 1108 | 559/559 | 100 | Z73326 | S288C |
| 13 | B | A | 4 | *Scedosporium prolificans* | 954 | 495/497 | 99.6 | AF022484 | CBS 114.90 |
| 36 | B | A | 4 | *Scedosporium prolificans* | 954 | 495/497 | 99.6 | AF022484 | CBS 114.90 |
| 25 | B | A | 3 | *A. fumigatus* | 1176 | 596/597 | 99.8 | AY214446 | ATCC 16907 |
| 11 | B | B | 5 | *Candida albicans* | 1063 | 536/536 | 100 | AF217609 | ATCC 28516 |
| 10 | B | B | 6 | *Candida glabrata* | 1221 | 634/639 | 99.2 | AY198398 | CBS 138 |
| 9 | B | B | 3 | *Candida parapsilosis* | 1031 | 520/520 | 100 | AY391843 | CBS 604 |
| 19 | B | B | 4 | *Candida krusei* | 876 | 480/483 | 99.4 | AF246989 | ATCC 6258 |
| 15 | B | B | 4 | *Candida krusei* | 926 | 480/483 | 99.4 | AF246989 | ATCC 6258 |
| 18 | B | D | 7 | *Candida parapsilosis* | 1031 | 520/520 | 100 | AY391843 | CBS 604 |
| 12 | SF | B | 7 | *Scedosporium prolificans* | 954 | 495/497 | 99.6 | AF022484 | CBS 114.90 |
| 12 | SF | C | 5 | *Scedosporium prolificans* | 954 | 495/497 | 99.6 | AF022484 | CBS 114.90 |
| 34 | B | D | 2 | *Candida albicans* | 1033 | 534/536 | 99.6 | AF217609 | ATCC 28516 |
| 43 | B | D | 3 | *Candida parapsilosis*[e] | 1031 | 520/520 | 100 | AY391843 | CBS 604 |
| 17 | B | D | 7 | *Candida parapsilosis* | 1031 | 520/520 | 100 | AY391843 | CBS 604 |
| 37 | PF | D | 2 | *Candida parapsilosis* | 1031 | 520/520 | 100 | AY391843 | CBS 604 |
| 40 | SB | E | 11 | *H. capsulatum* | 1070 | 552/556 | 99.3 | AF038353 | UAMH 7141 |
| 41 | B | A | 6 | *Candida guilliermondii*[f] | 1203 | 607/607 | 100 | AY939792 | ATCC 6260 |
| 42 | B | A | 5 | *M. pachydermatis* | 1039 | 534/536 | 99.6 | AY743637 | CBS 1879 |

B = venous blood (sample); SF = synovial fluid; PF = peritoneal fluid; VF = vitreous fluid; SB = skin biopsy; A = BacT/Alert FA; B = BACTEC Plus Aerobic/F (media); C = BACTEC Lytic/10 Anaerobic/F = D, BACTEC Peds Plus/F; E = BBL MGIT; ATCC = American Type Culture Collection (Manassas, VA); CBS = Centraal Bureau voor Schimmelcultures (Utrecht, the Netherlands); IFM = Institute for Food Microbiology (Chiba, Japan); UAMH = University of Alberta Microfungus Collection and Herbarium (Edmonton, AB, Canada); FEMS Yeast Res. = isolate recovered from fermented orange (Las Heras-Vazquez et al., 2003); S288C = *Saccharomyces cerevisiae* Genome Sequencing Project.

[a] Based on PCRS and phenotypic identification.

[b] BLAST results based on the criteria outlined in Materials and methods.

[c] NCBI BLAST terminology: normalized value of alignment derived from the raw alignment score (used to compare alignment scores from different searches).

[d] NCBI BLAST terminology: sequence length of the query (test sequence) compared with the subject (GenBank record).

[e] Mixed culture: *Candida parapsilosis* and *Candida albicans* isolated from subculture.

[f] Mixed culture: *Candida guilliermondii* and *Corynebacterium* sp. isolated from subculture.

36 h. The majority of fungi were identified by d-PCRS within 24 h of the initial positive microscopy result. The d-PCRS results for all fungi were obtained before the results of conventional identification, and in some cases, d-PCRS identification resulted in a change in patient management (data not shown).

Table 2
VITEK identification results of fungi requiring additional phenotypic testing after d-PCRS identification

| Patient | d-PCRS identification | VITEK identification | VITEK bionumber | Final identification established by: |
|---|---|---|---|---|
| 32 | *Candida dubliniensis* | 99% *Candida albicans*, <1% *Candida tropicalis* | 514455411 | Growth at 45 °C, dark-green colonies on CCAN, morphology on CMAT: chlamydospores in groups of 3–4 |
| 23 | *Candida kefyr* | 99% *Saccharomyces cerevisiae*, 1% *Candida kefyr* | 500200410 | ID32C (99.8% *Candida kefyr*, bionumber: 7220710001), morphology on CMAT |
| 38 | *Candida parapsilosis* | 75% *Candida albicans*, 14% *Candida parapsilosis* | 557457015 | Mauve colonies on CCAN, repeat VITEK identification (99% *Candida parapsilosis*, bionumber: 513447011) |
| 29 | *Candida albicans* | 79% *Candida guilliermondii*, 20% *Candida famata* | 577657615 | Germ-tube formation, repeat VITEK identification (99% *Candida albicans*, bionumber: 555457411) |
| 41 | *Candida guilliermondii* | 69% *Candida famata*, 30% *Candida guilliermondii* | 575777015 | ID32C (low discrimination 92.6% *Candida guilliermondii*, bionumber: 7377352117), morphology on CMAT |
| 3 | *Candida orthopsilosis* | 80% *Candida parapsilosis*, 15% *Candida tropicalis* | 557557015 | PCRS identification of the isolate grown from subculture |

### 3.4. BLAST similarity search results and sequence quality assessment

All test sequences from d-PCRS analysis showed >99% similarity when compared with known reference sequences using BLAST. Twenty test sequences demonstrated 100% sequence similarity to the reference sequence used for identification (44%). These included *Candida parapsilosis* ($n$ = 8), *Candida albicans* ($n$ = 7), *Candida orthopsilosis*, *Candida dubliniensis*, *Candida guilliermondii*, *Saccharomyces cerevisiae*, and *Candida kefyr*. Six test sequences demonstrated 99.8% sequence similarity to the reference sequence used for identification (13%). These included *Candida albicans* ($n$ = 5) and *A. fumigatus*. Six test sequences demonstrated 99.6% sequence similarity to the reference sequence used for identification (13%). These included *Scedosporium prolificans* ($n$ = 4), *Candida albicans*, and *M. pachydermatis*. The remainder of test sequences demonstrated 99.2% to 99.4% sequence similarity to the reference sequence used for identification (30%). The sequence test length remained relatively constant for test sequences identified as the same species.

### 3.5. Cost analysis: conventional versus d-PCRS

The average cost of performing conventional identification of fungi from blood cultures was AU$24.07 per isolate. This comprised reagents (AU$17.75) and labor (AU$6.32). The cost of performing d-PCRS identification (including the initial amplification step and sequencing reactions) was AU$24.54 per isolate. This comprised reagents (AU$18.00) and labor (AU$6.54).

## 4. Discussion

Given that methods for recovering fungi from blood, fluids, and tissues have improved with continuously monitored blood culture systems (Nolte et al., 1993; O'Hara et al., 2003; Thomson and Miller, 2003), it is reasonable to assume that uncommon fungi are likely to be encountered more frequently in the clinical microbiology laboratory. The routine identification of some fungi can be time consuming. Isolates from blood cultures are recovered by subculture and are identified using a range of culture-based methods. Rapid identification of fungi directly in blood cultures using molecular methods has been reported (Borst et al., 2001; Shin et al., 1999). However, the number of species identified by PCR-based methods is limited to the range of species-specific primers, nucleotide probes, or fluorogenic labels utilized in the assay. Hence, these methods usually target a small number of clinically important species. In contrast, broad-range PCR amplification and DNA sequencing of rRNA genes can potentially identify a wider range of fungi (Hall et al., 2003; Hall et al., 2004; Pryce et al., 2003). Although not as rapid as other molecular methods, a sequencing result can be obtained within 24–48 h from the first visible signs of growth (Hall et al., 2003; Henry et al., 2000; Pryce et al., 2003).

Until now, sequence-based approaches have not been evaluated for the direct identification of fungi in blood cultures. The MicroSeq D2 large-subunit ribosomal DNA sequencing kit (Applied Biosystems) has shown promise for identifying common clinical yeasts and filamentous fungi from solid media (Hall et al., 2003, 2004). However, less common yeasts and many clinically important filamentous fungi are not represented in MicroSeq sequence database, which may result in incorrect identification (Hall et al., 2003, 2004). In addition, the D2 large-subunit ribosomal DNA target may not demonstrate sufficient sequence variation to discriminate some species (Hall et al., 2004; Iwen et al., 2002). Furthermore, the cost of performing a sequence-based identification using this commercial system is high compared with phenotypic methods. Cost analysis comparing phenotypic testing to nucleic acid sequencing showed that the MicroSeq D2 identification was US$ 29.50 higher than the cost of using the API 20C AUX system (Hall et al., 2003).

We previously reported a rapid sequence-based approach for the accurate identification of 27 different species of fungi from the first visible signs of growth on solid media (Pryce et al., 2003). In the present study, we applied a similar approach for the rapid identification of fungi directly in liquid media. Depending on the time of day the culture

signaled positive, we obtained a d-PCRS identification within 24–36 h from the initial detection of fungi by Gram staining. The d-PCRS results agreed with the results obtained by conventional phenotypic methods to the species or genus level (Table 1). However, the d-PCRS identification of mixed cultures may be problematic. Other fungi not detected by d-PCRS may significantly alter patient management. Therefore, assessment of mixed cultures using appropriate culture methods is important. In contrast, the d-PCRS identification of fungi in mixed cultures containing bacteria (patient 41) may be advantageous. In this particular case, a slow-growing *Corynebacterium* sp. contaminated biochemical reactions, resulting in an ambiguous VITEK identification. Furthermore, the d-PCRS identification was useful to resolve ambiguous VITEK identifications and guide the selection of the most appropriate phenotypic tests to establish a final phenotypic identification (Table 2). The d-PCRS result of *Candida orthopsilosis* was of particular interest. The isolate grown from subculture was unable to be reliably identified. *Candida orthopsilosis* is a newly proposed species name to replace *Candida parapsilosis* group II and is morphologically indistinguishable from *Candida parapsilosis* group I and *Candida metapsilosis* (formerly *Candida parapsilosis* group III). There are currently no reliable phenotypic tests to differentiate these species (Tavanti et al., 2005). For other fungi, the d-PCRS method was the only effective means of species identification, particularly when a slow-growing fungus was encountered and only elementary distinctions such as septate hyphae were observed by microscopy. In these situations, nucleic acid sequencing may provide the greatest benefit to the laboratory and the clinician.

Overall, the broad-range primers V9D and LS266 primers worked well (PCR-A). However, these primers did not generate a PCR product from 1 blood culture containing *Cryptococcus neoformans*. Hence, primers ITS1 and ITS4 were used as an alternative (PCR-B) based on previous work in our laboratory (unpublished data). Both PCR methods target the entire ITS1 and ITS2 regions of the rRNA gene complex. These regions have been reported as promising targets for probe-based PCR assays and sequence-based assays for the identification and discrimination of many species of fungi (Lin et al., 2001; Pryce et al., 2003). The ITS1 and ITS2 regions combined are relatively short in length (~300–800 bp) and demonstrate significant sequence variation. In contrast, the 18S and the 28S rRNA genes of fungi are more highly conserved and larger in length (~1800 and 2900 bp, respectively), limiting the usefulness of these regions for species identification by sequencing. In some groups of fungi, species identification using ITS regions may also pose difficulties (Iwen et al., 2002). Within the genus *Fusarium* for example, reevaluation of the phenotypic positioning or the proposed revision of morphologic-based taxonomic schemes may be required. Whether sequence analysis of ITS regions will provide the ultimate sequence-based identification method needs to be established. In the present study and from previous work (Pryce et al., 2003), we have demonstrated the usefulness of the ITS regions as suitable targets for the sequence-based identification of a range of clinically important fungi. Furthermore, we have demonstrated comparable costs between the d-PCRS method and phenotypic methods of identification used in our laboratory, particularly identifications requiring additional or repeat phenotypic tests. From our assessment of total costs, screening positive blood cultures for *Candida albicans* using a rapid and specific real-time PCR assay, followed by d-PCRS identification if *Candida albicans* is not detected, may be a more cost-effective alternative for our laboratory.

Overall, the test sequences from each positive sample showed a high degree of similarity to each reference sequence used as a standard for d-PCRS identification (Table 1). The reference sequences used as standards for d-PCRS identification were selected from GenBank records based on strict criteria. Following these criteria, we were able to overcome some of the major limitations concerning the use of sequences in GenBank for organism identification. These include the use of incomplete sequences or sequences derived from nonreferenced or even misidentified cultures. We identified fungi by using the BLAST algorithm to compare our test sequences with sequences in the GenBank database. The output from BLAST arranges the sequences producing significant alignments by bit score followed by expect value. In our approach, the GenBank record with the highest bit score and expect value equal to zero from the reference sequences used as standards (Table 1) was recorded as the most likely species identification. Care should be taken although, as many species names that appear in the BLAST output may be incorrect, synonyms, or teleomorphs for a particular species.

Broth enrichment media inoculated with blood and other types of clinical samples are not normally considered to be ideal samples for PCR because of inhibitory substances such as sodium polyanetholesulfonate (SPS) (Fredricks and Relman, 1998). Various methods have been reported for the extraction of DNA from a variety of blood culture media (Chang et al., 2001; Fredricks and Relman, 1998, Iwen et al., 2004; Millar et al., 2000). Some of these methods are labor-intensive, and PCR inhibitors have been reported in DNA extractions from BACTEC and BacT/ALERT media using the QIAmp blood kit (Qiagen Corporation, Valencia, CA) (Fredricks and Relman, 1998) and the Roche High Pure PCR Template Preparation Kit (Millar et al., 2000). Steps to remove SPS by washing of inoculated BacT/ALERT medium by centrifugation before QIAmp DNA extraction are ineffective (Fredricks and Relman, 1998). DNA extraction of inoculated BacT/ALERT blood culture fluid based on a simple alkali wash method developed for BACTEC media (Kulski and Pryce, 1996) has been reported as the most sensitive, reproducible, and reliable method in a study evaluating 7 different DNA extraction methods (Millar et al., 2000). We developed a

similar approach to pretreat inoculated BacT/ALERT FA, BACTEC Plus Aerobic/F, Peds Plus/F, Lytic/10 Anaerobic/F, and BBL MGIT media with an alkali wash buffer before DNA extraction with a column-based kit. In addition, we removed charcoal and resins before DNA precipitation with isopropanol. This modification to the Roche high pure PCR preparation method is essential to remove these interfering substances before the DNA elution step. No PCR inhibition was observed in inoculated broth enrichment media positive for fungi. Furthermore, no PCR inhibition was observed in a separate PCR assay assessing the presence of PCR inhibitors in the negative controls. The column-based DNA extraction kit used provided sufficient DNA for PCR from all culture-positive samples and is well suited for routine diagnostic use. Current automated systems of nucleic acid extraction, such as the MagNA Pure LC instrument (Roche Diagnostics), may offer a less labor-intensive approach than column-based methods in the future.

The d-PCRS method described is an accurate and reliable tool for the identification of fungi directly in commercial broth enrichment media. Furthermore, it is possible that the d-PCRS method may be able to identify newly described species of fungi without the need to design new molecular approaches in the future. Moreover, this method may be useful in identifying fungi directly from clinical specimens. It is these types of situations where nucleic acid sequencing may provide the greatest benefit to the clinician. Further investigation is required to assess the clinical impact of rapid d-PCRS identification of fungi in the setting of a controlled clinical trial. Finally, more sequences are being deposited in GenBank and many have been identified by our laboratory as suitable reference sequences for identification of fungi, providing strict criteria are used for their inclusion.

## Acknowledgments

We thank the staff at the West Australian Genome Resource Centre at Royal Perth Hospital for their expertise, technical assistance, and performing the sequencing reactions for this study.

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## 2.4 **Publication 3 (Book chapter)**. Pryce, 2010. Universal detection and identification of fungi by PCR and DNA sequencing.

**Pryce TM**. 2010. Universal detection and identification of fungi by PCR and DNA sequencing. Chapter 70. In: Schuller M, Sloots T, James GS, Halliday CL, Carter IWJ. 2010. PCR for Clinical Microbiology: An Australian and International Perspective

Link: https://link.springer.com/chapter/10.1007/978-90-481-9039-3_70

As sole corresponding author and I wrote this chapter.

Clarivate journal impact factor from Journal Citation Reports: N/A (Textbook)

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# Chapter 70
# Universal Detection and Identification of Fungi by PCR and DNA Sequencing

**Todd M. Pryce**

## 70.1 Summary of Methods

This assay was developed to rapidly (within 24 h) detect and identify fungi from positive blood cultures and from clinical samples where initial laboratory evidence of fungal infection is suspected or the clinical pre-test probability of invasive fungal disease is high. Two PCR-DNA sequence based methods (PCRS) are described. The first method (PCRS-B) is used for the identification of fungi from culture plates and positive blood cultures. The second method (PCRS-D) is used for the detection and identification of fungi from microscopy-positive clinical samples such as fresh tissue or fluid. Requests for fungal PCR directly from clinical specimens are screened by a Clinical Microbiologist to determine whether it is worth testing based on laboratory and clinical evidence of invasive fungal disease.

Following sample extraction, DNA is amplified using a broad-range fungal PCR that targets the internal transcribed spacer (ITS) region of the rRNA. Amplified PCR products are purified and sequenced using automated methods and the derived sequence identified following comparison with reference sequences [3, 4].

## 70.2 Acceptable Specimens

Microscopy-positive clinical samples such as synovial fluid, peritoneal fluid, vitreous fluid, tissue biopsies and paraffin embedded sections are acceptable although fresh tissue or fluid in a sterile container are preferred. Additionally, the assay has been validated for use from a range of enrichment media used for the isolation of fungi in blood culture systems. These include BacT/ALERT FA media (BioMérieux, Marcy l'Etoile, France), BACTEC Plus Aerobic/F media (Becton

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M. Schuller et al. (eds.), *PCR for Clinical Microbiology*,
DOI 10.1007/978-90-481-9039-3_70, 

Dickinson, Microbiology Systems, Sparks, Md.), BACTEC Lytic/10 Anaerobic/F media (Becton Dickinson), BACTEC Peds Plus/F media (Becton Dickinson) and BBL MGIT media (Becton Dickinson) [4].

## 70.3 Unacceptable Specimens

Superficial swabs, or samples considered 'non-sterile' are not suitable for testing.

## 70.4 Sample Extraction

Nucleic acids are isolated from blood culture enrichment media using a universal DNA extraction procedure described previously [4].

The High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany) is used to extract DNA from clinical samples following the manufacturer's instructions. Nucleic acids are eluted in 50 μl of Elution buffer and stored at –70°C until use.

## 70.5 Primer Sequences

The primers V9D (5′-TTAAGTCCCTGCCCTTTGTA-3′) and LS266 (5′-GCATTCCCAAACAACTCGACTC-3′) [2] are used amplify an 800-1300 bp fragment that encompasses a portion of the 18S and 28S rRNA genes and the entire intervening ITS1, 5.8S and ITS2 rRNA gene regions. These primers bind to conserved regions, with corresponding positions to *Saccharomcyes cerevisiae* 18S (1609–1627) and 28S (287–266) rRNA genes.

## 70.6 PCR Amplification and Product Detection

Each PCR reaction is set-up in a 50 μl volume containing 1X FastStart Taq PCR Reaction Buffer (500 mM Tris-HCl, 100 mM KCl, 50 mM $(NH_4)_2SO_4$, pH 8.3) (Roche Diagnostics, Australia), 1.5 mM $MgCl_2$, 0.6 μM of each primer (V9D and LS266), 200 μM dNTPs, 2.25 U of FastStart Taq DNA Polymerase (Roche Diagnostics, Australia ), and 5 μl of DNA template. PCR amplification is performed in a MyCycler (Bio-Rad Laboratories, Australia) under the following conditions: 95°C for 9 min, followed by either 33 (PCRS-B) or 35 (PCRS-D) cycles of 95°C for 30 s, 62°C for 60 s and 72°C for 2 min, and a final extension of 72°C for 5 min. PCR-amplified products are detected by gel electrophoresis using a 2% (w/v) agarose gel stained with ethidium bromide.

## 70.7 DNA Sequencing

For both PCRS-B and PCRS-D, DNA sequencing is performed using primers V9D, LS266 and the internal primers ITS1 (5′-TCCGTAG-GTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) [2]. Sequencing primer concentrations are adjusted to 1 μM and all PCR products are sequenced using standard automated methods.

## 70.8 Sequence Assembly and Editing

Sequence electropherograms are visualised and edited using SeqScape Software Version 2.0 (Applied Biosystems) following DNA sequencing analysis and interpretation guidelines [5]. Sequence similarity searching is performed using the NCBI BLAST [1]. Test sequences are assigned a species identification using previously published criteria based on sequence length, similarity, type strain information, and current nomenclature [3, 4].

## 70.9 Quality Control and Validation Data

All DNA samples are tested both neat and diluted (1:10 with Elution buffer). A plasmid DNA construct derived from *Candida albicans* (ATCC 14053) amplified DNA is used as the positive control and Elution buffer is used as the negative control for both assays.

The analytical sensitivity of the PCRS-B and PCRS-D assays are 10 pg of *C. albicans* DNA ($2 \times 10^3$copies/μl) and 1 pg of *C. albicans* DNA ($2 \times 10^2$copies/μl), respectively.

The PCRS-B has successfully identified a wide range of fungal pathogens from 140 blood cultures, including *C. albicans* ($n$=14), *Candida parapsilosis* ($n$=8), *C. glabrata* ($n = 7$), *Candida krusei* ($n = 2$), *Scedosporium prolificans* ($n = 4$), and one each of *Candida orthopsilosis*, *Candida dubliniensis*, *Candida kefyr*, *Candida tropicalis*, *Candida guilliermondii, S. cerevisiae*, *Aspergillus fumigatus*, *Histoplasma capsulatum*, and *Malassezia pachydermatis*. All molecular identifications were in agreement with the morphological identification. Additionally, all culture negative blood cultures were negative by PCR and no PCR inhibition was detected ($n$=95).

The PCRS-D assay has been validated on a range of clinical specimens (including tissue, vitreous fluid and peritoneal dialysis fluid) where initial laboratory evidence of fungal infection is suspected, or the clinical pre-test probability of invasive fungal disease is high. A variety of fungal pathogens have been detected and identified including *C. albicans*, *C. parapsilosis*, *H. capsulatum*, *Cryptococcus neoformans* complex, *S. prolificans*, *Pseudoallescheria boydii*, *Aspergillus terreus*, *A. flavus*, *Rhizopus microsporus*, *Cunninghamella bertholletiae*, *Absidia corymbifera*, *Alternaria infectoria*, *Aureobasidium pullulans* and *Malassezia globosa*.

## 70.10 Assay Limitations

It is possible that DNA from some fungi may not be amplified using these broad range PCR assays due to polymorphisms in primer binding domains. To date we have found the assays cannot amplify *C. neoformans* DNA. Consequently when *C. neoformans* is suspected, a second PCR is performed (PCRS-C) and the primers V9D and LS266 are substituted with ITS1 and ITS4. A negative PCRS result cannot exclude the presence of fungi in a clinical sample. Other factors that may influence results include sampling, PCR inhibitors, contamination and non-specific interactions with human DNA. Non-specific interactions with human DNA can sometimes occur when the concentration of human DNA is high (tissues or fluids with a high inflammatory cell content i.e. pus), particularly in microscopy-negative samples where there may be no specific fungal target. In such cases, primers may bind non-specifically to human DNA and produce a high molecular weight PCR product. In these situations the presence of fungal DNA cannot be excluded until sequencing is performed. Finally, a direct PCRS result should always be interpreted in conjunction with other laboratory evidence or clinical evidence of disease.

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2.5 **Publication 4**. Pryce et al., 2003. Real-time automated polymerase chain reaction (PCR) to detect *Candida albicans* and *Aspergillus fumigatus* DNA in whole blood from high-risk patients.

**Pryce TM**, Kay ID, Palladino S, Heath CH. 2003. Real-time automated polymerase chain reaction (PCR) to detect *Candida albicans* and *Aspergillus fumigatus* DNA in whole blood from high-risk patients. Diagnostic Microbiology and Infectious Disease 47:487-96.

DOI link 10.1016/s0732-8893(03)00139-1

As corresponding first author, I conceptualised the study, methods and procedures. I collated all the samples for the investigation and performed all the laboratory testing and experiments. I wrote the protocol, collected and analysed the results and was the main person drafting the manuscript. All other authors either performed supervisory roles, provided clinical information and/or contributed to the writing and editing. All authors reviewed the manuscript prior to publication.

2024 Clarivate journal impact factor from Journal Citation Reports: 2.1

ELSEVIER

Diagnostic Microbiology and Infectious Disease
47 (2003) 487–496
Mycology

DIAGNOSTIC MICROBIOLOGY AND INFECTIOUS DISEASE

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# Real-time automated polymerase chain reaction (PCR) to detect *Candida albicans* and *Aspergillus fumigatus* DNA in whole blood from high-risk patients

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Received 27 January 2003; received in revised form 20 May 2003

**Abstract**

We report the development and evaluation of a real-time PCR assay using the LightCycler instrument for the detection of *C. albicans* and *A. fumigatus* DNA in whole blood. Recently published consensus criteria for the diagnosis of invasive fungal infection (IFI) were used for all patient samples. Unique and published primer pairs were developed and assessed for sensitivity, specificity, and reproducibility to detect *C. albicans* and *A. fumigatus* DNA in samples spiked with purified DNA, and whole blood samples from 8 high-risk patients and 45 negative controls. The real-time assay demonstrated an analytical sensitivity of 10 fg of purified *C. albicans* and *A. fumigatus* DNA and was found to be specific for each species. The standardized approach was highly reproducible and detected *C. albicans* and *A. fumigatus* DNA in two patients with proven IFI and in one patient with a possible IFI. In addition, we report for the first time the use of recently published international consensus criteria for the diagnosis of IFI in the evaluation of a mildy invasive fungal diagnostic assay. Standardized clinical criteria and a more standardized approach to detect fungal DNA in less invasive patient samples, may permit a more reliable comparison of future studies. A rapid real-time detection of fungal DNA in whole blood, combined with standard clinical markers of response, may be more useful for monitoring patients at risk of developing IFI than other diagnostic methods currently available. 

## 1. Introduction

Invasive fungal infections (IFIs) with yeasts and molds have emerged as the leading cause of morbidity and mortality in immunosuppresed patients, particularly those undergoing allogeneic bone marrow transplants and patients with hematologic malignancies receiving intensive cytotoxic chemotherapy (Denning et al., 1998). The survival of these patients depends on early diagnosis and prompt initiation of antifungal therapy, but conventional laboratory-based tests are rarely conclusive, because blood cultures lack sensitivity and invasive procedures are often required to obtain pathologic or microbiologic confirmation (Denning et al., 1998; Latge, 1999). The diagnosis of IFIs is therefore often only established at autopsy (Latge, 1999). Hence the development of reliable less invasive techniques to facilitate the early and reliable diagnosis of IFIs is currently under active investigation.

Detection of circulating fungal antigens is useful for the diagnosis of IFIs, particularly invasive aspergillosis (IA). Currently, there are a number of commercially available kits for the diagnosis of IA and candidemia: the latex agglutination test to detect galactomannan (Pastorex *Aspergillus* and Pastorex *Candida*, Sanofi Diagnostics Pasteur, Marnes-La-Coquette, France), a sandwich ELISA for the detection of galactomannan (Platelia *Aspergillus* and Platelia *Candida*, Sanofi Diagnostics Pasteur, Marnes-La-Coquette, France), and a plasma $(1 \rightarrow 3)$-$\beta$-D-glucan assay (Fungitec G test, Seikagaku Kogyo Corp., Tokyo, Japan). These techniques show good sensitivity and varying degrees of specificity in high-risk patients (Ascioglu et al., 2002; Maertens et al., 1999), but they may only yield positive test results at advanced stages of infection. In addition, the use of high-resolution CT of the chest has an emerging role in the noninvasive diagnosis of invasive pulmonary aspergillosis

Presented in part: Annual Scientific Meeting of the Australian Society for Microbiology, Perth, Australia, September 2001.

[1] Potential conflict of interest: Dr CH Heath, is a member of the Voriconazole Advisory Board, Pfizer (Australia) Pty.Ltd.

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0732-8893/03/$ – see front matter 
doi:10.1016/S0732-8893(03)00139-1

(IPA) (Caillot et al., 1997; Yeghen et al., 2000). The most characteristic findings on CT in IPA are a halo of ground-glass attenuation around focal nodules, the "halo sign", and the air-crescent sign. Both signs are relatively sensitive for the diagnosis of IPA in high-risk patients, but these signs can be relatively non-specific and have been reported in association with a wide range of other infective and non-infective diagnoses.

In addition to these non-invasive techniques, molecular techniques such as conventional PCR show great diagnostic potential with a high degree of sensitivity and specificity (Einsele et al., 1997; Hendolin et al., 2000; Loeffler et al., 1998; Van Burik et al., 1998; Williamson et al., 2000; Yamakami et al., 1996). Many published methods for conventional PCR include time consuming in-house DNA extraction protocols, requiring the use of gel-electrophoresis or other slow amplicon detection steps. Traditional PCR-gel electrophoresis also requires post-PCR manipulation which may lead to amplicon cross contamination (Loeffler et al., 1999), leading to false positive results. The use of standardized DNA extraction protocols and real-time PCR may address many of the limitations of conventional PCR. Recently, a quantitative real-time PCR assay using the LightCycler (LC) instrument (Roche Molecular Diagnostics, Mannheim, Germany) has been reported to show great potential for the rapid in vitro amplification and detection of *C. albicans* and *A. fumigatus* DNA in spiked blood samples and patient blood samples (Loeffler et al., 2000b). The use of real-time PCR technology with the LC can easily be incorporated into the routine diagnostic microbiology laboratory and turnaround times for results can be dramatically reduced (Palladino et al., 2001a; Palladino 2001b). Real-time PCR can also provide accurate and reproducible quantitation of fungal DNA in blood (Kami et al., 2001; Loeffler et al., 2000b), which may be useful in addition to standard clinical markers of response, in monitoring the outcome of the patient to antifungal therapy.

In this study we report the development of a new real-time PCR assay using the LC instrument with FRET and melting curve analysis to detect *C. albicans* and *A. fumigatus* DNA in whole blood. We also report the results of a preliminary evaluation of this assay in a number of high-risk patients. In addition we have used the recently published consensus criteria for the diagnosis of IFIs, and have therefore standardized the diagnosis of IFIs against agreed definitions (Ascioglu et al., 2002). The use of standardized definitions of IFIs has not been previously reported in the study of new less invasive fungal diagnostic techniques.

## 2. Materials and methods

### *2.1. Fungal cultures and growth conditions*

The following strains of yeasts were obtained from the American Type Culture Collection (ATCC): *Candida albicans* ATCC 14053, *C. parapsilosis* ATCC 22019, *C. krusei* ATCC 6528, *C. tropicalis* ATCC 750, *C. glabrata* ATCC 2238. The molds used in this study were obtained from the Royal Perth Hospital Mycology Culture Collection: *Aspergillus fumigatus* RMCC 75829, *A. niger* RMCC 75830, *A. flavus* RMCC 75831, *A. terreus* RMCC 75832, *A. nidulans* RMCC 75833. In addition, ten clinical isolates of *A. fumigatus* and *C. albicans* were included in this study. All fungi were grown on Sabouraud 2% dextrose agar for 72 h at 30°C. Suspensions of conidia or yeast were prepared with sterile water and were adjusted to 0.5 McFarland standard. The suspensions were filtered using a 10 $\mu$m syringe filter to remove clumps of cells and the number of colony forming units (CFUs) were calculated using a counting chamber. To avoid further growth, suspensions were adjusted without delay, to $1 \times 10^6$ CFU/mL in sterile water for the preparation of seeded samples and standard DNA templates.

### *2.2. DNA extraction from cultured cells (fungal DNA standards)*

DNA was isolated and purified from *Candida* spp. or *Aspergillus* spp. using the High Pure PCR Template Preparation Kit (Roche Molecular Biochemicals, Mannheim, Germany). Briefly, suspensions of either *Aspergillus* conidia or *Candida* cells were pelleted in a 1.5 mL microfuge tube at 3,000 $\times$ *g* for 5 min; then resuspended in 200 $\mu$L of 100 mM Tris buffer containing 10 U of lyticase (Sigma, Castle Hill, NSW, Australia). Samples were incubated for 30 min at 37°C. Nucleic acids were isolated from the treated samples following the manufacturers instructions. Purified DNA was then eluted from the filter tube with 200 $\mu$L of prewarmed (70°C) elution buffer containing 10 mM Tris (pH 8.5). The DNA concentration was determined using GeneQuant spectrophotometer (Amersham Pharmacia Biotech, Sydney, Australia). All samples were stored at −70°C until use. In addition, the lysing enzyme lyticase was assessed to ensure the enzyme preparation was free of endogenous fungal DNA that could cause a false-positive PCR result. A 10-fold concentrate of lyticase (1000 U) was extracted and stored as described above.

### *2.3. Extraction of fungal DNA from blood samples*

Extraction of nucleic acids from whole blood was performed with equipment dedicated to DNA extraction from clinical samples. Whole blood samples (9-mL EDTA) were separated into 1.5 mL microfuge tubes in 200 $\mu$L and 1 mL lots, and frozen at −70°C until use. Once thawed 10 U of lyticase was added to the 200 $\mu$L whole blood samples and incubated at 37°C for 30 min. Nucleic acids from all 200 $\mu$L whole blood samples were then isolated using the High Pure PCR Template Preparation Kit (Roche Molecular Biochemicals) according to the manufacturers instructions. All samples were stored at −70°C until use.

Table 1
Primers and probes used for 18S SSU rRNA gene amplification for detection of *Aspergillus fumigatus* and *Candida albicans* using the LC protocol

| Primers and probes | Sequence and label(s) (5′ → 3′)[a] | Melting Temperature (°C)[d] | Position[f] | GenBank accession no. | Product size (bp) |
|---|---|---|---|---|---|
| Primers | | | | | |
| F1-Forward[g] | ATT GGA GGG CAA GTC TGG TG | 59.6 | 543-562 | M60300 | 503 |
| F1-Reverse[g] | CCG ATC CCT AGT CGG CAT AG | 59.7 | 1046-1027 | M60300 | |
| PK-Forward | TGT TGC AGT TAA AAA GCT CGT AGT TG | 62.7 | 607-632 | M60300 | 336 |
| PK-Reverse | ATG CTT TCG CAG TAG TTA GTC TTC A | 60.1 | 943-919 | M60300 | |
| PK-Forward | TGT TGC AGT TAA AAA GCT CGT AGT TG | 62.7 | 607-632 | M60300 | 439 |
| F1-Reverse[g] | CCG ATC CCT AGT CGG CAT AG | 59.7 | 1046-1027 | M60300 | |
| Probes[g] | | | | | |
| *A. fumigatus* | [a]R-TGA GGT TCC CCA GAA GGA AAG GTC CAG C-P[b] | 71.1[e] | 708-681 | M60300 | |
| | F-GTT CCC CCC ACA GCC AGT GAA GGC | 71.2[e] | 734-711 | M60300 | |
| *C. albicans* | R-TGG CGA ACC AGG ACT TTT ACT TTG A-P | 62.9[e] | 713-737 | M60302 | |
| | F-AGC CTT TCC TTC TGG GTA GCC ATT | 63.7[e] | 687-710 | M60302 | |

[a] R, LC-Red 640, LightCycler Red 640 (Roche Molecular Biochemicals, Mannheim, Germany)
[b] P, phosphate
[c] F, LC fluorescein dye
[d] Based on nearest neighbour thermodynamic theory as described by Freier et al. (1986).
[e] Based on thermodynamic approach (TIB MOLBIOL, Berlin, Germany)
[f] Position refers to specific nucleotide locations of the primers and probes in the *A. fumigatus* and *C. albicans* 18S rRNA gene sequences.
[g] Sequences as described by Loeffler et al. (2000b).

### *2.4. Amplification and detection of fungal DNA using the LC instrument and FRET*

Sequence data from the 18S small-subunit (SSU) ribosomal RNA genes of the following fungi was obtained from GenBank database: *C. albicans*, accession number M60302; *C. glabrata*, accession number M60311; *C. parapsilosis*, accession number M60307; *C. krusei*, accession number M60305; *C. tropicalis*, accession number M60308; *A. fumigatus*, accession number M60300; *A. niger*, accession number X78538; *A. flavus*, accession number X78537; *A. terreus*, accession number X78540; and *A. nidulans*, accession number X78539. Sequences were aligned and compared using GeneDoc Multiple Sequence Alignment Editor and Shading Utility Version 2.6.001 (Pittsburgh Supercomputing Center (PSC), Carnegie Mellon University, University of Pittsburgh, Pittsburgh, PA, USA) (Nicholas and Nicholas, 1997). A consensus sequence was generated and was used as a basis for primer selection and development using Primer3 software (Whitehead Institute/MIT Center for Genome Research, Cambridge, MA, USA) (Rozen and Skaletsky, 1998). The internal probe binding site was excluded from the target sequence by marking the source sequence. Different primer pairs were generated and sorted according to quality and selected pairs were chosen for use by comparison of the statistical output. A number of primers were generated including primers that have been previously published (Einsele et al., 1997; Loeffler et al., 2000b). Primers (Gibco BRL, Life Technologies, Melbourne, Australia) used in this study are shown in Table 1. Oligonucleotide F1-R was also selected by Primer3 software to be an alternative to PK-R using PK-F as the forward oligonucleotide, which gave a 439 bp product.

### *2.5. Sequence dependent PCR using the LC instrument*

The LC DNA Master Hybridization Probes Kit (Roche Molecular Biochemicals) was used to detect target amplicons and the detection of *Candida albicans* and *A. fumigatus* DNA was performed in separate glass capillaries. Amplification mixtures were transferred to the glass capillary tubes containing 2 $\mu$L of 10 × LC FastStart enzyme (Roche Molecular Biochemicals) concentrate (containing Taq DNA polymerase, reaction buffer, dNTP mix with dUTP instead of dTTP, and 10 mM $MgCl_2$), 0.8 $\mu$L of 25 mM $MgCl_2$ stock solution, and sterile PCR grade water was added to a volume of 15 $\mu$L. Oligonucleotides were used at a concentration of 0.8 $\mu$M for *A. fumigatus* and 1.0 $\mu$M for *C. albicans* assay. Probes were used at concentrations of 0.5 $\mu$M for *A. fumigatus* and 0.8 $\mu$M for *C. albicans*. 5 $\mu$L of sample DNA was added to the amplification mixture to give a final volume of 20 $\mu$L. Samples consisted of fungal DNA standards, spiked fungal DNA standards, and seeded whole blood samples, high-risk patient samples and healthy negative controls. The loaded glass capillaries were filled using the LC Carousel Centrifuge (Roche Molecular Biochemicals). The carousel was transferred to the LC instrument and the following program was performed: an initial denaturation at 95°C for 10 min to activate the FastStart Taq polymerase, followed by a 45 cycle program consisting of heating to 95°C at 20°C/s with a 5 s hold, cooling to 62°C at 20°C/s with a 15 s hold and heating to 72°C at 20°C/s with a 25 s hold. Signal detection was performed at the end of the annealing step with a single fluorescence acquisition for each capillary. Following this melting curve analysis was performed to confirm positive results. The melting point of the *C. albicans* and *A. fumigatus* probe pairs was

determined by cooling to 55°C at 20°C/s with a 10 s hold and raising the temperature 0.2°C/s to 95°C. Fluorescence acquisition was performed continuously during the melting curve analysis. Finally, the capillary tubes were cooled to 40°C at 20°C/s with a 60 s hold. The LC system software (Roche Molecular Biochemicals) at the end of the run then calculates the crossing point ($C_p$). The $C_p$ is the number of cycles at which signal detection crosses the threshold of the crossing line.

### 2.6. In vitro examination of sensitivity, specificity and reproducibility of real-time PCR

To determine the sensitivity of the real-time PCR with FRET for the detection of amplified *Candida* or *Aspergillus* DNA, 100 pg ($1 \times 10^4$ genome copies) of standard DNA was serially diluted in water (assuming a single genome copy or 1 CFU is equivalent to approximately 10 fg of fungal DNA) (Sambrook et al., 1989). The diluted DNA standards were measured for each primer pair listed in Table 1. To determine reproducibility, 1 pg ($1 \times 10^2$ genome copies), and 100 fg ($1 \times 10^1$ genome copies) of standard DNA were measured eight times for each primer pair listed in Table 1. To determine specificity DNA from all fungi used in this study was also tested. To determine the efficiency of the DNA extraction method blood samples from healthy volunteers were seeded with serial dilutions ($1 \times 10^0 - 1 \times 10^4$ CFU) of either conidia or yeast cells. Cells were added to fresh whole blood samples and stored at −70°C; then thawed prior to DNA extraction to duplicate the method used for clinical samples. An evaluation of the analytical sensitivity of the PCR-FRET technique to detect fungal DNA in the presence of human DNA was also performed. Samples were spiked with serial dilutions of DNA within the same range as for seeded cells (100 pg-10 fg). The positive control used for all experiments consisted of 1 pg of purified fungal DNA ($1 \times 10^2$ genome copies). To detect PCR inhibition 1 pg of purified fungal DNA was added as an internal control to DNA extracts of whole blood samples from healthy negative controls.

### 2.7. Clinical samples and negative controls

All blood samples were collected from patients and volunteers' using 9-mL Vacuette tubes (Greiner Bio-One, Longwood, FL, USA). Samples were separated into sterile 1.5-mL microfuge tubes and kept at −70°C for retrospective analysis. To reduce the risk of contamination of samples with exogenous environmental fungi all fluid transfer was performed with aerosol-resistant pipette tips (Molecular Bio-Products, San Diego, CA, USA). This work was carried out in a separate research laboratory well isolated from the diagnostic mycology laboratory. Samples from 8 patients at high-risk for IFIs were collected and analyzed for the presence of *Candida albicans* or *Aspergillus fumigatus* DNA. Negative controls consisted of blood samples from forty-five healthy volunteers without clinical evidence of invasive fungal disease. All samples were obtained over a six-month period and information regarding the timing of specimen handling was recorded to monitor for potential exogenous environmental fungal contamination.

### 2.8. Statistical analysis

Statistical analysis of the $C_p$ values was performed using the Pearson's correlation coefficient.

## 3. Results

### 3.1. In vitro examination of sensitivity of the real-time PCR assay

The $C_p$ values of the serially diluted standard DNA were plotted against the logarithmic DNA concentration for each of the primers tested (data not shown). Good linearity was achieved for each assay tested and the correlation coefficient was greater than 0.98 for all tests. The analytical sensitivity of each primer pair was assessed using purified DNA from *C. albicans* and *A. fumigatus* in sterile water, purified DNA from *A. fumigatus* or *C. albicans* added to whole blood extracts from healthy volunteers, and blood from healthy volunteers spiked with *A. fumigatus* conidia or *C. albicans* blastoconidia. The F1-F/F1-R and the PK-F/F1-R oligonucleotide pairs tested demonstrated a sensitivity of 10 fg (1 genome copy) in purified *A. fumigatus* and *C. albicans* DNA, 100 fg ($1 \times 10^1$ genome copies) from purified DNA added to DNA extracts from healthy volunteers and 10 CFU from seeded blood samples. The PK-F/PK-R primer pair showed reduced sensitivity of 100 fg for purified *A. fumigatus* DNA. Concentrations less than 10 fg were unable to be detected (data not shown).

### 3.2. In vitro examination of the specificity of the real-time PCR assay

The specificity of the probes was assessed using DNA isolated from all fungi included in the study and human DNA from whole blood extracts. The probes hybridized to their specific templates only, irrespective of which primers were used. That is, the AF1 and AF2 probe pair, hybridized only to DNA amplified from *A. fumigatus*. In addition, the probes CA1 and CA2 hybridized only to DNA amplified from *C. albicans*. The probes did not hybridize with other *Candida* species, *Aspergillus* species, or human DNA from unseeded whole blood extracts. The results are in concordance with other investigators using the same oligonucleotide probes (Loeffler et al., 2000b). In addition, no fluorescent signals were observed when a 1000 U (10 × concentrate) of lyticase was tested, indicating the absence of contaminating *A. fumigatus* or *C. albicans* DNA in this commercial enzyme preparation.

### 3.3. In vitro examination of the reproducibility of the real-time PCR assay

Reproducibility was assessed for each primer pair using 1 pg and 100 fg amounts of purified *A. fumigatus* and *C. albicans* DNA. The coefficient of variation (CV) of the $C_p$ values for 1 pg and 100 fg of *A. fumigatus* DNA using the F1-F/F1-R and PK-F/F1-R primers were 33.16 ± 0.70, 33.18 ± 0.50, and 33.92 ± 5.98, 34.78 ± 4.89 (mean ± standard deviation), respectively. The CV of the $C_p$ values for 1 pg and 100 fg of *C. albicans* DNA for the primers F1-F/F1-R and PK-F/F1-R were 32.27 ± 0.34, 32.21 ± 0.36, and 33.92 ± 5.98, 35.05 ± 0.56, respectively. No fluorescence signals were detected in the reagent controls.

### 3.4. Evaluation of the PCR assay from whole blood samples from patients at high-risk of IFIs

The clinical features of the eight high-risk patients studied are summarized in Table 2. We used the recently published consensus criteria (Ascioglu et al., 2002) to standardize the diagnosis of IFIs. Three patients had proven IFIs and one patient had a possible IFI. Two patients had proven IA, one patient had candidemia with proven disseminated candidiasis and the fourth patient had possible IA. All four patients subsequently died; three deaths were directly attributable to IFIs. A patient with proven disseminated *A. fumigatus* infection (Patient 1, Table 2) had a positive real-time PCR result, but only one day before and on the day of death from IA, respectively. The PCR assay was negative on two occasions, 23 and 9 days before death, respectively. The real-time fluorescence output from the LC instrument of this patients' samples and controls using the PK-F and F1-R oligonucleotide primer pair and the *A. fumigatus* probes is shown in Fig. 1A. A patient with proven disseminated *A. terreus* infection (Patient 2; Table 2) did not have a positive real-time PCR result. The third patient with a positive *A. fumigatus* real-time PCR result was at high-risk of an IFI because of aggressive cytotoxic chemotherapy and underlying acute myeloid leukemia, and she had possible IA (Patient 3, Table 2). The patient with proven disseminated *C. albicans* infection and candidemia had a positive real-time PCR result for *C. albicans* DNA, but only one day before death. In this case, no blood samples were collected earlier than one day prior to death. Analysis from the F2 channel of the LC instrument showing fluorescence output from this patients' samples and controls using the PK-F and F1-R oligonucleotide primer pair and for the *C. albicans* probes is shown in Fig. 1B.

The remaining four patients at high-risk of IFI did not develop clinical or laboratory evidence of invasive fungal disease; two died of complications relating to their primary disease. They had negative PCR assays for *A. fumigatus* and *C. albicans* DNA and their deaths were not attributable to IFIs. However neither patient had a postmortem examination to unequivocally prove that they were free of IFIs prior to death. The two remaining patients survived, but had whole blood samples that were PCR positive for *A. fumigatus* DNA. In these cases there was no clinical or laboratory evidence of IA, although these patients were at high-risk of an IFI. The first patient had profound immunosuppression following cardiac transplantation and had active invasive CMV disease at the time the sample was collected, but did not receive antifungal prophylaxis or therapy. The second patient had prolonged and profound immunosuppression while receiving treatment for acute lymphocytic leukemia and had received antifungal prophylaxis with fluconazole (200 mg/day). At the time the sample was collected the patient was on empiric therapy with i.v. amphotericin B for febrile neutropenia non-responsive to broad-spectrum antibiotics. Neither of these patients went on to subsequently develop clinical or laboratory evidence of IFIs and both patients currently remain well. In both these cases it is not possible to distinguish between a false positive result or fungal DNAemia that was subclinical. The second patient may also have responded to antifungal treatment without a specific focus of infection having been identified.

### 3.5. Evaluation of the PCR assay from whole blood samples from negative controls

All whole blood samples taken from forty-five healthy healthy controls had negative PCR results following the protocol described in the methods.

## 4. Discussion

IFIs are life-threatening infections and most patients who succumb to invasive aspergillosis and disseminated candidiasis have an underlying immunosuppressive disease. In most cases the diagnosis of these infections is often just before death or at autopsy because current laboratory-based tests lack sensitivity and specificity (Hopfer, 1997). Blood cultures have poor sensitivity for the diagnosis of invasive fungal infections because fungemia is thought to occur intermittently with low numbers of viable fungal cells (<10 CFU/mL) circulating in the peripheral blood (Hopfer, 1997). Failure of blood cultures to detect circulating yeasts and molds may either be due to the inability of the fungi to grow or an inability for growth to be detected by automated blood culture systems. We therefore sought to evaluate a new PCR assay utilizing FRET and melting point analysis using the LC instrument for the early detection *C. albicans* and/or *A. fumigatus* DNA from whole blood samples of patients at high-risk of IFIs.

The real-time PCR assay targets *C. albicans* and *A. fumigatus* DNA in whole blood, as these two fungi are responsible for the majority of IFIs, particularly in those patients undergoing bone-marrow transplantation or aggressive cytotoxic chemotherapy with prolonged neutropenia. Fungal PCR on blood samples appears to be very sensitive,

Table 2
Clinical features of high-risk patients and samples tested using the LC protocol[a]

| Patient no. | Age, sex | Disease | Status | Immunosuppression | Diagnosis | Mycology | Sample | WCC $\times 10^9$/L | PCR | Outcome |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 44/F | End-stage renal failure, cadaveric renal transplantation | D+ 407 post-cadaveric renal transplantation | Prednisone, pulse steroids, ATG, CyA, MMF, $OKT_3$ | Proven, aspergillus trachoebroncitis and IPA at autopsy | *A. fumigatus* | A (D + 384)<br>B (D + 399)<br>C (D + 406)<br>D (D + 407) | 7.8<br>1.6<br>4.5<br>13.9 | −ve<br>−ve<br>+ve<br>+ve | Dead |
| 2 | 49/F | CML with blast transformation, AML FAB M7 | CR1, D+ 43 post-matched related donor allogeneic BMT, allograft failure, and PBSCT | Intinamb ICE, induction TBI, IDA-FLAG conditioning, GVHD prophylaxis, CyA, MMF | Proven, disseminated invasive apsergillus | *A. terreus* | A (D + 40) | <0.1 | −ve | Dead |
| 3 | 45/F | AML FAB M4 | IPA in 1999 cured with left lower lobectomy & antifungal therapy, CR4, relapse, D + 3 post-allogeneic BMT | ICE, induction TBI, IDA-FLAG conditioning, MUD | Proven, disseminated invasive candidiasis and candidemia | *C. albicans* | A (D + 2)<br>B (D + 2) | <0.1<br><0.1 | +ve<br>+ve | Dead |
| 4 | 28/F | AML FAB M2 | CR1, BMT delayed as pregnant, relapse, D + 21 post-allogeneic BMT | ICE, induction X2 Conditioning ICE MUD | Possible, multiple hepatosplenic lesions on CT | NG | A (D + 20) | 60.3 | +ve | Dead |
| 5 | 70/M | Burkitts' lymphoma | Inducation therapy | Hyper-CVAD, high-dose Ara-C, MTX, RX | No fungal infection detected | NG | A (D + 38) | NT | −ve | Dead |
| 6 | 26/F | Post-partum cardiomyopathy | D + 32 post-cardiac transplantation, prolonged LVAD support | Allograft rejection, prednisone, pulse steroids, ATG, CyA, MMF, FK-506, $OKT_3$, TLI | No fungal infection detected | NG | D + 32 | 4.4 | +ve | Alive |
| 7 | 31/F | ALL FAB L2 | Induction, consolidation, maintenance chemotherapy | UKALL XII protocol | No fungal infection detected | NG | D + 124 | 2.2 | +ve | Alive |
| 8 | 51/M | AML FAB M5 | Induction chemotherapy | Leukopheresis, ICE induction | No fungal infection detected | NG | D + 17 | 173 | −ve | Dead |

[a] Abbreviations: D+, days post-transplantation; AML, Acute myeloid leukemia; FAB, French American British Classification; M1-7, type of leukemia; CML, chronic myeloid leukemia; CR1, first complete remission; BMT, bone marrow transplant; PBSCT, peripheral blood stem cell transplant; ICE, idarubicin, cytarabine, etoposide; TBI, total body irradiation; IDA-FLAG, idarubicin, fludarabine, cytarabine; GVHD, graft versus host disease; ATG, anti-T globulin; CyA, cyclosporin A; MMF, mycophenylate mofetil; MUD, matched unrelated donor allogeneic bone marrow transplant; Hyper-CVAD, dexamethosone; cyclophosphamide, vincristine; Ara-C and Methotrexate; RX, local radiotherapy—20 Gray (5 fractions); LVAD, left ventricular assist device; FK-506, tacrolimus, $OKT_3$, monoclonal $CD_3$ antibodies; TLI, total lymphoid irradiation; ALL, acute lymphocytic leukemia; L2, pre-B cell ALL-CALLA +ve, common ALL antigen positive; UKALL XII protocol; NG, no growth.

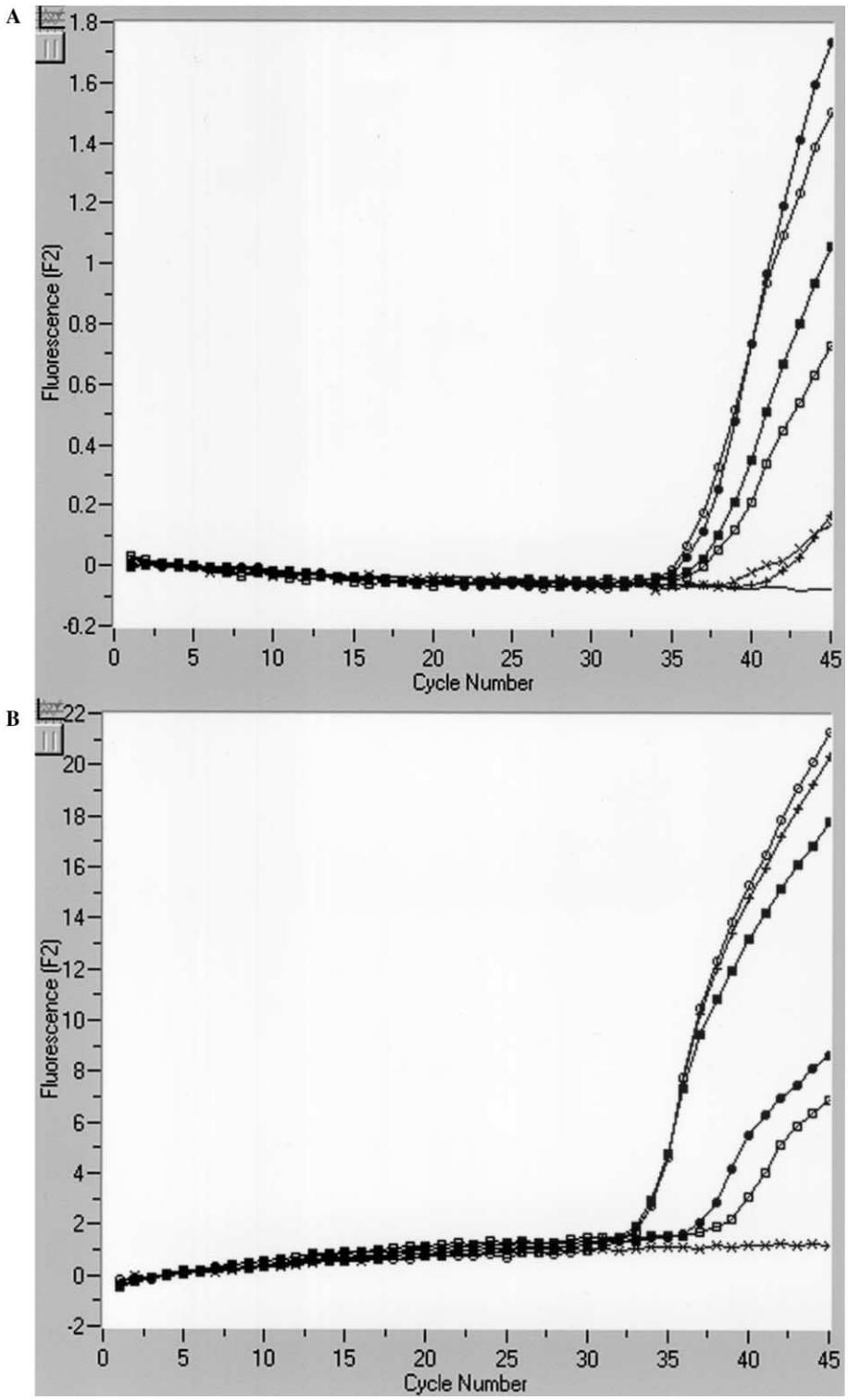


Fig. 1. (A) Real-time fluorescence output of patient samples and controls with *A. fumigatus* probes using the PK-F and F1-R oligonucleotide pair. The amplification plot was generated using the LC instrument and the LC software version 3.5. The *x* axis is the number of cycles and the *y* axis is the fluorescence output at 640 nm (F2 channel). The samples and $C_p$ values are as follows: Positive control (1 pg *A. fumigatus* DNA) (open circles), 35.50; Positive control duplicate (1 pg *A. fumigatus* DNA) (solid circles), 35.48; Patient 1 sample C (open squares), 38.79; Patient 1 sample C duplicate (solid squares), 36.38; Patient 1 sample D (large cross), 42.10; Patient 1 sample D duplicate (small cross), 41.92; Negative control (sterile double-distilled water) (solid line). (B) Real-time fluorescence output of patient samples and controls with *C. albicans* probes using the PK-F and F1-R oligonucleotide pair. The amplification plot was generated using the LC instrument and the LC software version 3.5. The *x* axis is the number of cycles and the *y* axis is the fluorescence output at 640 nm (F2 channel). The samples and $C_p$ values are as follows: Positive control (1 pg *C. albicans* DNA) (small cross), 33.10; Patient 3 sample A (solid circles), 36.19; Patient sample A with internal positive control (1 pg *C. albicans* DNA) (solid squares), 32.89; Patient 3 sample B (open squares), 38.00; Patient 3 sample B with internal positive control (1 pg *C. albicans* DNA) (open circles), 33.12; Negative control (sterile double-distilled water) (large cross).

possibly because it depends on the degree of angioinvasion, and therefore its negative predictive value is high. Fungal PCR has been performed on whole blood (Einsele et al., 1997; Lass-Florl et al., 2001; Loeffler et al., 2000a; Loeffler et al., 2000b), serum (Williamson et al., 2000; Yamakami et al., 1996), and plasma (Loeffler et al., 2000a). Comparisons between plasma and whole blood have been reported for the detection of *Aspergillus* spp. DNA (Loeffler et al., 2000b) which suggest that whole blood may be preferable to both serum or plasma. However, a study using the rabbit model of systemic candidiasis has suggested that serum may be preferable to whole blood (Bougnoux et al., 1999). There were however a number of limitations in this animal model study that may not be applicable to the dynamics of fungal DNA for fungal cells in the immunosuppresed human host. Whether serum would be more useful than whole blood in immunosuppressed patients remains unanswered. Therefore, although the optimal specimen in the immunosuppressed human host remains controversial, we chose to use EDTA-anti-coagulated whole blood for analysis, as an aliquot of this specimen contains viable and non-viable fungal cells and free fungal DNA if present. Although the use of whole blood samples may increase PCR inhibition compared with serum samples, we demonstrated no evidence of PCR inhibition in our study, suggesting successful removal of PCR inhibitory substances from whole blood.

In previously published studies on fungal PCR, a diversity of extraction methods, amplification protocols, sample volume, and primer selection, together with a range of source material studied, makes direct comparison of published studies difficult. To address this we chose a DNA extraction method based on elution of nucleic acids from fiber fleece of commercial spin columns. The use of commercial buffers and reagents ensures a certain degree of standardization that may make future comparisons between different PCR protocols more reliable. In our study we used a volume of 200 $\mu$L of whole blood, although a larger volume could further improve the assays sensitivity. However, there is a lack of suitable commercial methods of DNA extraction available for larger volumes of blood that meet the stringent degree of sterility or purity of buffers and reagents that is required to perform fungal PCR. In addition, improved reproducibility of conventional fungal PCR methods is needed. In our study, the crossing points and the signal intensity results from seeded and spiked whole blood samples highly reproducible following the approach described. Other workers using real-time PCR techniques (Kami et al., 2001; Loeffler et al., 2000b) have also shown improved reproducibility, when compared with conventional fungal PCR assays. Finally we standardized the diagnosis of IFIs by using the recently published agreed criteria, as evaluation of clinical studies have shown a poor correlation (Kappa statistic = 0.253) between studies before 1997, even when only proven cases of IFIs are considered (Ascioglu et al., 2001). The use of standardized criteria for the evaluation of new less invasive fungal diagnostic techniques has not been previously been reported.

The SSU ribosomal RNA genes of *A. fumigatus* and *C. albicans* are considered conserved multicopy genes that should enable increased analytical sensitivity. We found an analytical sensitivity of 10 fg (1 genome copy) for both the *A. fumigatus* and *C. albicans* assays, when purified DNA of these fungi were analyzed. The lower level of detection of most published fungal molecular reports varies from 10-100 genome copies, with few reports obtaining a sensitivity of 1 genome copy. In our study we used a 200 $\mu$L of volume of whole blood, though the spin columns can utilize up to 300 $\mu$L of whole blood. Aliquots greater than this amount cannot be processed unless multiple fractions are passed though the same column and may increases the risk of exogenous DNA contamination. The fiber fleece may also become saturated with human cellular DNA. Based on these limitations, if a whole blood sample contains 5 CFU/ml, then the final 50 $\mu$L eluate from the spin column may only contain a single genome copy. If only 5 $\mu$L of eluate is then used in the PCR master mix, the likelihood of the master mix containing a single genome copy, based on a 100% efficiency of DNA extraction, is only 10%. In addition, extraction of fungal DNA may not be 100% efficient, as not all human cellular DNA can be removed. Target DNA purified from clinical samples often co-precipitates with human DNA, which leads to nonspecific hybridization of oligonucleotides and mis-priming that may interfere with optimal PCR kinetics. Therefore the likelihood of fungal PCR products being formed may be significantly less than 10%. Hopefully further advances in fungal DNA extraction and purification will help resolve these problems.

Probe hybridization with two probes each specific for *A. fumigatus* and *C. albicans* DNA were used in this study as this enables a higher degree of specificity than genus-specific or panfungal probes (Van Burik et al., 1998). Another advantage of our approach is that additional probes can be developed to improve the range of detectable fungal pathogens. Panfungal probes are more likely to give false-positive results due to exogenous environmental fungal DNA or carry over amplicon contamination. In addition, broad-range primers may compromise the PCR with amplification of exogenous fungal DNA in addition to target fungal DNA. Contamination of fungal PCR assays has been documented with fungi including *Aspergillus fumigatus*, *Saccharomyces cerevisiae* and *Acremonium spp*. (Loeffler et al., 1999). The putative sources of exogenous fungal DNA include environmental air-borne fungal spores, and commercial products commonly used for fungal DNA extraction including PCR buffers or enzymes, such as zymolase and lyticase (Loeffler et al., 1999). Lyticase (Sigma) is a partially purified $\gamma$-irradiated powder from a culture of *Arthrobacter luteus* (Scott and Schekman, 1980). Loeffler et al. (1999) demonstrated contaminating fungal DNA with a band seen on gel electrophoresis from 5 U of lyticase, however they could not demonstrate contaminating fungal DNA in the

proteinase K fraction. We also examined commercially available lyticase for the presence of fungal DNA. Though we found contaminating fungal DNA in lyticase (Sigma) by gel-electrophoresis (data not shown); no hybridization to probes was detected using our assay on the LC. However despite the cost, we now routinely use recombinant lyticase to improve PCR efficiency, as exogenous fungal DNA may otherwise expend primers and dNTPs in the mastermix. Other possible sources of contamination that we have considered include; talcum powder from gloves, bench surfaces in the laboratory, paper sterilization bags, and reagents such as water, elution buffer and the ethanol solution used for DNA extraction. We have therefore introduced a number of precautions into the laboratory to address these potential sources of contamination. Since these additional precautions have been implemented we have not seen any evidence of fungal contamination in any of samples used in our real-time fungal PCR research program.

This study evaluated a relatively small number of high-risk patients. However these patients had been thoroughly assessed by a number of both non-invasive and invasive diagnostic techniques. Therefore, the clinical and laboratory diagnosis was clear in most of our patients, enabling at least a preliminary assessment of assay performance. Though 2 of the patients were not from the high-risk groups used to establish the agreed criteria for IFIs (Ascioglu et al., 2002), we believe that these criteria are likely to be applicable to a wider range of patients than those used for the development of these definitions. Based on analytical and clinical sensitivity, specificity and accuracy, our assay performed favorably when compared with other published assays (Kami et al., 2001; Loeffler et al., 2000b; Williamson et al., 2000).

In summary, we report the use of a real-time fungal PCR assay using the LC instrument in a diagnostic microbiology laboratory. The use of this technique allows both rapid diagnosis of IFIs and also allows for quantitation of fungal DNA that may be useful for monitoring response to antifungal therapy. Furthermore, the use of highly standardized DNA extraction techniques and the development of highly reproducible PCR assays should enable further progress to be made in the diagnosis of IFIs. In addition, we report for the first time the use of recently published consensus criteria for the diagnosis of IFIs in the evaluation of the performance of non-invasive fungal diagnostics. Standardized clinical criteria for the diagnosis of IFIs should now greatly improve the comparability of future studies of fungal diagnostics and trials of new antifungal therapies. In future studies we plan to prospectively evaluate our real-time PCR assay with a larger and more homogeneous group of high-risk patients with hematologic malignancies. The real-time PCR will be compared with the sandwich ELISA assay for the detection of galactomannan and regular high resolution CT scanning of the chest, to further evaluate the comparative clinical utility of these noninvasive tests.

## Acknowledgments

We thank A/Prof. James Flexman for critical review and comments of the manuscript. We also thank the staff in Molecular Laboratory, in the Department of Microbiology and Infectious Diseases, Royal Perth Hospital for invaluable technical assistance. This work was partly supported by a grant from AMGEN Australia Pty Ltd.

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## 2.6 Conclusion

Regarding the specific aims, the above studies showed that:

a) The ITS sequencing approach is more rapid and more accurate for the identification of fungi cultured from clinical samples, compared to traditional methods of identification.
b) The ITS sequencing approach is more rapid and more accurate for the identification of fungi directly from cultured enrichment media, dramatically improving the time to identification.
c) Targeted qPCR methods show utility for the detection of fungi directly from whole blood, albeit lacking in clinical sensitivity.

When read together, the body of work presented in **Chapter 2** is based on the premise that more rapid and definitive methods of identifying fungi are required. Traditional methods of fungal identification are slow and based on subjective interpretation of colony characteristics, microscopy morphology and physiological tests. These traditional methods require specialised staff trained in the use of mycological identification keys and culture techniques. **Publication 1** and **Publication 2** describe the development and application of an ITS PCR and sequencing approach for the identification of a wide variety fungi commonly, grown from either solid media or liquid enrichment media in the clinical diagnostic setting. **Publication 4** describes a species-specific qPCR method for the detection of *C. albicans* and *A. fumigatus* DNA directly in whole blood, capable of detecting *C. albicans* and *A. fumigatus* within 24 hours from sample receipt into the laboratory.

### 2.6.1 The results and impact of ITS PCR and sequencing for the universal identification of fungi from cultures and enrichment media

**Publication 1** describes a PCR method for the ITS regions with automated capillary sequencing for the accurate and timely identification of medically important fungi. This method addresses the challenges of traditional methods of identification, such as paucity and variability of microscopic features and slow culture-based identification techniques. A high overall agreement was observed comparing the phenotypic identification to the ITS sequence identification. However, two discordant identifications between traditional and sequencing methods were noted, highlighting the challenges of phenotypic identification. Both discordant results were from dermatophyte identification resulting from an incorrect identification and the variable surface colony characteristics of *T. interdigitale,* which may resemble *T. mentagrophytes* (52). Even today, the status of these taxa is a field of long-lasting debate, with the *T. interdigitale/T. mentagrophytes* species complex now defined as 9 species, including the hypervirulent (and antifungal resistant) *T. mentagrophytes-related* Indian strain *Trichophyton*

*indotineae* (137). These members of the *T. interdigitale/T. mentagrophytes* species complex cannot be resolved with modern-day methods such as MALDI-TOF and today still rely on ITS sequencing for definitive identification (138, 139).

Adding the identification resolving capabilities of the sequencing method, the molecular approach described was able to achieve a final identification within 48 hours of obtaining a pure culture, compared to 3-11 days for yeasts and 14-21 days for dermatophytes, albeit at additional overall cost. Regardless of the fungus encountered, identification costs for ITS sequencing remained fixed with a reliable identification not dependent on mycological expertise. The impact of ITS sequencing in our laboratory was demonstrated by the cessation of Dalmau plate culture and tedious sugar assimilation tests for yeast identification for all non-*Candida albicans* yeasts, including cessation of Trich trays for the identification of dermatophytes. Today, the ITS sequencing remains relevant in reference-level laboratories for all clinically significant yeasts and dermatophytes which cannot be reliably resolved by MALDI-TOF (52). However, new commercial molecular methods are being used for the direct detection and identification of common *Candida* spp. and dermatophytes in skin, hair and nail samples, greatly improving sensitivity and time to identification than culture (140). However, similar species-level challenges remain with definitive identification of *Trichophyton* spp. within *Trichophyton* complex groups: *T. rubrum* complex (*T. rubrum* and *T. soudanense)*, *T. mentagrophytes* complex (*T. mentagrophytes* and *T. interdigitale)* and *Candida spp.* lumped into two groups differentiating *C. albicans*, *C. guilliermondii*, *C. parapsilosis* complex and *C. glabrata.* Regardless, confirmation of dermatophyte infection allows authority for clinicians to prescribe the antifungal agent terbinafine at significantly reduced cost to the patient, subsidised by the Australian Pharmaceutical Benefits Scheme (140).

Similarly, **Publication 2** describes the ITS method applied mostly to blood cultures and other commonly used liquid-based media for the recovery of fungi from tissues and fluids. The key point of difference compared to **Publication 1**, is that the method was directly applied to blood culture fluid thereby saving time compared to subculture and identification by either conventional methods or the ITS sequencing method that was in routine use. We highlight the challenges with phenotypic methods and the inability to resolve *C. dubliniensis*, *C. kefyr*, *C. parapsilosis*, *C. guillermondii*, *C. orthopsilosis* and some strains of *C. albicans* using VITEK identification, consistent with other studies (117). We also demonstrate the impact to the laboratory and benefits to the clinician with an earlier identification for ITS compared to conventional tests, with similar laboratory costs for both methods. Although not presented, 29% (13/45) of fungi were identified that are intrinsically or more resistant to the azoles (*C. glabrata, C. krusei, S. prolificans*), which may have led to a change in antifungal therapy from

fluconazole to broader acting antifungal agents. In retrospect, a timelier implementation of CHROMagar in our laboratory based on earlier reports (119, 120), would have been advantageous, given that 53% (24/45) were *Candida* spp. that could have been presumptively identified with this media. Today, many laboratories combine initial microscopy with some defining phenotypic characteristics such as germ tube, CHROMagar for *Candidia* spp. and MALDI-TOF (135, 141). Further non-molecular enhancements for blood cultures include direct inoculation of CHROMagar, direct India Ink staining for *Cryptococcus* spp. and direct MALDI-TOF identification from the blood culture fluid (135, 141). Chromogenic medium for the identification of yeasts has also improved with several manufacturers offering a range of media, including CHROMagar Candida Plus for the presumptive identification of *C. auris* (142). The identification of this species is important as it has been recently labelled as a global threat by the U.S. Centers for Disease Control and Prevention (CDC) as it can cause invasive infections, demonstrates high rates of antifungal resistance and can spread rapidly in a health care facility (143). However, this agar is expensive and cannot resolve *C. vulturna* and *C. pseudohaemuloni* from *C. auris*, thereby requiring further confirmation with MALDI-TOF (135, 142).

Failing adequate species identification with the above, cultures can be tested with one of the many auto-microbic systems (e.g. Vitek 2 Yeast), or forwarded to a reference mycology laboratory for further identification (135, 141). In our laboratory, definitive identification is achieved with the sequenced-based method, with ITS considered universally robust to adequately identify a wide variety of fungi, or specific gene targets for more accurate identification of some genus groups (43, 50, 52, 129, 144). Despite these modern-day differences compared to routine methods in 2006, the direct sequence-based method simultaneously resolved three major challenges which are still relevant today: 1) nucleic acid extraction and fungal PCR amplification from a variety of blood culture media, including tissues and fluids; 2) detection and identification of the fungus circumventing time delays due to culture; 3) provide a broad-range species identification to the clinician to assist with appropriate antifungal selection. Hence, both methods described in **Publication 1** and **Publication 2** are still in routine use in our laboratory today with more than 200 requests per year for fungal identification. However, the diagnostic landscape has changed with the implementation of chromogenic medium for yeasts and MALDI-TOF for many common yeasts, dermatophytes and other fungi. Similarly, new qPCR assays such as the AusDiagnostics dermatophyte panel, have redefined rapid diagnostics for superficial mycoses. Today, most requests for ITS PCR and sequencing are for featureless fungi or difficult-to-identify fungi, or as a pathogen exclusion tool whereby common microscopy features are shared between a saprophyte and a potential pathogen. The requests for direct ITS PCR and sequencing from tissues and fluids remain, with more than 100 requests per year from within PathWest, or external customers. The utility of the assay has been greatly improved using

a different combination of primers described in **Publication 1** and **Publication 2** (V9D and ITS4) for PCR tissues and fluids, which mitigates nonspecific human DNA amplification as described with V9D and LS266 (Pryce, 2006, unpublished data). Modern improvements to the approach include the cessation of capillary-based Sanger sequencing to long-read next-generation sequencing, with a new automated bioinformatic pipeline.

### 2.6.2 The results and impact of *C. albicans* and *A. fumigatus* detection in whole blood

Despite the success of the ITS regions for the broader identification of fungi from cultures, sensitivity and non-specificity problems were encountered with the use of ITS to detect fungi directly from whole blood. Rather than breadth of species detection, we focussed on sensitivity with improved specificity and optimised a real-time 18S PCR from whole blood for two common fungal species in the context of urgent life-threatening potential fungal infection. **Publication 4** describes the development and evaluation of a real-time PCR assay using the LightCycler instrument for the detection of *C. albicans* and *A. fumigatus* DNA directly in whole blood. This approach addressed and highlighted the sensitivity challenges with early detection of fungaemia. These challenges included a sensitive sample preparation technique combined with a sensitive and specific qualitative PCR. We also resolve the nonspecific challenges faced with ITS PCR using the 18S rRNA targets. Unique and published 18S primer pairs and probes were developed and assessed for sensitivity, specificity and reproducibility. Here, a high degree of analytical sensitivity, specificity and reproducibility was achieved. Although analytical sensitivity was addressed (1 genomic copy), limitations and additional future challenges were discovered with clinical samples, impacting on clinical sensitivity and specificity. These included endogenous (reagents) and exogenous (environment) sources of contamination and the volume of blood that can be processed for efficient nucleic acid recovery; the likelihood of a PCR reaction containing a single genomic copy was less than 10%. Overall, we report an analytically sensitive and standardised method for detection of the two most common cause of invasive fungal infection (IFI) at the time of this investigation, albeit with low numbers of patients. Our assay performed favourably in terms of analytical sensitivity and specificity to other investigations at the time (133, 145, 146). The results of **Publication 4** showed that detection of fungal DNA for *C. albicans* and *A. fumigatus* from whole blood is achievable, albeit at a suboptimal clinical sensitivity, as evidenced in Table 2 of **Publication 4**, with a high degree of patient mortality in our cohort.

We still face significant challenges in the clinical sensitivity of diagnostic tests for invasive fungal disease, despite many assays showing promise that report a high degree of analytical sensitivity.

Detection of fungaemia is also problematic as deep tissue infections may not result in fungaemia; invasive tissue biopsies are still required to establish a diagnosis. Some standardised serological tests are showing promising results for diagnosing invasive aspergillosis (IA), such as galactomannan enzyme immunoassay and glucan detection systems, however, sensitivity and specificity vary within the studies and are dependent on the patient population involved (48, 147). In contrast, there is a lack of standardised molecular-based assays for IA, which still struggle in terms of clinical sensitivity (48). However, the European Aspergillus PCR Initiative working party of the International Society of Human and Animal Mycoses) was formed in 2006 with the aim of establishing a standard for PCR methodology for the diagnosis of invasive aspergillosis, which led to a number of recommendations for invasive IA and standards for various parts of the assays for IA PCR, rather than a standard PCR assay (148). The advances in the standardisation of clinical criteria and laboratory procedures show that PCR is now sufficiently robust for routine *Aspergillus* diagnostics. As a consequence, there are several commercial assays for the diagnosis of IA, with some having the added advantage of detecting Cyp51A gene associated with azole resistance such as the FungiPlex *Aspergillus* Azole-R assay (149). However, sensitivity (47%-94%) and specificity (88%-99%) of these PCR-based assays are dependent on the specimen used for PCR, such as serum or bronchoalveolar lavage (149).

Whilst the analytical sensitivity of PCR cannot be disputed (single copy gene detection), the challenge is increasing the concentration of the target prior to PCR. This could be achieved by enriching target DNA prior to PCR (target capture) whilst removing unwanted human DNA that would otherwise compete for nucleic acid capture (via MGP's). Examples of human DNA competing with target DNA for reagent resources, which can be learnt from next-generation sequencing (NGS) technologies, are likely to point to the over-abundance of human DNA whilst targeting a few genomic copies of the target; depletion of human DNA (150) or using nucleic acid capture or baits for target enrichment (151), may be the key to resolving this challenge in the future. However, these technologies have only been applied to NGS approaches, not prior to qualitative or quantitative PCR methods for the detection of fungaemia. As targeted NGS methods progress and continue to be improved, the nature of their clinical utility will be better understood. Until significant progress can be made with clinically sensitive and specific molecular approaches, the clinician is still faced with a high degree of uncertainty from diagnostic tests for the diagnosis of fungaemia.

# Chapter 3: *Neisseria gonorrhoeae* NAAT specificity and predicting AMR

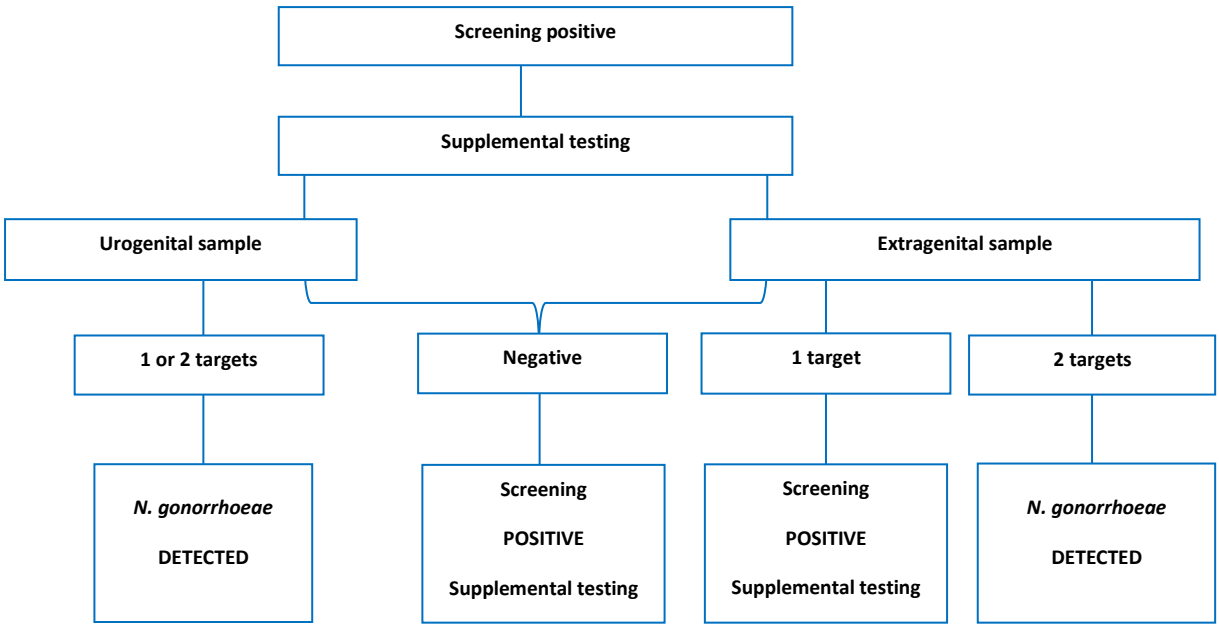
## 3.1 Introduction to prior publications

*N. gonorrhoeae* infections are a major cause of sexually transmitted infections (STIs) worldwide with a WHO estimate of 87 million new infections per year (152). In Australia the annual number of *N. gonorrhoeae* notifications has increased steadily from 15,012 in 2013 to 40,541 in 2023 (153). Infections caused by *N. gonorrhoeae* and associated complications are well-documented (154, 155). In Australia, *N. gonorrhoeae* infections are disproportionately distributed, with a much higher prevalence in Indigenous Australian populations compared to non-Indigenous Australians (156). NAATs have largely replaced culture as the primary method for gonorrhoea diagnosis, with many commercial tests offering sensitive and cost-effective diagnostic testing strategies for easy-to-collect non-invasive specimens such as urine and self-collected swabs, which are commonly collected for STI investigations (157). The other important advantage is the ability to simultaneously detect other causes of STIs, such as *C. trachomatis*; for example, 22% of all NG-positive samples detected by our laboratory are also positive for *C. trachomatis* (158). Many commercial CT/NG assays are available to suit different laboratory requirements, with many continuing to progress to more efficient workflows.

Despite the above advantages, NG-NAATs have been plagued with specificity problems associated with cross-reaction with commensal *Neisseria* species. The problem has been most pronounced for extragenital sites, such as oropharyngeal swabs, where commensal *Neisseriae* are ubiquitous (72, 159-161). For example, an historical report of a first-generation commercial assay demonstrated a false positive rate of 94% for oropharyngeal samples (162). Other examples include false-positive results or false-negative results due to target drop-out have also been reported (60, 163, 164). The first Australian Public Health Laboratory Network (PHLN) guidelines for the use and interpretation of nucleic acid detection tests for *N. gonorrhoeae* testing in Australia were published in 2005 (76). The primary concern of the PHLN recommendations focussed on reducing the likelihood of the laboratory issuing false-positive results. As a consequence, supplemental testing was embedded into Australian routine molecular diagnostics (165), with our laboratory performing supplemental testing since 2006. Another important reason to optimise the specificity of NG-NAAT is the potential serious social consequences of a false positive result, that can lead to issues like relationship breakdowns or sexual assault allegations (166, 167).

As screening assays improved in sensitivity and specificity over time, a new issue emerged with one of the 2005 recommendations: “If a sample is positive in a screening assay but a suitable supplemental assay is negative, then the result should be issued as negative”. I highlighted this issue in 2012 at the Australian Society for Microbiology (ASM) Annual Scientific meeting proceedings titled “Confirmatory rates of *Neisseria gonorrhoeae* from urogenital and non-urogenital sites: need to review current guidelines for *N. gonorrhoeae* confirmation”, where supplemental testing may lead to false negative results (168). Furthermore, data collated from quality assurance programs showed that supplemental testing may lead to false-negative results for samples with low *N. gonorrhoeae* load, i.e., supplemental testing was now leading to sensitivity problems. A subsequent review of the guidelines was undertaken by the National Neisseria Network and published in 2015 focusing on false-negative results (159). Our interpretation of these revised guidelines are shown in Figure 12.

Figure 12. Reporting of *N. gonorrhoeae* supplemental testing results. Reporting of *N. gonorrhoeae* supplemental testing results in our laboratory based on the PHLN guidelines (76, 159). Figure created using PowerPoint. Pryce TM (2024).




The review concluded that supplementary testing remains best practice, but recommended negative supplementary results from NG-positive urogenital screening results should not be reported as negative, without an appropriate explanatory comment indicating that gonococcal infection cannot be excluded; the requirement for supplemental testing should not be relaxed even for urogenital samples. Given the improved sensitivity and specificity of later generation NAATs (163), laboratories may need to change or improve the performance of their supplementary testing methods to negate

the potential negative impacts from suboptimal supplementary test performance. This additional workload and expense of supplementally testing can however be quite onerous for laboratories and has driven the ongoing debate whether supplemental testing is required for certain NG-NAAT platforms and certain sample types (160, 169-171). Despite many second second-generation assays demonstrating substantially less cross-reactivity with non-gonococcal *Neisseria* species than the earlier generation assays (163), reports of non-specificity issues continued. It should be noted though, there was no commercial test available in Australia prior to 2017 which was validated by the manufacturer for testing extragenital sites (**Chapter 1**, Table 3). Given the widespread concerns over the potential for false-positive results, many laboratories have been particularly vigilant when assessing the sensitivity and specificity of new screening assays and supplemental tests. The prior published work presented in this chapter highlights the scientific rigor of these evaluations (79, 158, 161).

Although the history of supplemental testing focussed on addressing specificity issues of screening assays, a new role was emerging; detection of AMR markers. Given heightened concerns over *N. gonorrhoeae* AMR (172), the integration of reliable genotypic AMR markers into NG-NAATs, allows for individualised therapy and enhanced antimicrobial stewardship (173, 174). Also given the decline in culture and the subsequent reduction of antimicrobial resistance data (172), including limitations in conducting *N. gonorrhoeae* AMR surveillance in remote settings, integration of reliable molecular AMR markers into routine supplemental assays has already embedded itself as being a useful addition to enhance culture-based surveillance (175). More recently, these rapid AMR tools provide new potential beyond surveillance, including the ability to directly predict successful treatment options in a timely manner and reduce AMR selection pressure. Currently there are many in-house assays (and some commercial assays) to predict antimicrobial resistance to ciprofloxacin, azithromycin and third-generation cephalosporins (defined as high priority targets) (176). However, the lack of accessible commercial AMR assays impinges the uptake AMR testing in clinical laboratories, as in-house tests require significant development, in-house validation, more oversight and considerably more work effort.

In this chapter, I describe three publications where we address these ongoing issues on *N. gonorrhoeae* non-specificity, including the challenges of validating new assays and assessing new molecular AMR markers. The keys aims are:

a) Investigate the analytical specificity of several commercial screening assays for *N. gonorrhoeae*, including in-house and commercial supplemental assays.
b) Re-investigate a historical case of oropharyngeal non-specificity.
c) Investigate a new supplemental testing approach for the detection of *N. gonorrhoeae*, which also includes a molecular AMR target to predict the susceptibility to ciprofloxacin.
d) Investigate the analytical sensitivity of all these assays in relation to specificity.

In the first publication, we compared two second-generation commercial screening assays; RealTime CT/NG (m2000) and cobas 4800 CT/NG (c4800) in combination with our routine in-house dual-target (*opa*/*porA*) supplemental assay (NG-Duplex), including a third assay, cobas 6800 CT/NG (c6800), as the first screening assay available in Australia with an oropharyngeal and anorectal testing claim.

Our objectives here were:

a) Ascertain whether there were any significant differences in confirmatory rates, or analytical sensitivity in changing from one platform to another, assessed by sample type.
b) To determine if there were any sample types for which we could appropriately limit supplemental testing in the future.

In brief, we retrospectively compared m2000/NG-Duplex results from 2015-2016 with c4800/NG-Duplex results from 2016-2017 and re-tested the 2016-2017 samples using m2000/NG-Duplex, with discordant results resolved with Xpert CT/NG (Xpert). Next, we prospectively tested 400 c4800-positive samples from routine testing in 2017-2018, then tested these retrospectively with the c6800. These separate investigations were pooled in one large study, to define the specificity and sensitivity of these different testing platforms. Importantly, we generated a large supplemental file with all reported $C_q$ values for all assays to assist other laboratories with their own investigations and comparisons. This study is presented in **Publication 5** titled "Second- and third-generation commercial *Neisseria gonorrhoeae* screening assays and the ongoing issues of false-positive results and confirmatory testing (79).

In the second publication, we investigated the improved specificity claims of the c6800 test, in light of a documented c4800 false positive result with an *Neisseria macacae* strain recovered from an oropharyngeal swab (72). Given the low c4800 confirmation rates for oropharyngeal samples observed in **Publication 5** (62-65%), we speculated that the c6800 may still be prone to cross-reaction,

with the previously described *N. macacae* species since both the c4800 and c6800 use the same target (177). We obtained the original *N. macacae* strain with objectives to:

a) Re-affirm the *N. macacae* species identification.
b) Re-test the isolate using c4800 to confirm non-specificity of the c4800 DR-9 target.
c) Test the isolate with c6800 to assess the specificity of the c6800 DR-9 target.
d) Test the isolate using Xpert as a molecular comparator.

In brief, we re-tested the *N. macacae* strain alongside *N. gonorrhoeae* as a standard comparator with the c4800, c6800 and Xpert. This study is presented in **Publication 6** titled "A previously documented *Neisseria macacae* isolate providing a false-positive result with Roche cobas 4800 CT/NG does not cross-react with the later generation cobas 6800 CT/NG assay" (161).

A new challenge faced with the c6800 test was the inaccessibility of the extracted nucleic acids and the subsequent inability to access these for supplemental testing. In the third publication, we investigated the sensitivity and specificity of the c6800 again using a larger sample set and re-validated a new nucleic acid extraction method for the subsequent supplemental testing. At the time, co-author and colleague A/Professor David Whiley was collaborating with SpeeDx to develop a new supplemental test, namely *opa*/*porA*/*gyrA ResistancePlus*GC (RP-GC), which includes a clinically useful and reliable AMR marker for ciprofloxacin susceptibility. We compared the RP-GC assay from c4800 nucleic acids (in routine use) to RP-GC from MagNA Pure 96 (MP96) nucleic acids, as the new c6800 workflow. Additionally, we evaluated Xpert as an additional supplemental testing strategy for c6800 positive/RP-GC negative samples, to generate a workflow with the c6800 that would maximise the NG confirmatory rates and improve specificity for our clinicians.

Our objectives here were to:

a) Re-assess the sensitivity and specificity of the c6800 test for all sample types.
b) Evaluate RP-GC as an NG supplemental test using the MP96 nucleic acid extraction method.
c) Evaluate Xpert as an additional NG supplementary test.
d) Evaluate the sensitivity and specificity of the *gyrA* determinant.
e) Understand the basis of any identified specificity differences (e.g., investigating analytical sensitivity using quantitative PCR).

In brief, we retrospectively retested NG-positive (n = 300) and -negative (n = 150) samples with c4800, c6800, c4800-RP-GC, and MP96-RP-GC. Selected samples were also tested with Xpert for discrepant analysis. Additionally, we transformed the c6800 qualitative test into a quantitative assay using in-house standard curves and performed detection limit studies to determine analytical sensitivity. Finally, the RP-GC assay *gyrA* status was compared to ETEST ciprofloxacin susceptibility for recovered isolates (n = 63). As for **Publication 5**, we generated a large supplemental file with all reported $C_q$ and quantitative results to assist the other laboratories with their own investigations and comparisons. This study is presented in **Publication 7** titled "Maximizing the *Neisseria gonorrhoeae* confirmatory rate and the genotypic detection of ciprofloxacin resistance for samples screened with cobas CT/NG" (158).

Overall, these 3 publications regarding *N. gonorrhoeae* PCR testing have evolved alongside our changing needs in the diagnostic laboratory due to the biology of the *Neisseria* species and emerging issues of AMR. Despite the improvements to successive generations of testing platforms, the on-going need for supplemental testing remains, especially for oropharyngeal samples. Future assays targeting *Neisseria gonorrhoeae* are likely to face similar problems with specificity and evolving AMR, given the historical problems encountered with this challenging organism.

## 3.2 **Publication 5**. Pryce et al., 2021. Second- and third-generation commercial *Neisseria gonorrhoeae* screening assays and the ongoing issues of false-positive results and confirmatory testing.

**Pryce TM**, Hiew VJ, Haygarth EJ, Whiley DM. 2021. Second- and third-generation commercial *Neisseria gonorrhoeae* screening assays and the ongoing issues of false-positive results and confirmatory testing. European Journal of Clinical Microbiology and Infectious Diseases 40:67-75.

DOI link 10.1007/s10096-020-04004-5

As corresponding first author, I conceptualised the study, methods and procedures. I wrote the protocol, tested samples, analysed the results and was the main person drafting the manuscript. DW assisted with result analysis. All other authors either assisted with testing, assisted collating testing data and/or contributed to the writing and editing. All authors reviewed the manuscript prior to publication.

2024 Clarivate journal impact factor from Journal Citation Reports: 3.7

European Journal of Clinical Microbiology & Infectious Diseases (2021) 40:67–75
https://doi.org/10.1007/s10096-020-04004-5

ORIGINAL ARTICLE

# Second- and third-generation commercial *Neisseria gonorrhoeae* screening assays and the ongoing issues of false-positive results and confirmatory testing

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Received: 18 June 2020 / Accepted: 30 July 2020 / Published online: 7 August 2020


## Abstract

Supplementary nucleic acid amplification tests for *Neisseria gonorrhoeae* (NG) are widely used to circumvent specificity problems often associated with extragenital sites. This study was prompted by our observations and concerns from local sexual health physicians over increased discrepancies between Roche cobas 4800 CT/NG (c4800) and our in-house supplementary NG-PCR (NG-duplex) for oropharyngeal samples, when compared with Abbott RealTime CT/NG (m2000) performed prior. Here, we investigated these differences. Three banks of NG-positive samples were used. Bank 1 ($n = 344$) were screened using m2000. Banks 2 ($n = 344$) and 3 ($n = 400$) were screened using c4800. Remnant nucleic acids from all banks were tested using NG-duplex as part of routine testing. Bank 2 samples were further tested using m2000, some selectively tested using Cepheid Xpert CT/NG. Bank 3 samples were further tested using cobas CT/NG (cobas 6800 system). Confirmatory rates were significantly ($p < 0.0001$) higher for m2000 compared with c4800, with oropharyngeal samples the key difference. However, we also showed that our NG-duplex failed to confirm some true-positive NG samples. Using an expanded gold standard, confirmatory rates for m2000 and c4800 exceeded 90% for all anatomical sites with the exception of c4800 for oropharyngeal specimens at 78%. The observed discrepancies were due to a combination of c4800 producing false-positive results for oropharyngeal samples as well as sensitivity issues related to the NG-duplex assay. The data highlight the ongoing need for NG supplemental nucleic acid testing for oropharyngeal samples but also emphasise the need for careful selection of supplementary methods.

**Keywords** *Neisseria gonorrhoeae* · Commercial screening assays · False-positive results · Confirmatory testing

## Introduction

Globally there are an estimated 78 million cases of gonorrhoea occurring each year [1]. Accurate diagnosis and subsequent treatment with effective antimicrobials are the mainstay of gonorrhoea management. Microbiological diagnosis of *Neisseria gonorrhoeae* (NG) infection has historically been performed using microscopy and culture; however, over the last two decades, these techniques have been replaced by more sensitive nucleic acid amplification tests (NAAT). Despite the various advantages of using NAAT technology for NG detection, NG-NAATs—particularly earlier generation tests—have been plagued by specificity problems associated with cross-reaction with commensal *Neisseria* species. The problem has been most pronounced testing oropharyngeal swabs, where commensal *Neisseria* species are ubiquitous [2–9]. Most commercial NG-NAATs are approved for testing urogenital samples but do not have specific claims for extragenital sites such as oropharyngeal and anorectal swabs. Nevertheless, due to superior sensitivity, NAATs are used widely and recommended for screening oropharyngeal and anorectal swabs, particularly in high-risk patients [9, 10].

To circumvent NG-NAAT specificity problems and help facilitate testing of extragenital sites, supplementary testing (whereby samples testing positive in a screening NG-NAAT are confirmed by a second NAAT) has been advocated by

**Electronic supplementary material** The online version of this article (https://doi.org/10.1007/s10096-020-04004-5) contains supplementary material, which is available to authorized users.

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many jurisdictions to minimise the potential harm associated with NG-NAAT false-positive results. The strategy has been routinely used by diagnostic laboratories worldwide for many years with recommendations for NG supplementary testing, for example, having been in place in Australia since 2005 [5, 8]. The additional workload and expense of supplementary testing can however be quite onerous for laboratories and has driven ongoing debate whether or not supplementary tests are required for certain NG-NAAT platforms [3, 4, 11, 12]. Subsequent improvements in technology and specificity have also contributed to this debate. For example, while there is one documented report of the cobas 4800 CT/NG test (c4800; Roche Diagnostics) cross-reacting with a commensal *Neisseria macacae* strain [7], the c4800 has demonstrated marked improvement in NG analytical specificity compared with the earlier generation COBAS AMPLICOR CT/NG method [6]. Moreover, the third-generation cobas CT/NG test on the cobas 6800 system (c6800; Roche Diagnostics), which targets the same NG *DR-9* region as the c4800, has improved sensitivity compared with c4800 and claims for both oropharyngeal and anorectal specimens (CE/IVD marked kits).

Australian guidelines for the use and interpretation of NG-NAATs advocate the use of supplemental confirmatory test for all sample types, especially extragenital samples [5, 8]. Our laboratory has been performing in-house NG supplemental testing for 14 years to address ongoing concerns with commercial assay specificity. We have used several different screening platforms, starting with the Abbott RealTime CT/NG assay/m2000 system (m2000) before moving to the c4800 (current) and more recently evaluating the c6800 for NG detection. The in-house supplemental PCR (NG-duplex) has not changed since implementing m2000. Throughout these changes, we have continued to report both the screening and supplementary results from each specimen to clinicians. This study was prompted by our observations and concerns raised by our local sexual health clinicians over increasing numbers of discrepancies between the screening and supplementary results, particularly since we ceased m2000 screening. Specifically, we aimed to (a) ascertain whether there were any significant differences in confirmatory rates between the three different platforms, (b) understand the basis of any identified differences and (c) to determine whether there were any sample types for which we could appropriately limit supplementary testing.

## Materials and methods

### Study overview

Three banks of samples were used for this study (Fig. 1 and Supplementary Material). All samples were received between 2015 and 2018 and the majority were collected from patients at two large specialist sexual health clinics in Perth, Western Australia. The NG prevalence during the study periods were 1.62% (2015–2016), 1.81% (2016–2017) and 1.65% (2018–2019). Bank 1 samples (collected in years 2015–2016) were tested using m2000 and NG-duplex (described below), whereas Bank 2 (collected in years 2016–2017) samples were all tested using c4800, NG-duplex and then subsequently using m2000. The Cepheid Xpert CT/NG test (Xpert) was then selectively applied to discordant samples from Bank 2. Bank 3 samples (collected in years 2017–2018) were tested by c4800, c6800 and NG-duplex. NG culture data from bacteriological investigations was also available via routine testing for a limited number of samples from all three sample banks. All sample banks tested had a similar sample type distribution.

### Sample banks

Bank 1 (Table 1 and Supplementary Table 1) consisted of 344 consecutively collected samples that tested positive for NG by m2000 via routine CT/NG testing. The samples were collected during the time m2000 was our primary NAAT assay used for NG testing. Bank 1 samples were preserved in multi-Collect media (Abbott Molecular) and were prospectively tested with m2000. As part of routine reflex testing, Bank 1 samples were also tested using NG-duplex.

Bank 2 (Table 1 and Supplementary Table 2) consisted of an identical number ($n = 344$) of consecutively collected samples testing positive using c4800. The samples were collected and preserved in cobas PCR media (Roche Diagnostics) and were prospectively tested when c4800 replaced the m2000 in our laboratory for routine NG screening. DNA extracts from the c4800 were reflexed to the NG-duplex as part of routine testing. For the purposes of this study, Bank 2 samples were retrospectively tested using m2000, all within 12 months of sample collection. Discordant results between m2000, c4800 or NG-duplex were further tested using the Xpert assay. It should be noted that the cobas PCR media is not an approved collection media nor validated for either the m2000 or Xpert.

Bank 3 (Table 1 and Supplementary Table 3) consisted of 400 additional consecutively collected samples testing positive by c4800. Samples were preserved in cobas PCR media and were prospectively tested as part of routine testing with c4800, and reflexed to NG-duplex. For this research study, the samples were retrospectively tested with c6800. All retrospective testing was performed in a timely fashion, prior to cobas PCR media expiry and within 12 months of sample collection.

### NG supplemental testing

Australian Public Health Laboratory Network (PHLN) guidelines for the use and interpretation of nucleic acid tests for the detection of NG were followed [5, 8]. As per these PHLN guidelines, all samples screened as NG-positive were retested using an alternative supplemental method (NG-duplex confirmatory assay). This assay is an in-house dual-target PCR

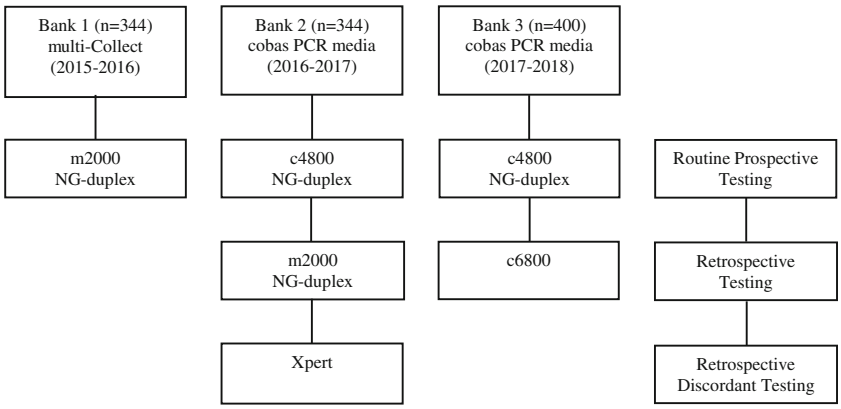


**Fig. 1** Study overview of the sample banks tested with m2000, c4800, NG-duplex, c6800 and Xpert

targeting the *opa* and *porA* genes of *N. gonorrhoeae* [13]. The method has been used in our laboratory since 2012 and was implemented for confirmation of NG-positive nucleic acids from m2000. The PHLN guidelines state that samples testing NG-positive for any screening assays "should also be positive on a reliable supplemental assay before a positive result is reported". For extragenital infections, which are more prone to cross-reaction with commensal species, a higher stringency approach is taken by the PLHN whereby extragenital samples should be positive by "three separate gene" targets before a positive result is reported. To address this in our laboratory, we issue NG-positive results (confirmed) for any urogenital sample that test positive in the screening assay (e.g. c4800) and then provide a positive result for any target in the NG-duplex (either *opa* or *porA* or both). However, for extragenital sites, we only issue a NG-positive result if the sample is positive in the screening assay as well as both targets (i.e. both *opa* and *porA*) in the NG-duplex. For all samples that do not confirm by NG-duplex, an NG screening-positive result is issued along with the number of targets detected using NG-duplex.

## Routine culture

At the time of sample collection for PCR, 185 swabs were collected for routine NG culture for Bank 1, 184 swabs for Bank 2 and 156 swabs for Bank 3. Culture methods are described in the Supplementary Material.

## Detection limit studies

The analytical sensitivity of m2000, c4800, NG-duplex, Xpert and c6800 for the detection of NG was performed using both cobas PCR media and multi-Collect media. The detection

**Table 1** Summary of all results from this study

| Sample bank | Routine testing results | | | | | | | Additional testing results | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | No. of samples | m2000 | c4800 | NG-duplex | Bacterial culture | | | m2000 | c6800 | Xpert positive | Xpert negative |
| | | | | | NG isolated | Not isolated | Not performed | | | | |
| Bank 1 ($n = 344$) | 328 | Positive | n/a | Positive | 101 | 74 | 153 | n/a | n/a | n/a | n/a |
| | 16 | Positive | n/a | Negative | 0 | 10 | 6 | n/a | n/a | n/a | n/a |
| Bank 2 ($n = 344$) | 291 | n/a | Positive | Positive | 94 | 53 | 144 | Positive | n/a | 6 | 1 |
| | 5 | n/a | Positive | Positive | 0 | 1 | 4 | Negative | n/a | 2 | 3 |
| | 26 | n/a | Positive | Negative | 0 | 19 | 7 | Negative | n/a | 1 | 25 |
| | 22 | n/a | Positive | Negative | 5 | 12 | 5 | Positive | n/a | 17 | 5 |
| Bank 3 ($n = 400$) | 343 | n/a | Positive | Positive | 97 | 32 | 214 | n/a | Positive | n/a | n/a |
| | 2 | n/a | Positive | Positive | 0 | 1 | 1 | n/a | Negative | n/a | n/a |
| | 2 | n/a | Positive | Positive | 0 | 0 | 2 | n/a | Inhibited | n/a | n/a |
| | 6 | n/a | Positive | Negative | 1 | 0 | 5 | n/a | Negative | n/a | n/a |
| | 47 | n/a | Positive | Negative | 3 | 22 | 22 | n/a | Positive | n/a | n/a |

*n/a*, not applicable

limit study was performed to investigate if cobas PCR media and multi-Collect media performed equally well for the detection of NG in an oropharyngeal matrix across different assays at the same dilution. In addition, we performed the detection limit studies to compare the results to the package insert claims with the respective collection media. In brief, a ten-fold dilution series of a quantified culture of *N. gonorrhoeae* ATCC 49226 was diluted in a matrix of pooled NG-negative throat specimens in both cobas PCR media and multi-Collect media. Both sets of dilutions were tested in duplicate for all screening assays on the same day (m2000, c4800, Xpert and c6800). In addition, nucleic acids from m2000 and c4800 were also tested with the NG-duplex for comparison on the day of screening.

## Results

The summaries of all results for each sample bank are summarised in Tables 1, 2, 3, and 4. Individual results for all three sample banks (Banks 1, 2 and 3) are provided in the Supplementary Material (Supplementary Tables 1, 2 and 3, respectively). The detection limit investigation is also provided in the Supplementary Material (Supplementary Table 4).

### m2000 and c4800 confirmation rates using NG-duplex PCR (Banks 1 and 2)

Tables 2 and 3 show m2000 and c4800 data from routine testing for Bank 1 ($n = 344$) and Bank 2 ($n = 344$) respectively, including the number of samples confirmed by NG-duplex. The results for m2000 show an overall confirmation rate 95% (328/344 samples), with the lowest rate observed for endocervical swabs at 92% (11/12 samples). Confirmation rates for oropharyngeal and anorectal samples were 93% (82/88) and 92% (60/65), respectively. Culture results were available for 10 of the 16 samples that failed to confirm in the NG-duplex (4 oropharyngeal and 6 anorectal) and *N. gonorrhoeae* was not isolated.

The results for the c4800 (Table 3) show an overall confirmation rate of 86% (296/344), which was significantly ($p < 0.0001$; Fisher's exact test) lower than that observed for m2000 above. The c4800 confirmatory rates for all urogenital samples exceeded > 97%; however, the confirmatory rates for non-urogenital sites were 62% (59/95) for oropharyngeal and 80% (40/50) for anorectal samples. Culture results were available for 36 of the 48 samples that failed to confirm in the NG-duplex (29 oropharyngeal and 7 anorectal) and 5 of these samples (3 oropharyngeal and 2 anorectal) were culture positive.

### Retrospective testing of Bank 2 with m2000 and Xpert

Bank 2 samples (in cobas PCR media) were retested with m2000, and any discordant samples between any NAAT were retested by Xpert (Tables 1 and 3). In total, 93% (319/344) of the c4800 positive samples were positive by one or more methods (NAAT or culture). Notably, 23 of the 48 samples positive by c4800 but negative by NG-duplex provided positive results in m2000 and/or Xpert. A total of 25 samples were positive by the c4800 but negative by all other NAATs as well as bacterial culture (where available; Table 1). These samples are detailed in Bank 2 data; 21/25 were oropharyngeal samples. A direct comparison between the c4800 and m2000 (excluding the NG-duplex results) showed 91% agreement (313/344). The m2000 results demonstrated high agreement with the c4800 for urogenital (97%; 183/189) and anorectal samples (96%; 48/50); however, as per above, agreement was only 76% (72/95) for oropharyngeal samples with 23 oropharyngeal samples testing negative by m2000.

**Table 2** Results for 344 samples preserved in multi-Collect (Bank 1) tested with m2000 and NG-duplex

| Sample type | m2000 | | NG confirmed (%)[b] | Final decision (%)[c] |
|---|---|---|---|---|
| | Pos | *DC* value[a] | | |
| Urine | 102 | 13.18 (19.29, 0.11) | 100 (98.0) | 100 (98.0) |
| Urethral | 57 | 13.35 (19.02, 0.69) | 56 (98.2) | 56 (98.2) |
| Vaginal | 19 | 10.22 (17.23, 0.02) | 18 (94.7) | 18 (94.7) |
| Endocervical | 12 | 10.06 (18.02, 1.28) | 11 (91.7) | 11 (91.7) |
| Unspecified | 1 | 11.18 (11.18, 11.18) | 1 (100) | 1 (100) |
| Oropharyngeal | 88 | 7.86 (17.49, 0.04) | 82 (93.2) | 86 (97.7) |
| Anorectal | 65 | 10.03 (16.81, 0.91) | 60 (92.3) | 65 (100) |
| Total | 344 | | 328 (95.3) | 337 (98.0) |

[a] Mean (min, max)

[b] Based on NG-duplex results (urogenital: *opa* or *porA* positive; non-urogenital: *opa* and *porA* positive)

[c] Based on positive results for either *opa*, *porA* or culture

**Table 3** Results for 344 samples preserved in cobas PCR media (Bank 2) tested with c4800 and NG-duplex

| Sample type | c4800 | | NG confirmed (%)[b] | Final decision (%)[c] |
|---|---|---|---|---|
| | Positive | $C_T$ value [a] | | |
| Urine | 111 | 28.73 (22.40, 40.00) | 110 (99.1) | 110 (99.1) |
| Urethral | 35 | 29.43 (25.70, 41.10) | 34 (97.1) | 34 (97.1) |
| Vaginal | 23 | 30.84 (26.40, 37.60) | 23 (100.0) | 23 (100.0) |
| Endocervical | 20 | 32.07 (26.40, 40.00) | 20 (100.0) | 20 (100.0) |
| Unspecified | 10 | 29.61 (26.40, 33.30) | 10 (100.0) | 10 (100.0) |
| Oropharyngeal | 95 | 36.02 (28.50, 41.70) | 59 (62.1) | 74 (77.9) |
| Anorectal | 50 | 31.55 (24.50, 40.80) | 40 (80.0) | 48 (96.0) |
| Total | 344 | | 296 (86.0) | 319 (92.7) |

[a] Mean (min, max)

[b] Based on NG-duplex results (urogenital: *opa* or *porA* positive; non-urogenital: *opa* and *porA* positive)

[c] Based on positive results for either *opa*, *porA*, m2000, Xpert or culture

## Reassessing NG-duplex data for Banks 1 and 2

To determine whether the PHLN requirements for positivity in three gene targets were impinging upon NG-duplex confirmatory rates for extragenital samples, we re-examined the NG-duplex *opa* and *porA* amplification results for all extragenital specimens in Banks 1 and 2. For Bank 1, there were 11 extragenital specimens (6 oropharyngeal and 5 anorectal) of 16 total specimens that were m2000 positive and not confirmed by NG-duplex. Of these 11 samples, 9 (4 oropharyngeal and 5 anorectal) had amplification evident in the NG-duplex *opa* reaction but were considered "not confirmed" because of a lack of signal in the *porA* target.

For Bank 2, there were 46 extragenital specimens (36 oropharyngeal and 10 anorectal) of 48 total specimens that were c4800 positive and not confirmed by NG-duplex. Of these 46 samples, there were 23 samples (8 anorectal and 15 oropharyngeal) that were positive by m2000, and/or Xpert, with 5/17 also being culture positive; 20 of these 23 specimens had amplification evident in the NG-duplex *opa* reaction but were considered "not confirmed" because of a lack of signal in the *porA* target. No NG-duplex *opa* or *porA* amplification was

**Table 4** Results for 400 samples preserved in cobas PCR media (Bank 3) tested with c4800, NG-duplex and c6800

| Sample type | c4800 | | NG confirmed (%)[b] | c6800 | | | | Final decision (%)[c] |
|---|---|---|---|---|---|---|---|---|
| | Pos | $C_T$ value [a] | | Pos (%) | $C_T$ value[a] | Neg | Inhibited | |
| Urine | 119 | 28.60 (20.1, 40.9) | 116 (97.5) | 118 (99.2) | 24.40 (18.29, 38.13) | 1 | 0 | 116 (97.5) |
| Urethral | 40 | 28.72 (24.9, 34.2) | 40 (100) | 40 (100) | 21.65 (19.14, 26.49) | 0 | 0 | 40 (100) |
| Endocervical | 18 | 31.62 (22.6, 39.0) | 17 (94.4) | 18 (100) | 26.52 (20.26, 38.49) | 0 | 0 | 17 (94.4) |
| Vaginal | 16 | 31.12 (24.4, 40.4) | 14 (87.5) | 15 (93.8) | 27.57 (17.33, 41.77) | 0 | 1 | 15 (93.8) |
| Unspecified | 4 | 30.73 (22.6, 36.7) | 4 (100) | 4 (100) | 25.99 (15.57, 34.25) | 0 | 0 | 4 (100) |
| Oropharyngeal | 103 | 36.13 (28.3, 43.1) | 67 (65.0) | 101 (98.1) | 30.05 (11.57, 38.84) | 2 | 0 | 87 (84.5) |
| Anorectal | 97 | 31.42 (17.7, 42.7) | 85 (87.6) | 91 (93.8) | 24.86 (11.70, 36.94) | 5[d] | 1 | 91 (93.8) |
| Peritoneal fluid | 2 | 33.00 (30.6, 35.4) | 2 (100) | 2 (100) | 27.23 (25.46, 29.00) | 0 | 0 | 2 (100) |
| Eye | 1 | 21.70 | 1 (100) | 1 (100) | 15.31 | 0 | 0 | 1 (100) |
| Total | 400 | | 346 (86.5) | 390 (97.5) | | 8 | 2 | 373 (93.2) |

[a] Mean (min, max)

[b] Based on NG-duplex results (urogenital: *opa* or *porA* positive; non-urogenital: *opa* and *porA* positive)

[c] Based on positive results for either *opa*, *porA* or culture

[d] Two samples CT positive ($C_T$ 31.5, $C_T$ 18.63)

observed for any of the remaining 23 extragenital specimens (2 anorectal and 21 oropharyngeal) that were c4800 positive and negative by all other methods (NAATs and culture).

Based on above, we recalculated the confirmation rates for the m2000 and c4800 tests from Banks 1 and 2 using a revised reference standard: samples were considered true-positive if they were positive in any other test (NAAT and/or culture), including either target (*opa* or *porA*) in the NG-duplex. The revised confirmation rate (Tables 3 and 4) for the m2000 was 98% (337/344) and was still significantly ($p = 0.002$; Fisher's exact test) higher than that of the c4800 revised rate at 93% (319/344). Again, the key difference related to oropharyngeal samples: 98% (86/88) for m2000 and 78% (74/95) for c4800 ($p = 0.00003$; Fisher's exact test).

### Comparison of c4800 and c6800 results (Bank 3)

Samples from Bank 3 ($n = 400$; Tables 1 and 4) showed 98% (390/400) agreement for NG detection in both c4800 and c6800, with only 10 c4800-positive samples (6 anorectal, 2 oropharyngeal, 1 urine and 1 vaginal) failing to provide positive results in the c6800; 8 were negative in c6800 and 2 samples (1 vaginal and 1 anorectal) were inhibited. Of the 390 samples positive by both methods, 47 failed to confirm in the NG-duplex (8 anorectal, 1 endocervical, 34 oropharyngeal, 3 urine and 1 vaginal). When the NG-duplex data were further assessed for the extragenital samples, 6/8 anorectal samples and 18/34 oropharyngeal samples provided amplification in either the *opa* or *porA* targets of the NG-duplex. Three of the 34 oropharyngeal samples were culture positive, including one that was negative in both NG-duplex targets. Overall, 101 oropharyngeal specimens were positive by both c4800 and c6800, and of these 86 (85%) produced amplification in either or both NG-duplex targets and/or were positive by culture (culture results available for 57/101 samples).

### Further analysis of low-load c4800 positive samples

Based on c4800 cycle threshold ($C_T$) values for Bank 2 data in the supplementary file, we observed that samples failing to confirm as NG-positive by any method, all had c4800 NG $C_T$ values of 35 cycles or higher. We therefore compared the confirmation rate of oropharyngeal samples ($n = 60$) to all other samples ($n = 34$) with $C_T$ values $\geq 35$ cycles. The spread of $C_T$ values for both groups was similar; ranging from 35.2 to 41.7 (mean 38.2) cycles for oropharyngeal samples and 35.0 to 41.1 (mean 37.7) cycles for all other samples. Despite this, we observed that c4800-positive oropharyngeal swab specimens were significantly less likely to be confirmed by any method (39/60; 65%) compared with the combined results of all other sample types (30/34; 88%; $p = 0.02$; Fisher's exact test).

### Detection limit investigation

The results for the NG detection limit studies comparing cobas PCR media and multi-Collect media for all assays are shown in Table 5 and Supplementary Table 4. Notably, for cobas PCR media, NG was detected in both replicates at 100 CFU/ml for c4800 and 1000 CFU/ml for NG-duplex (from c4800 extraction). For multi-Collect media, NG was detected in both replicates at 10 CFU/ml for m2000 and 1000 CFU/ml for NG-duplex (from m2000 extraction). Overall, the c4800 was no more sensitive than m2000 for the detection of *N. gonorrhoeae* ATCC 49226, and the NG-duplex provided equivalent performance using extracts from both the c4800 and m2000 platforms. The limits of detection for each commercial assay and associated collection device media were consistent with the package insert claims.

## Discussion

Overall, our results conclusively show how NG-NAAT confirmatory rates significantly decreased ($p < 0.0001$) when we moved from using m2000 to the c4800 for NG-NAAT screening, and not surprisingly were most evident for oropharyngeal samples. However, the reasoning for the differences was not as clear cut as we initially thought, and actually involved issues relating to both our screening and supplementary methods.

False-positive NG results for oropharyngeal samples have previously been reported for c4800 [7]; hence, we initially assumed that this would be the primary reason for the drop in confirmation rates from the m2000 to c4800. Another contributing factor we considered was a reduction in NG-duplex sensitivity when performed from c4800 nucleic acid extracts compared with m2000 extracts; however, our investigations comparing the lower limit of detection of NG-duplex using DNA extracts from both systems did not support this (see Table 5 and Supplementary Table 4). Nevertheless, the results clearly indicated a key factor contributing to the problem was a failure of our in-house PCR to confirm many true c4800 NG-positive specimens. In order to achieve optimal specificity for extragenital specimens, we employed the "positive in three gene targets" rule as per local NG-NAAT guidelines [5], but this in practice lowered sensitivity. A key example of this related to the 36 c4800-postive oropharyngeal samples from Bank 2 reported as "not confirmed"; 13/36 had amplification observed in one of the two targets (*opa* but not *porA*) and all 13 samples were positive by one or more of the additional methods retrospectively applied to the samples as part of this research study (e.g. m2000 and/or Xpert). These 13 samples were clearly c4800 true-positives (rather than false-positives) and reflected a simple but critical flaw in our original testing algorithm. It should also be noted that the failure of the NG-

**Table 5** Detection limit investigation for all assays and targets

| Assay | Limit of detection for each media type[a] | | | |
|---|---|---|---|---|
| | Media | CFU/ml | Media | CFU/ml |
| c6800 | cobas PCR media | 1 | multi-Collect | 0.1 |
| c4800 | cobas PCR media | 10 | multi-Collect | 10 |
| NG-duplex (*opa*) | cobas PCR media[b] | 100 | multi-Collect[b] | 100 |
| NG-duplex (*porA*) | cobas PCR media[b] | 1000 | multi-Collect[b] | 1000 |
| NG-duplex (*opa* and *porA*) | cobas PCR media[b] | 1000 | multi-Collect[b] | 1000 |
| m2000 | cobas PCR media | 10 | multi-Collect | 10 |
| NG-duplex (*opa*) | cobas PCR media[c] | 1 | multi-Collect[c] | 1 |
| NG-duplex (*porA*) | cobas PCR media[c] | 10 | multi-Collect[c] | 1000 |
| NG-duplex (*opa* and *porA*) | cobas PCR media[c] | 10 | multi-Collect[c] | 1000 |
| Xpert (NG2) | cobas PCR media | 1 | multi-Collect | 1 |
| Xpert (NG4) | cobas PCR media | 10 | multi-Collect | 1 |
| Xpert (NG2 and NG4) | cobas PCR media | 10 | multi-Collect | 1 |

[a] Lowest CFU/ml detectable in both replicates (Supplemental Material - Detection Limit Investigation)

[b] DNA extract from the c4800 was tested by NG-duplex

[c] DNA extract from the m2000 was tested by NG-duplex

duplex to detect these true-positives occurred despite the assay passing all appropriate internal quality control processes as well as external quality assurance panels. Thus, this is a timely reminder that in some circumstances potential assay performance problems can only be identified when data are analysed at a population level. A notable example of the latter was the *Chlamydia trachomatis* identified in Sweden in 2006 following an unexpected 25% decrease in *C. trachomatis* infections; the variant had a deletion in sequence targets of the *C. trachomatis* tests manufactured by Abbott and Roche [14]. In fact, NG is well recognised for its genetic diversity and NG variants producing false-negative results for either the *porA* or *opa* PCR assays (but not both targets simultaneously) have previously been observed in the Australian population at low prevalence [15]. Therefore, sequence variation may have accounted for some of the observed false-negative results.

From a public health perspective, the above results provide a noteworthy example of how supplementary NG-NAAT testing can in some circumstances do more harm than good. In the above cases, the testing errors could conceivably have led to infected individuals (in the absence of other clinical signs or risk factors) failing to receive treatment. Fortunately, however, we believe our decision to report both screening and supplementary results would have helped alleviate this, as the discrepancy and thus inconclusiveness of the testing would have been flagged to local clinicians. For example, local clinicians have, to date, remained suspect over oropharyngeal samples providing NG screening-positive/supplementary negative (both targets) in high-risk patients, and so have asked such patients whether they would be still be willing to receive NG treatment. For oropharyngeal samples providing positive results for only one supplementary target (*opa* or *porA*), clinicians have (justifiably based on the data from this study) been more confident of a true-positive result and have recommended treatment. Having identified these issues, we are now changing our NG-NAAT algorithm, and based on these data will report on extragenital sites as NG-detected (confirmed) if they provide positive results in *either* NG-duplex target (rather than needing to be positive in both). While only applied to select samples in the study, our data also suggest the Xpert assay may be a more suitable method for confirmation of c4800 results.

Notwithstanding the above, and taking into account all available testing data in this study, we still found the confirmation rate (based on "final decision data") for oropharyngeal samples in the c4800 remained unacceptably low at 78%. This is well below the recommended threshold of 90% [5, 8] and is consistent with, albeit slightly lower than, the 80% to 88.5% c4800 oropharyngeal confirmation rates previously reported in other studies [4, 8]. In contrast, all other sites exceeded 90% in the c4800 (again largely consistent with previous findings of [4, 8]), and all urogenital and extragenital sites exceeded 90% in the m2000. Thus, while these data may lend further weight ceasing supplementary NG-NAAT testing in certain circumstances, our data do not support ceasing confirmatory testing oropharyngeal samples on the c4800. Given the similarities in test performance and (to our knowledge) the fact the same sequence targets are used by the c4800 and c6800 NG

methods, these results also, in our opinion, raise questions over whether the c6800 NG assay should be used for screening of oropharyngeal specimens in the absence of a reliable supplementary test. Interestingly, unlike the Australian kit insert, the c6800 version of the NG assay in the USA does not state oropharyngeal and anorectal claims. Thus, in our opinion, further studies are needed to investigate c6800 performance, and we will continue to utilise supplementary testing of c6800 NG-positive oropharyngeal samples in our laboratory despite the fact the c6800 assay, approved for use in Australia (CE/IVD marked), has an oropharyngeal claim.

There are some limitations of this study. Firstly, cobas PCR media is not an approved collection media for m2000 or Xpert, and so the possibility of false-negative results in these methods caused by the use of the non-validated media cannot be excluded. Likewise, not all of the testing was conducted at the same time, with some of the retrospective testing conducted 3 to 6 months after the initial samples were collected. According to the manufacturer, all samples collected in cobas PCR media and tested with the c6800 are however stable for 12 months at 2–30°C. Based on this, we infer that the samples were adequately preserved for other testing methods. Additionally, while a previous study has shown evidence of a commensal *Neisseria* strain cross-reacting with the c4800 [7], there was no evidence of such strains uncovered by our culture testing. In fact, extensive culture-based investigations were performed (data not shown), such as re-examining NG-negative oropharyngeal cultures that were c4800 NG-positive, and testing a sweep of the primary inoculum and colonies with c4800. However, we did not detect any c4800 NG-positive material. Finally, we did not sequence positive samples or isolates to investigate the potential for false-negative results arising from sequence variants.

Prompted by concerns raised by our local clinicians, here we again show the challenges of teasing out true-positive from true-negative results utilising later generation NG-NAATs. Overall, our data highlight the ongoing challenges associated with using *N. gonorrhoeae* NAATs, and how notable differences in performance are still evident even using the later generation NG-NAAT methods. Specifically, the data question the need for supplemental confirmatory testing for urogenital and potentially rectal samples for the c4800/c6800 and m2000 assays, but indicate supplemental testing is still warranted for oropharyngeal samples. The data also highlight the need for careful selection of supplementary methods. Moreover, our experiences here further highlight the need for maintaining a solid rapport between the laboratory and the clinicians receiving the results, particularly so as to minimise any potential negative impact that changes in testing practice may have upon patient management.

**Acknowledgements** We acknowledge the dedication of all staff: Sexual Health Clinic Royal Perth Hospital, South Terrace Sexual Health Clinic Fremantle Hospital, Fiona Stanley Hospital Sexual Health, Magenta Clinic Northbridge, Department of Clinical Microbiology, PathWest Laboratory Medicine WA, Fiona Stanley Hospital, Murdoch. We thank A/Prof Mark Chatfield from The University of Queensland for assistance with the statistical analyses and Arron Radinger for prevalence data (Department of Clinical Microbiology, PathWest Laboratory Medicine WA, Fiona Stanley Hospital, Murdoch).

**Authors' contributions** TP: Conceptualisation, formal analysis, investigation, writing–original draft, review and editing.
VH: investigation, writing–review and editing.
EH: investigation, writing–review and editing.
DW: conceptualisation, formal analysis, writing–review and editing.

**Funding information** All funding was internal (PathWest Laboratory Medicine WA) and there were no external funding sources.

**Data availability** Supplementary material provided as a supplementary file (entire data for this study).

## Compliance with ethical standards

**Conflict of interest** TP: Nil
VH: Nil
EH: Nil
DW: reports research funding from SpeeDx Pty Ltd., and SpeedDx was not part of this study whatsoever.
We confirm that Abbott, Roche or Cepheid had no role in the design of this study, the collection, analysis, interpretation of data, or the write-up of the manuscript.

**Ethical approval** Not applicable: according to the National Statement on Ethical Conduct in Human Research 2007 (Updated May 2015) by the National Health and Medical Research Council, Australian Research Council and Australian Vice-Chancellors' Committee.

**Consent to participate** Not applicable (as above)

**Consent for publication** Not applicable (as above)

**Code availability** Not applicable

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3.3 **Publication 6**. Pryce et al., 2023. A previously documented *Neisseria macacae* isolate providing a false-positive result with Roche cobas 4800 CT/NG does not cross-react with the later generation cobas 6800 CT/NG assay.

**Pryce TM**, Bromhead C, Whiley DM. 2023. A previously documented *Neisseria macacae* isolate providing a false-positive result with Roche cobas 4800 CT/NG does not cross-react with the later generation cobas 6800 CT/NG assay. European Journal of Clinical Microbiology and Infectious Diseases 42:121-123.

DOI link 10.1007/s10096-022-04519-z

As corresponding first author, I conceptualised the study, methods and procedures. I prepared all the samples for the investigation and performed all the laboratory testing and experiments. I wrote the protocol, collected and analysed the results and was the main person drafting the manuscript. All other authors contributed to the writing and editing. All authors reviewed the manuscript prior to publication.

2024 Clarivate journal impact factor from Journal Citation Reports: 3.7

European Journal of Clinical Microbiology & Infectious Diseases (2023) 42:121–123
https://doi.org/10.1007/s10096-022-04519-z

LETTER TO THE EDITOR

# A previously documented *Neisseria macacae* isolate providing a false-positive result with Roche cobas 4800 CT/NG does not cross-react with the later generation cobas 6800 CT/NG assay

**Todd M. Pryce[1] · Collette Bromhead[2] · David M. Whiley[3]**

Received: 15 August 2022 / Accepted: 31 October 2022 / Published online: 14 November 2022


Many diagnostic nucleic acid tests (NAATs) for *Neisseria gonorrhoeae* (NG), particularly earlier generation tests, have been beset with specificity problems associated with cross-reaction with commensal *Neisseria* species. The problem has been most pronounced when testing oropharyngeal swabs, where commensal *Neisseria* species are ubiquitous [1–7]. NAATs are used widely and recommended for screening for NG urogenital infection, and more recently for oropharyngeal and anorectal swabs, particularly in high-risk patients [8, 9]. However, until recently, many diagnostic nucleic acid tests (NAATs) for NG were not validated nor marketed for testing extragenital sites, including oropharyngeal swabs because of the risk of false-positive results. To help facilitate testing in these sites, supplementary testing (whereby samples testing positive in a screening NAAT are confirmed by a second NAAT) has been advocated in many jurisdictions. This conservative strategy has been in routine use in Australia since 2005 and in New Zealand since 2012 [1, 4].

The first confirmed clinical NG false-positive result using the cobas 4800 CT/NG assay (c4800; Roche Molecular Systems) was reported from a pharyngeal swab from a patient in New Zealand in 2012 [6]. A commensal *Neisseria* strain was subsequently recovered from the oropharynx of the patient by bacterial culture and a cross-reaction with the c4800 assay was confirmed, with a pure suspension of the isolate testing positive for the NG target of the c4800 assay. A precise species could not be ascertained for the strain; however, it was categorically not *N. gonorrhoeae*. The isolate was initially identified as *Neisseria macacae* by MALDI-TOF MS (Bruker Daltonics), *Neisseria flava* by partial 16S rDNA sequencing and *Neisseria subflava* biovar *perflava* via phenotypic testing performed at the Environmental and Science Research (ESR) reference laboratory (Porirua, New Zealand; where it was lyophilised for long-term storage and designated as *Neisseria macacae* ESR 4671). The isolate and recovered c4800 nucleic acid tested negative with Abbott Realtime CT/NG assay (m2000; Abbott Molecular) and an in-house *opa*/*porA* assay (NG-duplex), respectively, providing clear evidence of a c4800 NG false-positive result. The authors acknowledged at the time of the investigation that oropharyngeal swabs were not validated by the manufacturer, but concluded that the ongoing need for routine supplemental testing of extragenital sites should be maintained.

Roche has since released the third-generation cobas CT/NG test on the cobas 6800 system (c6800; Roche). The assay was released in Australia in 2017, and unlike the previous c4800 assay, included an additional claim for testing oropharyngeal and anorectal specimens (CE-IVD 10/01/2017 version 1.0). In 2022, the c6800 US-IVD marked assay was revised by the manufacturer (US-IVD 01/02/2022, version 4.0) to include oropharyngeal and anorectal swab specimens in the instructions for use. In a recent study conducted in Western Australia, we evaluated the c6800 assay (CE-IVD, 10/01/2017 version 1.0 and 18/01/2018 version 2.0) for NG detection and specifically compared NG confirmation rates across various sample types with those of the c4800 [10]. The CE-IVD c6800 assay demonstrated improved sensitivity compared to c4800 and showed that supplemental confirmatory testing may not be required for urogenital and potentially rectal samples for the either c4800 or c6800. However, our previous data indicated that supplemental testing is still warranted for oropharyngeal samples for both assays owing to suboptimal confirmation rates (< 90%) [10]. Based on the similar c4800 and c6800 performance data, and specifically

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**Table 1** N. *macacae* ESR 4671 and *N. gonorrhoeae* ATCC 49226 serial dilutions tested with cobas 4800, cobas 6800 and Xpert

| Colony forming units/mL | $\log_{10}$ CFU/mL | Isolate | cobas 4800 testing (swab protocol) | | | | cobas 6800 testing (swab protocol) | | | | Xpert testing | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Replicate 1 ($C_q$) | Replicate 2 ($C_q$) | Mean ($C_q$) | Qualitative result | Replicate 1 ($C_q$) | Replicate 2 ($C_q$) | Mean ($C_q$) | Qualitative result | N2 target ($C_q$) | N4 target ($C_q$) | Qualitative result |
| 1.50E+08 | 8.18 | *N. macacae* ESR 4671 | 27.8 | 27.7 | 27.8 | Positive | Negative | Negative | N/A | Negative | Negative | Negative | Negative |
| 1.50E+07 | 7.18 | *N. macacae* ESR 4671 | 27.7 | 28.1 | 27.9 | Positive | Negative | Negative | N/A | Negative | Negative | Negative | Negative |
| 1.50E+06 | 6.18 | *N. macacae* ESR 4671 | 28.5 | 28.6 | 28.6 | Positive | Negative | Negative | N/A | Negative | Negative | Negative | Negative |
| 1.50E+05 | 5.18 | *N. macacae* ESR 4671 | 30.8 | 31.0 | 30.9 | Positive | Negative | Negative | N/A | Negative | Negative | Negative | Negative |
| 1.50E+04 | 4.18 | *N. macacae* ESR 4671 | 34.1 | 34.3 | 34.2 | Positive | Negative | Negative | N/A | Negative | Negative | Negative | Negative |
| 1.50E+03 | 3.18 | *N. macacae* ESR 4671 | 37.4 | 38.1 | 37.8 | Positive | Negative | Negative | N/A | Negative | Negative | Negative | Negative |
| 1.50E+02 | 2.18 | *N. macacae* ESR 4671 | 39.7 | 41.8 | 40.8 | Positive | Negative | Negative | N/A | Negative | Not tested | Not tested | Not tested |
| 1.50E+01 | 1.18 | *N. macacae* ESR 4671 | Negative | Negative | Negative | Negative | Negative | Negative | N/A | Negative | Not tested | Not tested | Not tested |
| 1.50E+00 | 0.18 | *N. macacae* ESR 4671 | Negative | Negative | Negative | Negative | Negative | Negative | N/A | Negative | Not tested | Not tested | Not tested |
| 1.50E-01 | -0.82 | *N. macacae* ESR 4671 | Negative | Negative | Negative | Negative | Negative | Negative | N/A | Negative | Not tested | Not tested | Not tested |
| 1.50E+08 | 8.18 | *N. gonorrhoeae* ATCC 49226 | 15.7 | 15.8 | 15.8 | Positive | 11.87 | 11.91 | 11.89 | Positive | Not tested | Not tested | Not tested |
| 1.50E+07 | 7.18 | *N. gonorrhoeae* ATCC 49226 | 19.0 | 19.0 | 19.0 | Positive | 15.00 | 14.99 | 15.00 | Positive | Not tested | Not tested | Not tested |
| 1.50E+06 | 6.18 | *N. gonorrhoeae* ATCC 49226 | 22.3 | 22.3 | 22.3 | Positive | 18.58 | 18.31 | 18.45 | Positive | Not tested | Not tested | Not tested |
| 1.50E+05 | 5.18 | *N. gonorrhoeae* ATCC 49226 | 25.9 | 25.6 | 25.8 | Positive | 22.02 | 21.81 | 21.92 | Positive | 19.8 | 19.5 | Positive |
| 1.50E+04 | 4.18 | *N. gonorrhoeae* ATCC 49226 | 29.4 | 29.3 | 29.4 | Positive | 25.50 | 25.51 | 25.51 | Positive | 23.3 | 23.2 | Positive |
| 1.50E+03 | 3.18 | *N. gonorrhoeae* ATCC 49226 | 32.9 | 33.1 | 33.0 | Positive | 29.08 | 29.60 | 29.34 | Positive | 26.3 | 25.5 | Positive |
| 1.50E+02 | 2.18 | *N. gonorrhoeae* ATCC 49226 | 36.2 | 36.5 | 36.4 | Positive | 32.68 | 32.80 | 32.74 | Positive | 29.8 | 29.5 | Positive |
| 1.50E+01 | 1.18 | *N. gonorrhoeae* ATCC 49226 | 37.5 | 38.4 | 38.0 | Positive | 35.09 | 35.71 | 35.40 | Positive | 33.1 | 32.6 | Positive |
| 1.50E+00 | 0.18 | *N. gonorrhoeae* ATCC 49226 | Negative | Negative | Negative | Negative | Negative | 36.10 | 36.10 | Positive | Negative | 36.1 | Negative [a] |
| 1.50E-01 | -0.82 | *N. gonorrhoeae* ATCC 49226 | Negative | Negative | Negative | Negative | Negative | Negative | N/A | Negative | Negative | Negative | Negative |

[a]Both Xpert N2 and N4 targets are required to be positive to return a NG-positive result

the similar low confirmation rates for oropharyngeal samples, we speculated that a problem may lay with the fact that (according to the kit inserts) both the c4800 and c6800 assays target the NG *DR-9* region, and hence the c6800 may still be prone to cross-reaction with the previously described *N. macacae* strain.

In this study, we re-tested *N. macacae* ESR 4671 under controlled experimental conditions in this follow-up investigation. The original isolate was recovered from lyophilised storage and subcultured to CHOC agar (Oxoid; Path-West Media) at 37°C in 5% $CO_2$ for 48 h. We also included *N. gonorrhoeae* ATCC 49226 as a reference strain to evaluate the relative cycle of quantitation ($C_q$) of the *N. macacae* isolate compared to NG when tested with the c4800 method. A 0.5 McFarland standard (equivalent to $1.5 \times 10^8$ colony forming units/mL; CFU/mL) was prepared in 0.85% saline. Ten-fold serial dilutions were prepared from this standard in cobas PCR media using a pooled oropharyngeal matrix over the range of $1.5 \times 10^7$ CFU/mL to an endpoint of $1.5 \times 10^{-1}$ CFU/mL. NG testing was conducted on the c4800, c6800 and the Xpert CT/NG test (Xpert; Cepheid). The standards from each strain were tested in duplicate with the c4800 and c6800 test using the swab testing protocol. The qualitative and $C_q$ results for each assay are shown in Table 1. The results highlighted in Table 1 demonstrate that all *N. macacae* dilutions are positive for c4800 and negative for c6800, providing clear evidence that the *N. macacae* isolate does not cross react with the c6800 test. The c6800 showed improved sensitivity by 1 $\log_{10}$ dilution (1.5 CFU/mL compared to 15 CFU/mL) when compared to the c4800, consistent with previous lower-limit of detection investigations [10]. To ensure the correct isolate was purified for this testing, an extract of *N. macacae* isolate was characterised by full-length 16S rDNA sequencing and the isolate demonstrated a 100% match (1423/1423 bp) to *N. macacae* ATCC 33926 (GenBank reference CP09424.1). The isolate was also retested with Bruker MALDI-TOF MS and identified as *N. macacae*, consistent with previous observations. In addition, negative results were obtained for all *N. macacae* dilutions using the Xpert assay.

Based on testing of the *N. macacae* isolate, we conclude that the c6800 assay has improved analytical specificity for *N. gonorrhoeae* over the former c4800 assay. It is unclear what components of the c6800 have been modified, either in assay design or PCR stringency; however, concerns related to c6800 non-specificity based on the c4800 *N. macacae* report are not warranted in this particular case. Notwithstanding these results, we continue to recommend supplemental testing for oropharyngeal sites providing NG-positive results from c4800 and c6800 assays. More broadly, it should be noted that the appropriateness of NG confirmatory testing depends on the population prevalence and the frequency of inaccurate results, whilst balancing the additional cost to the public healthcare system and the potential harm of false-positive results to individual patients.

**Acknowledgements** We thank Olivia R. Foti for performing the cobas testing and collation of results.

**Author contribution** TP: conceptualisation, formal analysis, investigation, writing—original draft. CB: conceptualisation, formal analysis, writing—review and editing. DW: conceptualisation, formal analysis, writing—review and editing.

**Funding** All funding was internal (PathWest Laboratory Medicine WA) and there were no external funding sources.

**Data availability** Table 1 provides all data for this study.

**Code availability** Not applicable.

## Declarations

**Ethics approval** Not applicable as is a quality exercise: according to the National Statement on Ethical Conduct in Human Research 2007 (updated May 2015) by the National Health and Medical Research Council, Australian Research Council and Australian Vice-Chancellors' Committee.

**Consent to participate** Not applicable (as above).

**Consent for publication** Not applicable (as above).

**Conflict of interest** TP: none. CB: reports educational funding from Roche Diagnostics New Zealand Ltd. DW: reports research funding from SpeeDx Pty Ltd.

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## 3.4 **Publication 7**. Pryce et al., 2023. Maximizing the Neisseria gonorrhoeae confirmatory rate and the genotypic detection of ciprofloxacin resistance for samples screened with cobas CT/NG.

**Pryce TM**, Foti OR, Haygarth EJ, Whiley DM. 2023. Maximizing the *Neisseria gonorrhoeae* confirmatory rate and the genotypic detection of ciprofloxacin resistance for samples screened with cobas CT/NG. Journal of Clinical Microbiology 62(1), e0103923.

DOI link 10.1128/jcm.01039-23

As corresponding first author, I conceptualised the study, methods and procedures. I wrote the protocol, tested selected samples, analysed the results and was the main person drafting the manuscript. DW assisted with result analysis. All other authors either assisted with testing, collated testing data and/or contributed to the writing and editing. All authors reviewed the manuscript prior to publication.

2024 Clarivate journal impact factor from Journal Citation Reports: 6.1

Journal of Clinical Microbiology



# Maximizing the *Neisseria gonorrhoeae* confirmatory rate and the genotypic detection of ciprofloxacin resistance for samples screened with cobas CT/NG

**Todd M. Pryce,[1] Olivia R. Foti,[1] Erin J. Haygarth,[1] David M. Whiley[2]**

**AUTHOR AFFILIATIONS** See affiliation list on p. 13.

**ABSTRACT** Supplementary nucleic acid amplification testing for *Neisseria gonorrhoeae* (NG) is widely used to circumvent specificity problems associated with extragenital sites. Here, we compared different supplementary approaches for confirming NG-positive samples from the cobas 4800 CT/NG (c4800) and cobas 6800 CT/NG (c6800) assays using the *ResistancePlus*GC (RP-GC) assay, which in addition to detecting NG, also predicts ciprofloxacin susceptibility via NG *gyrA* characterization. Two different nucleic acid extraction techniques were investigated for RP-GC detection; extracts from c4800 (c4800-RP-GC) and MagNA Pure 96 (MP96-RP-GC). NG-positive ($n$ = 300) and -negative ($n$ = 150) samples in cobas PCR media from routine c4800 testing were retrospectively retested with c4800, c6800, c4800-RP-GC, and MP96-RP-GC. Selected samples were also tested with Xpert CT/NG (Xpert) for discrepant analysis. The *gyrA* status was compared to ETEST ciprofloxacin susceptibility or non-susceptibility for recovered isolates ($n$ = 63). Extragenital confirmatory rates were higher for MP96-RP-GC (131/140; 93.6%) compared to c4800-RP-GC (126/146; 86.3%), albeit not significantly ($P$ = 0.6677). Of 9 samples testing positive by c6800 and negative by MP96-RP-GC, 7/9 (77.8%) were also negative by Xpert. By contrast, the number of samples returning a valid *gyrA* status was significantly ($P$ = 0.0003) higher for MP96-RP-GC (270/293; 92.2%) compared to c4800-RP-GC (245/298; 82.2%). The overall MP96-RP-GC *gyrA* status correlated 98.4% (61/62) with the reported ciprofloxacin sensitive (35/36; 97.2%) or non-susceptible (26/26; 100%) phenotype. Improved RP-GC confirmatory rates and reported *gyrA* status were observed using MP96 nucleic acids compared to c4800 extracts. The data further highlight the ongoing need for NG supplemental testing for oropharyngeal samples.

**KEYWORDS** *Neisseria gonorrhoeae*, confirmatory testing, *gyrA*, ciprofloxacin susceptibility

Sexually transmitted infections caused by *Neisseria gonorrhoeae* (NG) are estimated to account for 87 million new infections per year (1). Transmission by direct mucosal contact can lead to asymptomatic or symptomatic infections in the urethra, endocervix, rectum, and pharynx. Nucleic acid amplification tests (NAATs) have largely replaced culture as the primary method for gonorrhea diagnosis (2). Following the implementation of NAATs for NG screening, specificity problems associated with cross-reaction with commensal *Neisseria* species have widely been reported. The problem has been most pronounced in testing oropharyngeal swabs, where commensal *Neisseria* species are ubiquitous (3–5). Earlier generation commercial assays lacked specific claims for testing oropharyngeal samples; however, third-generation commercial NG-NAATs have progressed to include performance claims for extragenital sites, such as oropharyngeal and anorectal swabs (2, 6, 7).

**Editor** Daniel D. Rhoads, Cleveland Clinic, Cleveland, Ohio, USA

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David M. Whiley's conflict of interest: reports research funding from SpeeDx Pty Ltd.

**Received** 9 August 2023
**Accepted** 27 October 2023
**Published** 12 December 2023

Supplementary testing (whereby samples testing positive in a screening NG-NAAT are confirmed by a second NAAT) has been widely implemented by laboratories to address the issues of non-specificity (8–10). Our laboratory recently evaluated two versions of the cobas CT/NG assay (Roche Molecular Systems Inc., Branchburg, NJ, USA) using the cobas 4800 (c4800) and cobas 6800 (c6800) testing platforms. We showed that supplemental confirmatory testing may not be required for urogenital sites but is warranted for oropharyngeal samples for both assays owing to suboptimal confirmation rates (7). Another recent c6800 study has shown suboptimal confirmatory rates for oropharyngeal samples and advocated secondary testing for oropharyngeal samples in line with the UK guidelines (11).

NG infection is frequently treated empirically upon clinical presentation; however, the efficacy of antimicrobial therapy is threatened by the development of successive NG antimicrobial resistance (AMR) (12, 13). Given the decline in culture and the subsequent reduction of antimicrobial susceptibility data (14), the integration of reliable genotypic AMR markers into NG NAATs allows more appropriate and personalized therapy to combat the growing state of AMR (15). Currently, the prediction of AMR based on genetic changes is most accurate for fluoroquinolones (15–19). Codon 91 gyrase A (*gyrA*) testing has shown to be a reliable predictor of ciprofloxacin resistance in NG and tests targeting this marker may reduce the use of ceftriaxone (20). Considering the pharynx has been suggested as an important site for AMR development due to non-gonococcal *Neisseria* species at the pharyngeal mucosa (21), a dual-purpose NG supplemental test that includes AMR markers would be ideal, particularly for oropharyngeal sites where specificity issues have been a concern.

This study was prompted by our previous concerns relating to suboptimal oropharyngeal confirmatory rates with an in-house supplemental assay detecting *opa* and *porA* (7). The *ResistancePlus*GC assay (RP-GC) (SpeeDx, Eveleigh, NSW, Australia) is a commercial supplemental test that simultaneously detects NG (dual target detection of *opa* and *porA*), the *gyrA* 91S (wild type), or the *gyrA* 91F mutation associated with resistance to ciprofloxacin. This test can be performed from nucleic acid recovered from the cobas 4800 (c4800-RP-GC) or Roche MagNA Pure 96 (MP96-RP-GC) nucleic acids following the methods issued by the manufacturer. Given RP-GC shares the same targets as our in-house assay and co-detects an AMR target, we aimed to assess the suitability for NG confirmation in our population. However, the challenge with NG confirmation following c6800 screening compared to c4800 is the lack of accessible DNA extracts from the c6800 system. Hence, we also sought to compare the MP96 and c4800 extraction methods for RP-GC detection for all samples preserved in cobas PCR media. Xpert CT/NG (Xpert) (Cepheid, Sunnyvale, CA, USA) testing was selectively applied to samples to better understand confirmation data and bacterial culture results were used to assess RP-GC *gyrA* results.

## MATERIALS AND METHODS

### Study overview and sample testing

A study overview is presented in Fig. 1. Consecutive NG-positive ($n$ = 300) and -negative ($n$ = 150) samples preserved in cobas PCR media (Roche) were collected in the years 2021–2022, primarily from two large specialist sexual health clinics in Perth, Western Australia. The samples were routinely tested using c4800 following the instructions issued by the manufacturer (Roche) and were selected for this study based on these routine testing results. Samples were stored at room temperature in accordance with the instructions issued by the manufacturer. For this study, all samples were retrospectively retested in a blinded fashion with c4800 and c6800. RP-GC was performed from nucleic acids recovered from c4800 and from MP96 according to the methods issued by the manufacturer (SpeeDx). Briefly, 200 µL of the sample was extracted with MP96 using the MagNA Pure 96 DNA and Viral NA Small Volume Kit and the Pathogen Universal 200 protocol (Roche). An elution volume of 50 µL was used. A volume of 5 µL of either

c4800 or MP96 nucleic acids was added to 15 µL of RP-GC master mix. Amplification and detection were performed using the LightCycler 480 (II) instrument using the PCR cycling conditions issued by the manufacturer (SpeeDx). Result analysis was performed using RP-GC (LC480) software version 1.0 with no modifications to target calling. The final NG supplemental result (confirmed or not confirmed) was interpreted according to the NG supplemental testing algorithm described below. Xpert was performed using 1 mL of cobas PCR media on selected samples tested with c6800 according to the NG supplemental testing algorithm below. We note that cobas PCR media has not been validated by the manufacturer for Xpert. However, for consistency, we used the same sample volume of cobas PCR media as stated for the manufacturer's recommended Xpert urine and vaginal/endocervical collection devices. All retesting was performed on two separate days with a single amplification lot number for each respective assay (c4800, c6800, RP-GC, and Xpert), with RP-GC supplemental testing performed from stored nucleic acids (4°C) within 24 hours of retesting. All retesting of cobas PCR media was performed within 12 months of the sample collection date in line with the manufacturer's recommendations. NG culture data from bacteriological investigations were also available via routine testing for a limited number of samples. Routine media included blood agar, CHOC, and GC Lect (Oxoid; PathWest Media). Samples were cultured at 37°C in 5% CO2 for 48 hours. All NG isolates recovered from culture were identified using BD BBL Oxidase (Becton, Dickinson and Company, USA), MALDI Biotyper (Bruker Daltonik, GmbH), and VITEK 2 NH card (bioMérieux, France). Culture swabs were collected either at the time of sample collection for molecular testing, or subsequently within 12 days of the NG screening result. Susceptibility to ciprofloxacin was performed on all isolates using ETEST Ciprofloxacin (ETEST; bioMérieux, Marcy-l'Etoile, France) according to the instructions for use and interpreted using the Clinical and Laboratory Standards Institute (CLSI) guidelines (22). Culture results are described in the Supplementary Material.

## NG supplemental testing

As per Australian Public Health Laboratory Network (PHLN) guidelines for the use and interpretation of nucleic acid tests for the detection of NG, all samples were subjected to confirmatory testing (5, 8). For this study, all results (screen and confirmatory) were interpreted as per the assay kit inserts. Note that the RP-GC utilizes both *opa* and *porA* for

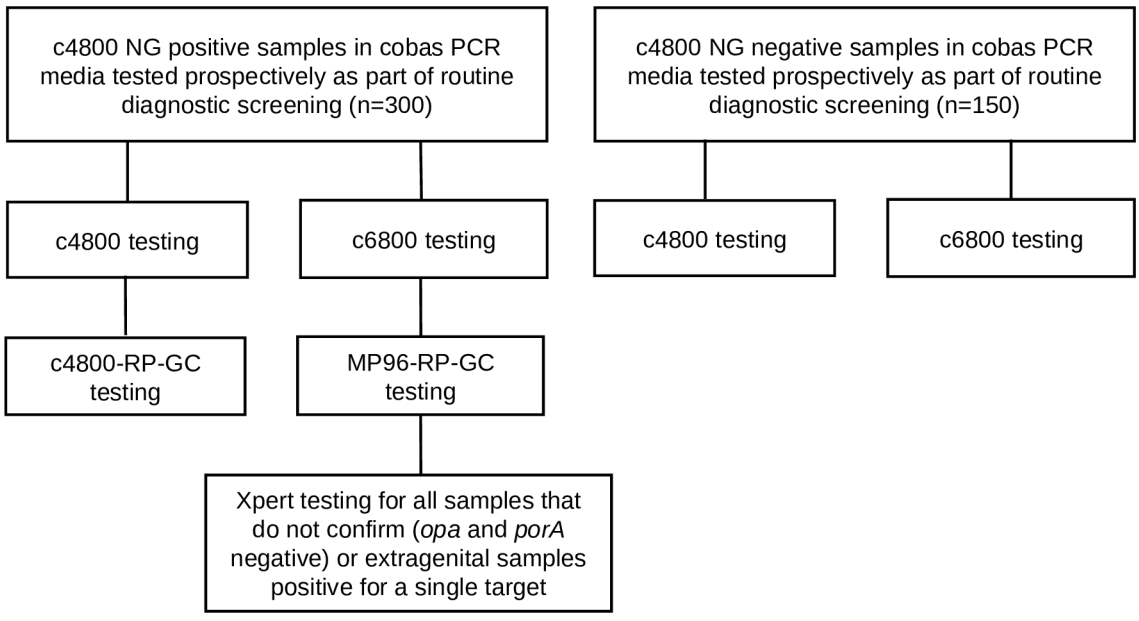


**FIG 1** Study overview of the positive and negative samples tested with c4800, c6800, c4800-RP-GC, M96-RP-GC, and Xpert.

NG detection and that samples, irrespective of the sample site, are considered positive for NG if either the *opa* or *porA* targets provide positive results.

In addition to RP-GC, we also conducted a pilot study using Xpert as an additional supplemental test for the c6800. For this testing, we performed Xpert for all samples that did not confirm using the above c6800/MP96-RP-GC workflow. We also applied Xpert to any extragenital samples that were confirmed by the MP96-RP-GC but were only positive by one RP-GC NG target (*opa* or *porA* positive; single confirmatory target positive) noting that pharyngeal samples are particularly prone to producing false-positive results in NG NAAT methods (5, 8). Xpert testing was recorded as detected or not detected according to the manufacturer's instructions. All results for all samples are detailed in the Supplementary Material.

### Detection limit studies

The analytical sensitivity of c4800, c6800, c4800-RP-GC, MP96-RP-GC, and Xpert for the detection of NG was compared. In brief, a 10-fold dilution series of a quantified culture of *N. gonorrhoeae* ATCC 49226 (American Type Culture Collection, Manassas, VA, USA) was diluted with a matrix consisting of pooled NG-negative oropharyngeal specimens in cobas PCR media ($n$ = 40). The dilutions covered the range of 1.00E+06 to 1.00E-01 colony forming units per milliliter (CFU/mL). The standards were tested in triplicate using c6800 and a quantitative standard curve was prepared from the mean cycle of quantitation ($C_q$) at each dilution. Dilutions over the range of 1.00E+04 to 0.1 CFU/mL were also tested in triplicate for each assay (except Xpert due to cost implications). Results are expressed as $\log_{10}$ CFU/mL or CFU/mL, with the latter rounded to the nearest whole CFU. Results were also interpreted following the NG supplemental testing criteria above. The results are shown in the Supplementary Material.

### Data analysis

Two-tailed *P*-values from Fisher's exact test are reported for all comparative analyses, except the agreement between the reported *gyrA* status and susceptibility testing where overall agreement is reported.

## RESULTS

### Supplementary material

The number of sample types, qualitative and quantitative results, including $C_q$ values and culture results for all 450 samples tested are shown in the Supplementary Material. All results, tables, and statistical analyses presented in this study have been interpreted from the data in the Supplementary Material.

### Evaluation of cobas screening results compared to RP-GC and Xpert

A summary of the screening results and the NG confirmatory rates tested with c4800, c6800, c4800-RP-GC, MP96-RP-GC, and Xpert is shown in Table 1. From 300 NG-positive archival samples, c4800 returned 298 NG-positive results. The two (previously c4800 positive) samples providing negative results in the c4800 in this retrospective testing (sample S50, oropharyngeal and sample S224, vagina; see Supplementary Material) tested positive with c6800 ($C_q$ values = 37.18 and 36.64, respectively) and quantified to 4 and 5 CFU/mL respectively, indicating detection at the reported lower limit of detection for c6800 (1.0 CFU/mL). Both samples confirmed with *opa* and *porA* using MP96-RP-GC; however, sample S50 was *opa* positive and *porA* negative, and sample S224 was negative for both *opa* and *porA* using c4800-RP-GC. By contrast, the c6800 returned 293 NG-positive results, with seven (previously c4800 positive) samples providing c6800 negative results (samples numbers were S5, S23, S48, S74, S110, S124, and S180). These samples were all oropharyngeal samples with c4800 $C_q$ values ranging from 31.8 to 38.4 cycles. All were *opa* and *porA* negative for both c4800-RP-GC and MP96-RP-GC. In

addition, all were c4800-RP-GC *opa* and *porA* negative at the time of the initial c4800 routine screening (data not shown). These seven discrepant samples were tested with Xpert to investigate. All were reported as not detected with Xpert; however, samples S5, S23, and S48 (all from the same patient) were positive for the NG4 Xpert target only. The NG4 $C_q$ values were 37.5, 32.7, and 33.7 cycles respectively. For interest, we also show the results for *Chlamydia trachomatis* (CT) for all samples, with 19.0% (57/300) and 22.0% (66/300) reporting a CT-positive result for c4800 and c6800, respectively. Both c4800 and c6800 returned 150 negative results for all NG-negative archival samples.

### Comparison NG confirmatory rates

Table 1 shows the NG confirmatory rates according to sample type for the samples that tested positive for c4800 and c6800. The urogenital NG confirmatory rate for c4800-RP-GC was 97.4% (148/152) compared to MP96-RP-GC of 100% (153/153). This improvement was not statistically significant ($P$ = 0.0605). The extragenital NG confirmatory rate was higher for MP96-RP-GC at 93.6% (131/140), compared to 86.3% (126/146) for c4800-RP-GC; however, the difference was not statistically significant ($P$ = 0.6677). The oropharyngeal sample NG confirmatory rates were 90.2% (74/82) for MP96-RP-GC compared to 80.7% (71/88) for c4800-RP-GC, albeit again not statistically significant ($P$ = 0.6520). The increase in NG confirmatory rates for MP96-RP-GC was primarily caused by improved detection of *porA* from MP96 nucleic acids compared to c4800 nucleic acids. Combining urogenital and extragenital samples, the confirmatory rate for MP96-RP-GC was 96.9% (284/293) compared to 91.9% (274/298) for c4800-RP-GC ($P$ = 0.6796). In all, 13 extragenital samples were c6800 positive that were single target positive or did not confirm using MP96-RP-GC. These were tested with Xpert to investigate and four were positive (S31, S144, S248, and S287). The remaining nine samples were Xpert negative (all were negative for the Xpert NG2 and NG4 targets), with one sample (S42) returning an invalid result due to failure of the sample adequacy control. Combining all confirmed extragenital results from c6800, MP96-RP-GC, and Xpert, the final extragenital confirmatory rate was 96.4% (135/140) with a total overall confirmatory rate of 98.3% (288/293).

### Comparison of c4800-RP-GC and MP96-RP-GC *gyrA* results with ETEST results

Table 2 shows c4800-RP-GC and MP96-RP-GC *gyrA* results compared to ETEST for isolates recovered from culture ($n$ = 63). From these isolates, 58.7% (37/63) were ETEST susceptible and 41.3% (26/63) were non-susceptible. The qualitative ETEST results closely correlate with the reported percentage for c4800-RP-GC and MP96-RP-GC according to the *gyrA* status, with MP96-RP-GC closest at 52.9% (155/293) for *gyrA* S91 and 39.2% (115/293) for *gyrA* S91F. The number of samples reporting a conclusive *gyrA* result for urogenital samples tested with MP96-RP-GC was 96.1% (147/153) compared to 90.8% (138/152) for c4800-RP-GC. This improvement using the MP96 extraction was not statistically significant ($P$ = 0.0682). Fewer *gyrA* indeterminate results were observed for MP96-RP-GC ($n$ = 6, 3.9%) compared to c4800-RP-GC ($n$ = 14, 9.2%). The number of samples reporting a conclusive *gyrA* result for extragenital samples tested with MP96-RP-GC was 87.9% (123/140) compared to 73.3% (107/146) for c4800-RP-GC. This improvement was statistically significant ($P$ = 0.0027). Fewer *gyrA* indeterminate results were observed for MP96-RP-GC ($n$ = 17, 12.1%) compared to c4800-RP-GC ($n$ = 39, 26.7%). Overall, the number of samples reporting a conclusive *gyrA* result for all samples was 92.2% (270/293) for MP96-RP-GC compared to 82.2% (245/298) for c4800-RP-GC. The improvement was statistically significant ($P$ = 0.0003).

The RP-GC *gyrA* testing results were concordant with available culture data for 61/62 samples. An isolate recovered from a urethral swab at the same day and time of sample collection for molecular screening (sample S176) was discordant with the *gyrA* status for both c4800-RP-GC and MP96-RP-GC compared to susceptibility testing. The sample was determined to be resistant by RP-GC but ciprofloxacin susceptible by culture. This sample was a high titer sample (5.3 $\log_{10}$ CFU/mL) with an observed *gyrA* S91F $C_q$ of 16.7 with a nominal *gyrA* S91 $C_q$ of 28.4. Other samples from the same patient on the same day

**TABLE 1** Summary of the screening and NG confirmatory results for all samples tested with c4800, c6800, c4800-RP-GC, MP96-RP-GC, and Xpert

| Sample type | Total[a] | c4800 results | c4800-RP-GC results | | | c6800 results | MP96-RP-GC results | | | Xpert testing results | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | NG positive[b] | NG confirmed (%)[c] | | NG not | NG positive[e] | NG confirmed (%)[c] | | NG not | Xpert positive | Xpert negative |
| | | | Dual target | Single target | confirmed (%)[d] | | Dual target | Single target | confirmed (%)[d] | (%) | (%) |
| Urine | 139 | 86 | 83 (96.5) | 0 | 3 (3.5) | 86 | 86 (100) | 0 | 0 | –[h] | – |
| Vaginal | 37 | 27 | 27 (100) | 0 | 0 | 28 | 28 (100) | 0 | 0 | | – |
| Urethral | 36 | 26 | 25 (96.2) | 0 | 1 (3.8) | 26 | 26 (100) | 0 | 0 | – | – |
| Endocervical | 18 | 13 | 13 (100) | 0 | 0 | 13 | 13 (100) | 0 | 0 | – | – |
| Total urogenital | 230 | 152 | 148 (97.4) | 0 | 4 (2.6) | 153 | 153 (100) | 0 | 0 | | |
| Oropharyngeal | 133 | 88 | 58 (65.9) | 13 (14.8) | 17 (19.3) | 82 | 70 (85.4) | 4 (4.9) | 8 (9.7) | 4 (4.8) | 8 (9.7) |
| Anorectal | 85 | 56 | 54 (96.4) | 0 | 2 (3.6) | 56 | 55 (98.2) | 0 | 1 (1.8) | 0 | 1 (1.8)[g] |
| Joint fluid | 1 | 1 | 1 (100) | 0 | 0 | 1 | 1 (100) | 0 | 0 | – | – |
| Eye | 1 | 1 | 0 | 0 | 1 (100) | 1 | 1 (100) | 0 | 0 | – | – |
| Total extragenital | 220 | 146 | 113 (77.4) | 13 (8.9) | 20 (13.7) | 140 | 127 (90.7) | 4 (2.9) | 9 (6.4) | 4 (2.9) | 9 (6.4) |
| Sample total | 450 | 298 | 261 (87.6) | 13 (4.4) | 24 (8.0) | 293 | 280 (95.6) | 4 (1.3) | 9 (3.1) | 4 (1.4) | 9 (3.1) |

[a]Number of c4800 NG-positive ($n$ = 300) and NG-negative ($n$ = 150) samples collected for this study.
[b]Based on c4800 screening.
[c]Based on RP-GC testing; dual target, samples positive for *opa* and *porA*; single target, samples positive for *opa* or *porA*.
[d]Based on RP-GC testing, samples negative for *opa* and *porA*.
[e]Based on c6800 screening.
[f]Based on Xpert testing according to the instructions for use.
[g]Xpert sample adequacy control failed and the final Xpert NG result reported as invalid (NG2 and NG4 targets were negative).
[h]–, refers to none tested.

**TABLE 2** Summary of the c4800, c6800, c4800-RP-GC *gyrA*, and MP96-RP-GC *gyrA* results compared ciprofloxacin ETEST results from recovered isolates

| Sample type | c4800 results | c4800-RP-GC results | | | c6800 results | MP96-RP-GC results | | | Ciprofloxacin ETEST results from isolates | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | NG positive[b] | *gyrA* S91 (%)[c] | *gyrA* S91F (%)[c] | *gyrA* ind. (%)[c] | NG positive[d] | *gyrA* S91 (%)[c] | *gyrA* S91F (%)[c] | *gyrA* ind (%)[c] | No. isolates | S (%)[e] | R/NS (%)[e] | Concordance (%)[f] |
| Urine | 86 | 51 (59.3) | 27 (31.4) | 8 (9.3) | 86 | 55 (64.0) | 27 (31.4) | 4 (4.7) | 11 | 8 (72.7) | 3 (27.3) | 100.0 |
| Vaginal | 27 | 15 (55.6) | 9 (33.3) | 3 (11.1) | 28 | 17 (60.7) | 9 (32.1) | 2 (7.1) | 6 | 3 (50.0) | 3 (50.0) | 100.0 |
| Urethral | 26 | 15 (57.7) | 10 (38.5) | 1 (3.8) | 26 | 15 (57.7) | 11 (42.3) | 0 | 18 | 10 (55.6) | 8 (44.4) | 94.4[g] |
| Endocervical | 13 | 7 (53.8) | 4 (30.8) | 2 (15.4) | 13 | 7 (53.8) | 6 (46.2) | 0 | 5 | 4 (80.0) | 1 (20.0) | 100.0 |
| Total urogenital | 152 | 88 (57.9) | 50 (32.9) | 14 (9.2) | 153 | 94 (61.4) | 53 (34.6) | 6 (3.9) | 40 | 25 (62.5) | 15 (37.5) | 97.5[g] |
| Oropharyngeal | 88 | 25 (28.4) | 30 (34.1) | 33 (37.5) | 82 | 29 (35.4) | 37 (45.1) | 16 (19.5) | 10 | 4 (40.0) | 6 (60.0) | 100.0 |
| Anorectal | 56 | 28 (50.0) | 23 (41.1) | 5 (8.9) | 56 | 31 (55.4) | 24 (42.9) | 1 (1.8) | 13 | 8 (61.5) | 5 (38.5) | 100.0 |
| Joint fluid | 1 | 0 | 1 (100.0) | 0 | 1 | 0 | 1 (100.0) | 0 | 0 | - | - | - |
| Eye | 1 | 0 | 0 | 1 (100.0) | 1 | 1 (100.0) | 0 | 0 | 0 | - | - | - |
| Total extragenital | 146 | 53 (36.3) | 54 (37.0) | 39 (26.7) | 140 | 61 (43.6) | 62 (44.3) | 17 (12.1) | 23 | 12 (52.2) | 11 (47.8) | 100.0 |
| Sample total[a] | 298 | 141 (47.3) | 104 (34.9) | 53 (17.8) | 293 | 155 (52.9) | 115 (39.2) | 23 (7.8) | 63 | 37 (58.7) | 26 (41.3) | 98.4[g] |

[a]From all positive samples collected for this study ($n$ = 300).
[b]Number of NG-positive samples from c4800 testing.
[c]*gyrA* status based on RP-GC testing (*gyrA* S91, wild type associated with ciprofloxacin sensitivity; *gyrA* S91F, mutation associated with ciprofloxacin resistance; *gyrA* ind., *gyrA* status indeterminate).
[d]Number of NG-positive samples from c6800 testing.
[e]Number of isolates recovered from culture and ciprofloxacin ETEST results; S = sensitive, R/NS = resistant/non-susceptible.
[f]Percent concordance comparing *gyrA* status and ciprofloxacin ETEST results.
[g]Urethral sample S176 was *gyrA* S91F positive with a ciprofloxacin ETEST minimum inhibitory concentration of 0.064 (S). Isolate recovered from culture also tested as *gyrA* S91F positive.

(urine sample S175 and oropharyngeal sample S177) were also *gyrA* S91F positive for both c4800-RP-GC and MP96-RP-GC. The isolate demonstrated a ciprofloxacin ETEST MIC result of 0.064, which is considered sensitive according to CLSI guidelines. The isolate was recovered from −80°C glycerol storage and repeat ciprofloxacin ETEST was performed with consistent results (MIC 0.064). The original sample and the recovered isolate were retested with MP96-RP-GC. In both cases, the *gyrA* S91F mutation was detected. The reported *gyrA* status correlated with the susceptibility result for all other isolates ($n$ = 62; 26 non-susceptible and 36 susceptible). The overall agreement between the reported *gyrA* status and susceptibility testing was 98.4% (confidence interval estimate at 95% confidence of 91.5%–99.7%).

## Analysis of positive c4800 and c6800 samples that were RP-GC and Xpert negative

Table 3 shows the qualitative and quantitative results for eight samples that were c4800 and c6800 positive that were negative for c4800-RP-GC, MP96-RP-GC, and Xpert targets. From the Supplementary Material, Sample S115 was culture negative, and all other samples were not cultured. Samples S42 and S115 demonstrated quantitative NG results of <10 CFU/mL. Samples S192 and S257 demonstrated quantitative NG results of 10–100 CFU/mL. Samples S35, S214, and S223 demonstrated quantitative NG results of 100–1000 CFU/mL. Sample S205 was the highest titer demonstrating a quantitative NG result of 2,459 CFU/mL. Sample S42 (anorectal) demonstrated a late Xpert sample processing control (SPC) $C_q$ value (40.5), possibly indicating the presence of interfering substances. This sample also failed to amplify the sample adequacy control (SAC), which may be a consequence of interfering substances or lack of exogenous human DNA. All other samples demonstrated nominal internal control (IC) and SPC $C_q$ values when compared to the results for oropharyngeal samples. The average $C_q$ and standard deviation of the MP96-RP-GC IC for all c6800 positive oropharyngeal samples was 24.41 ± 0.66 $C_q$. Similarly, anorectal samples were 24.62 ± 0.66 $C_q$ and all urogenital samples were 24.50 ± 0.66 $C_q$. Excluding the seven oropharyngeal samples presented in Table 3, all other oropharyngeal samples with a quantitative NG result >1 CFU/mL ($n$ = 74) confirmed with MP96-RP-GC (*opa* and *porA*) ($n$ = 70, 94.6%), with the remaining confirmed results ($n$ = 4) demonstrating a positive result with a single confirmatory target (*opa*), with three samples (S31, S144, S287) positive for Xpert.

## Analysis of the detection limit results using quantitative analysis

The results for the c6800 NG standard curve and detection limit studies comparing c4800, c6800, c4800-RP-GC, MP96-RP-GC, and Xpert are shown in the Supplementary Material. The NG standard curve of NG ATCC 49226 was log-linear with an $R^2$ value of 0.9994. The regression formula for calculating the $\log_{10}$ CFU/mL value for any given sample $C_q$ value was $y = -0.2904x + 11.371$. The standard curve covered the quantitative range typically observed for all clinical samples (Supplementary Material). All three replicates at 1 CFU/mL were positive which correlates with the reported lower limit of detection for c6800 according to the manufacturer. The detection limit studies showed c6800 to be 2 log dilutions more sensitive than c4800 (all three replicates positive), with c6800 at 1 CFU/mL compared to 100 CFU/mL for c4800. The MP96-RP-GC showed to be 1 log dilution more sensitive than c4800-RP-GC (all three replicates *opa* and *porA* positive), with MP96-RP-GC at 100 CFU/mL and c4800-RP-GC at 1000 CFU/mL. Overall, *opa* was more sensitive than *porA* for c4800-RP-GC and MP96-RP-GC. Xpert was 1 log dilution less sensitive than c6800, with Xpert at 10 CFU/mL compared to c6800 at 1 CFU/mL. The limits of detection for each commercial assay are similar to the package insert claims.

## Routine culture

All culture results are shown in the Supplementary Material. Culture requested at the time of collection represented 25.7% (77/300) of the total compared to PCR-only requests at 74.3% (223/300) of the total. Subsequent culture requests 1–11 days after

**TABLE 3** Summary of positive c4800 and c6800 samples that were negative for all RP-GC and Xpert targets

| Sample | Patient | Sample type | c4800 results | c4800-RP-GC results[b] | | c6800 results | | MP96-RP-GC results[d] | | Xpert results[e] | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | NG $C_q$[a] | *opa* and *porA* | IC $C_q$[a] | NG $C_q$[a] | NG CFU ($log_{10}$)/mL[c] | *opa* and *porA* | IC $C_q$[a] | NG2 and NG4 | SAC $C_q$[a] | SPC $C_q$[a] |
| S35 | P23 | Oropharyngeal | 35.7 | Negative | 24.62 | 30.27 | 381 (2.58) | Negative | 24.62 | Negative | 22.9 | 30.8 |
| S42 | P27 | Anorectal | 38.4 | Negative | 25.62 | 38.50 | 2 (0.19) | Negative | 25.62 | Negative[f] | Failed[f] | 40.5 |
| S115 | P86 | Oropharyngeal | 37.7 | Negative | 24.45 | 37.55 | 3 (0.47) | Negative | 24.45 | Negative | 28.4 | 32.1 |
| S192 | P142 | Oropharyngeal | 38.2 | Negative | 24.69 | 35.11 | 15 (1.18) | Negative | 24.69 | Negative | 29.7 | 31.6 |
| S205 | P153 | Oropharyngeal | 33.6 | Negative | 24.14 | 27.48 | 2,459 (3.39) | Negative | 24.14 | Negative | 21.2 | 30.5 |
| S214 | P153 | Oropharyngeal | 35.6 | Negative | 24.54 | 29.00 | 891 (2.95) | Negative | 24.54 | Negative | 24.1 | 30.7 |
| S223 | P153 | Oropharyngeal | 35.9 | Negative | 24.49 | 31.50 | 167 (2.22) | Negative | 24.49 | Negative | 23.3 | 31.6 |
| S257 | P181 | Oropharyngeal | 36.7 | Negative | 24.25 | 33.76 | 37 (1.57) | Negative | 24.25 | Negative | 24.1 | 31.5 |

[a]$C_q$, cycle of quantitation.
[b]RP-GC results from c4800 nucleic acids (IC, internal control).
[c]From NG standard curve (Supplementary Material); CFU, colony forming units.
[d]RP-GC results from MP96 nucleic acids (IC, internal control).
[e]Xpert results from cobas PCR media (NG2 and NG4, chromosomal NG targets; SAC, sample adequacy control; SPC, sample process control).
[f]Xpert sample adequacy control failed and the final Xpert NG result was reported as invalid (NG2 and NG4 targets were negative).

the screening swab was collected accounted for 6.7% (20/300) of the total. The total recovery rate from culture was 70.0% (64/97) with 63.6% (49/77) recovered from the time of collection and 75% (15/20) from subsequent culture requests.

## DISCUSSION

Our results conclusively show an increase in the overall NG confirmatory rate for MP96-derived nucleic acids compared to c4800 nucleic acids, with oropharyngeal samples as the key point of difference. Although the increase in overall NG confirmatory rate was not statistically significant, we demonstrated a significant increase ($P$ = 0.0003) in reporting a valid *gyrA* status, with fewer indeterminate results for MP96-RP-GC compared to c4800-RP-GC. We conclude that both increases are related to the improved sensitivity of MP96-RP-GC as evidenced in the detection limit studies. The increase in sensitivity may be due to higher nucleic acid yield or more optimal PCR performance. Regardless, the improvement in MP96-RP-GC test performance is welcomed for a number of reasons: (i) the c6800 has no retrievable nucleic acids for supplemental testing; therefore, separate extraction is mandatory, (ii) a more sensitive NG confirmatory test is beneficial, particularly for oropharyngeal samples, with the improved analytical sensitivity of c6800 compared to c4800, and (iii) additional sensitive or resistant *gyrA* results further improve the clinical utility of the RP-GC assay.

We demonstrate that the c6800 followed by the MP96-RP-GC testing is an improved supplemental testing method compared to c4800-RP-GC with a 100% confirmatory rate for urogenital samples and 93.6% for extragenital samples. As with our previous study and others (7, 11), we conclude that supplemental testing is not required for c6800 for urogenital samples, but oropharyngeal samples should undergo secondary testing, in line with current Australian and UK guidelines (5, 10). At the time of our previous study, the US version of the c6800 CTNG test did not have an oropharyngeal or anorectal claim, whereas the CE/IVD marked kits approved for use in Australia and the UK were approved for use for these sample types. Recent versions are now harmonized and include both oropharyngeal and anorectal samples. Despite this, our data show that there are still some unexplained occurrences of oropharyngeal positive samples which cannot be confirmed using other NG-NAATS. This has been previously demonstrated in our earlier study and other investigations (7, 11). Another evaluation has shown discrepant c6800 positive and c4800 negative results for oropharyngeal samples; however an independent supplemental NAAT was not used to investigate these discordant results (6).

Based on our data, it is unlikely that the discrepancies are due to the differences in the analytical sensitivity of c6800 compared to Xpert. The claimed sensitivity of c6800 irrespective of sample type is 1 CFU/mL which was confirmed experimentally in this study. This same detection limit has been confirmed by others (6). Furthermore, the claimed sensitivity of Xpert with a pooled pharyngeal swab matrix sensitivity is 6.4 CFU/mL. Again, this was confirmed experimentally in this study, with Xpert 10-fold less sensitive than c6800. In both cases, we used the same ATCC strain used by the manufacturers. Finally, we developed an in-house method for the quantification of NG using a pooled oropharyngeal swab matrix. The standard curve was applied to c6800 for the limit of detection studies and all clinical samples tested. Although the standard was prepared using an oropharyngeal matrix and then applied to non-oropharyngeal samples, the manufacturer has stated a limit of 1 CFU/mL for all sample types tested, with the ability to detect *N. gonorrhoeae* strains below 1 CFU/mL. Therefore, we consider our quantitative approach valid and worst-case scenario in terms of encountering interfering substances and other non-gonococcal *Neisseria* species in the oropharyngeal matrix. Based on the premise that c6800 and MP96-RP-CG detected and confirmed all urogenital samples and 93.6% of extragenital samples greater than 2 CFU/mL, of which three were positive with Xpert, we conclude that the majority of samples presented in Table 3 should have at least flagged positive for a single target with an alternative NAAT based on the NG load. Furthermore, we expected the samples with quantitative values between 100 and 2500 CFU/mL to confirm with MP96-RP-GC and/or detected with Xpert.

We acknowledge that cobas PCR media is not a validated reagent for Xpert. However, Xpert also uses a similar guanidinium chloride-based media for urine. Therefore, we consider cobas PCR media compatible with Xpert and have shown that cobas PCR media performs well for oropharyngeal samples.

In terms of improved specificity, we have shown that a previously documented case of *N. macacae* providing false-positive results with c4800 does not cross-react with the later generation c6800 assay (23). From 300 c4800 NG-positive samples collected from initial screening, we observed seven samples that were c6800 negative. Not surprisingly, all were oropharyngeal samples. By contrast, an earlier Roche-funded study comparing c4800 and c6800 did not encounter any c4800-positive oropharyngeal samples with c6800-negative results (6). Our discordant cases were c4800-RP-GC, MP96-RP-GC, and Xpert negative. Three were positive for the Xpert NG4 target; however, this target has been shown to cross-react with non-gonococcal *Neisseria* species (24, 25). These discrepancies may be examples of the aforementioned c4800 non-specificity, but we were unable to recover an isolate from culture to confirm. We conclude some improvement in specificity with the c6800 test compared to c4800 from this observation alone. We also demonstrate improvement in the oropharyngeal NG confirmatory rate using MP96-RP-GC combined with Xpert. Following this approach, the number of oropharyngeal samples was unable to be confirmed accounting for 8.5% of the total (7/82). By contrast, in a recent report using c6800 as the screening test followed by a supplemental test targeting the *pilin inversion* (*pivNG*) gene using the cobas omni channel, the investigators were unable to confirm 23.7% (27/114) c6800-positive oropharyngeal samples with *pivNG* and Xpert combined, despite reporting equal detection limits in the supplemental material for *pivNG* compared to c6800 (albeit with commercial control material in either cobas PCR media or water) (11). The investigators state sample volumes of 400 µL for the omni channel *pivNG* and 300 µL for Xpert. By contrast, we use 200 µL for MP96 and 1 mL for Xpert. We note that the first version of Xpert CT/NG (301–0234, Rev. B, January 2013) and subsequent versions available to our laboratory have consistently stated a sample input volume of 1 mL. Following our approach, an overall confirmation rate of 98.3% was achieved with supplemental assays analytically less sensitive compared to c6800 when tested with pooled oropharyngeal matrix. The detection limits we observed are also similar to those reported by the manufacturer, using an oropharyngeal matrix. As a diagnostic strategy, we consider maximizing the NG confirmatory rate and reporting an AMR marker as a more advantageous trade-off than reduced sample handling and *pivNG* testing with the omni channel.

The RP-GC assay utilizes *PlexZyme* technology (26) to simultaneously detect *opa* and *porA* with *gyrA* S91 (wild type) or *gyrA* S91F (mutant) in a single PCR. We also observed an improvement in the *gyrA* status reporting for RP-GC using MP96 nucleic acids compared to c4800 nucleic acids, with fewer *gyrA* indeterminate results for MP96-RP-GC. Given that in 2021, 47% of all reported NG isolates are ciprofloxacin susceptible in Australia and even higher in metropolitan (63.6%) and remote (96.4%) areas of Western Australia (27), a large proportion could be potentially treated with ciprofloxacin, despite being unsuitable for empirical treatment on the basis of being well above the WHO 5% resistance threshold (28). Current and historical recommended first-line antimicrobials in many countries for NG exceed the 5% resistance threshold (13). Given that greater than 5% resistance to ceftriaxone could be reached by 2030 based on mathematical models (29), improved strategies for continued antimicrobial stewardship and new diagnostic tests to allow resistance-guided therapy for the 50–70% of isolates susceptible to ciprofloxacin are needed (15, 30). When compared with the corresponding bacterial culture results, the positivity of the *gyrA* S91 or *gyrA* S91F at the time of NG supplemental testing, correctly predicted NG susceptibility to ciprofloxacin in 62 of 63 samples (98.4%). The discrepant sample was further investigated. The original sample was retested with MP96-RP-GC with consistent results. Subsequent subculture from storage and retesting isolate with ETEST and MP96-RP-GC revealed consistent results. We confirmed the *gyrA* S91F-resistant mutation for the isolate with an ETEST MIC of 0.064 (sensitive) according

to CLSI criteria (≤0.06 reported as sensitive). It is unlikely that the discrepancy is due to different strains and the reason for the ciprofloxacin susceptible result is unknown. Nevertheless, we do not see a "major error" (false resistance) as a problem as this simply means ciprofloxacin would be ruled out for this patient. A "very major error" (false susceptibility) where ciprofloxacin was inappropriately ruled in would have otherwise been a problem.

Our study has limitations. Although our sample size for positive samples collected over a year of testing was sufficient ($n$ = 300), the number of negative samples tested was half ($n$ = 150) and not reflective of the positive and negative prevalence of the population tested. Determining c6800 performance and NG confirmation with MP96-RP-GC compared to c4800 using a much larger negative sample set screened with c4800 would be ideal to determine the true number of c6800-positive samples compared to c4800-negative samples, given the increase in the analytical sensitivity of c6800 compared to c4800. This would be of value for oropharyngeal samples in particular, given we may encounter more NG-positive samples that are RP-GC and Xpert negative. Similarly, a larger number of ciprofloxacin susceptibility results compared to *gyrA* would have been ideal; although ETEST concordance with the *gyrA* status was high, only 22% (63/298) of c6800-positive samples had isolates available for ciprofloxacin susceptibility testing. It should also be noted that our statistical approaches considered all patient samples to be independent and did not consider the broader infected patient status; however, this was considered necessary for the study given the objectives focused on maximizing detection of NG and AMR within individual samples and not collectively for all samples from an individual patient. This is further supported by the fact that as per routine practice, all samples testing positive for NG from any site are sent for NG confirmation and this is irrespective of results of any other sites from the same patient. Finally, we have already acknowledged that cobas PCR media is not a manufacturer-validated reagent for Xpert; however, based on this study and our previous work (7), cobas PCR media has no discernible impact on Xpert test performance. Although all samples in cobas PCR media were retested within 12 months (based on the confirmed stability studies of the manufacturer), similar stability for Xpert is assumed.

In conclusion, this is the first study to show the clinical performance of the RP-GC assay in conjunction with c4800 and c6800 CT/NG. We demonstrate exceptionally high NG confirmatory rates for urogenital and extragenital samples combining RP-GC with MP96 extraction. In addition, we also show comparable sensitivity of *gyrA* detection compared to NG confirmation while confirming the reliability of *gyrA* to predict ciprofloxacin susceptibility (19). We also highlight the ongoing need for supplemental testing for oropharyngeal samples and the importance of genotypic AMR detection, as a progressive step toward specific individualized treatment to reduce the growing state of AMR in gonococcus.

## ACKNOWLEDGMENTS

We acknowledge the dedication of all staff: Sexual Health Clinic Royal Perth Hospital, South Terrace Sexual Health Clinic Fremantle Hospital, Fiona Stanley Hospital Sexual Health, Magenta Clinic Northbridge, Department of Clinical Microbiology, PathWest Laboratory Medicine WA, Fiona Stanley Hospital, Murdoch. We thank A/Prof Mark Chatfield from The University of Queensland for assistance with the statistical analyses.

T.P. conceptualized the study, prepared the methodology, performed the formal analysis, performed the investigation, and wrote and edited the manuscript. O.F. and E.H. performed the investigation, prepared the methodology, and performed the data collation, review, and editing of the manuscript. D.W. performed the formal analysis and review and editing of the manuscript. D.W. reports research funding from SpeeDx Pty Ltd., and SpeedDx was not part of this study whatsoever. We confirm that Roche, Cepheid, or SpeeDx had no role in the design of this study, the collection, analysis, interpretation of data, or the write-up of the manuscript.

All funding was internal (PathWest Laboratory Medicine WA) and there were no external funding sources.

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## ETHICS APPROVAL

All patient samples tested in this study, including initial and subsequent samples, were collected as part of routine clinical care, where CT/NG screening was requested. This laboratory quality exercise is exempt from human research ethics approval [according to the National Statement on Ethical Conduct in Human Research 2007 (Updated May 2015) by the National Health and Medical Research Council, Australian Research Council, and Australian Vice-Chancellors' Committee].

## ADDITIONAL FILES

The following material is available online.

### Supplemental Material

**NG confirmation supplemental material (JCM01039-23-S001.xlsx).** Excel NG supplemental file.

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## 3.5 Conclusion

Regarding the specific aims, the above studies have shown that:

a) Specificity issues remain for oropharyngeal samples in second- and third-generation assays, which can be mitigated by appropriate supplemental testing, although supplemental testing may not be required for urogenital sites.
b) Some improvement to DR-9 specificity is evident with the c6800 assay.
c) A new supplemental testing approach combining RP-GC and Xpert, not only maximises the NG confirmatory rate and reportable results, but also adds a sensitive and specific AMR marker, thereby adding clinical utility.

However, oropharyngeal sites are still challenging, as highlighted by the above studies demonstrating that:

d) Using $C_q$ analysis and a finer resolution of analytical sensitivity using quantitative PCR, we quantify the *N. gonorrhoeae* load-related to oropharyngeal infections and show examples of high *N. gonorrhoeae* load that do not correlate with a positive supplemental result.

When read together, these studies highlight the complexities and challenges of assay specificity with this organism. All the challenges presented in **Chapter 3** focus on evaluating new screening and supplemental assays for gonococcal diagnosis, whilst addressing the changing PHLN guidelines concerning issues of *N. gonorrhoeae* non-specificity, which have plagued NG-NAAT's for decades. However, supplemental testing represents additional work and cost to the laboratory with nucleic acid retrieval or separate extraction, assay set-up, testing, result interpretation, additional quality control and quality assurance testing. Historically, this added workload was clinically justified given the poor specificity of earlier tests. Today, this justification has diminished given the improved specificity of modern NG-NAATs, essentially redesigned to mitigate the potential for false-positive results (79, 158, 161, 178). Additionally, these modern NG-NAAT have improved sensitivity that is problematic for supplemental tests (particularly in-house NAATs) that fail to match the sensitivity of the screening tests, resulting in screening-positive/supplemental-false-negative results. Furthermore, closed NAAT systems and platform-specific screening collection devices complicate the workflow for supplemental testing, such as the need for a separate nucleic acid extraction, adding to test turnaround time and cost. With these three publications, we collectively address these issues, whilst highlighting the complex ongoing issue of non-specificity in oropharyngeal samples.

### 3.5.1 The impact of changing screening and supplemental testing methods

**Publication 5** conclusively shows how NG confirmation rates significantly decreased ($p<0.0001$) when we changed from m2000 to c4800 and then to the c6800 for NG-NAAT screening, and not surprisingly were most evident for oropharyngeal samples. However, the reasoning for the observed differences were not as clear-cut was we initially thought. The discrepancies were due to a combination of false positives for oropharyngeal samples, potentially caused by the *N. macacae* issue as previously described in New Zealand (72), but additionally the c4800 had lower sensitivity for the NG-Duplex *porA* target, due to the extraction method for c4800 nucleic acids. Alternatively, the poor sensitivity of *porA* detection may be due to *N. gonorrhoeae* variants, with known genetic diversity in our Australian population (179), and **Publication 5** is a reminder that assay performance problems may only become evident when data is analysed at a population level (as opposed to analytical sensitivity and specificity comparisons during assay evaluation on selected organism strains). A classic historical example of this was reported in 2006, when failure to detect a variant of CT due to a deletion in the cryptic plasmid used as the PCR target (Swedish variant), resulted in an unexpected 25% decrease in the detection of CT infections, when analysed on the Abbott and Roche assay platforms (180). Fortunately, our decision to report both screening and supplemental testing results openly allows clinicians to offer tailored treatment options for patients. For example, clinicians may interpret a positive supplemental test result from an oropharyngeal site as a true *N. gonorrhoeae* infection that needs treatment, but those for patients with an NG-positive oropharyngeal sample that returns a negative supplemental test, the clinician may choose to discuss with the patient the need for treatment, based on patient's clinical history.

### 3.5.2 The ongoing problem of oropharyngeal samples

Overall, these studies demonstrated a confirmatory rate of 78% for oropharyngeal samples, consistent with other c4800 studies (160), but below the recommended 90% threshold for all sample sites (159). For this reason, supplemental testing for oropharyngeal samples should continue, but the scientific justification to continue supplemental testing for urogenital sites is under question, with our high urogenital confirmatory rates exceeding 97%. The overall results from the c4800/NG-Duplex and c6800 comparison were highly concordant, although we detected a few c4800-positive oropharyngeal and anorectal samples that were negative on c6800, despite the 1 CFU/mL sensitivity of the c6800 test, an issue not reported by the assay manufacturer (177). It is possible that these are examples of the *N. macacae* non-specificity or other non-specificity. Regardless, testing the *N. macacae* isolate

with c6800 as described in **Publication 6** showed improved specificity for this strain only. We concluded that a more extensive c6800 evaluation is required to assess extragenital specificity, by investigation using other molecular assays.

Further addressing and linking to the above, our more recent in-depth investigation of the c6800 assay (n=300) using quantitative PCR analysis in **Publication 7**, revealed a small number of oropharyngeal samples with earlier $C_q$ values than described in **Publication 5** that did not test positive by RP-GC or Xpert. These samples should have readily confirmed if they were truly positive for *N. gonorrhoeae*; a finding additionally supported by our manufacturer-independent lower-limit-of-detection studies published in **Publication 5 and 7**. Hence, based on these data we showed that c6800 may still be prone to false-positive results for oropharyngeal samples. In fact, a recent example of c6800 positive oropharyngeal sample demonstrating a $C_q$ of 23.7 (equivalent to 31,600 CFU/mL) was RP-GC and Xpert negative (T. Pryce, unpublished work). Unfortunately, an accompanying oropharyngeal swab was not submitted for culture. Hence, we continue to recommend supplemental testing for extragenital samples for purposes of enhancing specificity. Notwithstanding this recommendation, we also re-affirmed that (as per above) *N. gonorrhoeae* load related issues may impede accurate confirmatory testing (181), and that the load-related inability to confirm a screening result must be taken into consideration in the ongoing debate whether or not confirmatory testing is required for oropharyngeal samples. Also note that while we suggest supplemental testing *may* not be required for urogenital samples, we continue supplemental testing for all sample types by default for another reason: AMR detection (outlined below).

### 3.5.3 Maximising the *N. gonorrhoeae* confirmation rates and the reporting of ciprofloxacin resistance

In **Publication 7** we resolved the problem concerning restricted access to nucleic acids with the c6800 system and validated a new *opa*/*porA* supplemental test to fulfill the strict PHLN supplemental testing requirements. Furthermore, we enhanced the clinical utility of the supplemental test as RP-GC via NG *gyrA* characterization. Our objects were met - we focussed on maximising the NG confirmatory rate, bringing the sensitivity of the combined RP-GC and Xpert supplemental testing strategy close to the sensitivity of the c6800 test, providing a greater number of definitive and reportable results for our clinicians. Extragenital confirmatory rates were higher for MP96-RP-GC (93.6%) compared to c4800-RP-GC (86.3%). Although not statistically significant, this subtle improvement makes a big impact in terms of improved confidence of *N. gonorrhoeae* detection for the patient and clinician. Although testing c4800 nucleic acids was a decision of convenience at the time, this study highlights that an

improvement to the NG confirmatory rate may have been possible with a separate MP96 extraction when we were routinely using the c4800 screening assay. Although small improvements to sensitivity may be possible by optimising the MP96 protocol, this additional developmental work is not required as the Xpert test confirms the majority, achieving a final confirmation rate of 98% across all samples. To our knowledge, no other studies have reported confirmation rates of this magnitude. For example, a recent manufacturer-funded study was unable to confirm 24% of c6800-positive oropharyngeal samples using a supplemental test targeting the pilin inversion gene (pivNG) using the on-board cobas omni channel and Xpert combined (182). Whilst the lack of sensitivity of in-house pivNG may be assay dependent, the low confirmation of results for Xpert sharply contrasts to our study, caused by the reduced volume used for Xpert.

Additionally, our MP96-RP-GC optimisation had a flow-on effect to the *gyrA* sensitivity, improving the number of reportable *gyrA* valid results from MP96 extracted samples compared to c4800 extracted samples. A high degree of specificity (98.4%) was also established with the reported ciprofloxacin sensitive (35/36; 97.2%) or non-susceptible (26/26; 100%) phenotype from culture. An important point of our work is that the RP-GC supplemental test can be used with or without *opa/porA* (depending on local supplemental testing requirements) for the reliable prediction of ciprofloxacin resistance. Until now, there has been a lack of accessible commercial assays for AMR prediction, and we have contributed to the scientific literature promoting their use. A recent UK study performed RP-GC only for those patients that returned an NG-positive result which did not receive empirical treatment on clinical presentation (183). In this targeted approach, the investigators performed RP-GC for the *gyrA* result only on selected patients and waited for the results prior to treatment with either ciprofloxacin or an alternative treatment. Here, the investigators reduced the *gyrA* testing laboratory workload, but this came with the trade-off with needing more staff to review patient treatment records. In contrast, our laboratory tests all positives as the RP-GC assay also serves as the confirmatory test. For both targeted and non-targeted approaches, the most common scenario leading to ciprofloxacin use would be a patient recalled for treatment from a positive asymptomatic screen. However, our decision to test all positive samples and report the *gyrA* status, with a focus on maximising sensitivity and specificity, whilst providing the fastest test turnaround time possible, assists the clinicians with their treatment decisions for some patients. We did not assess if ciprofloxacin was used based on the results of the RP-GC assay, however a clinical audit is planned to investigate the impact of our highly efficient testing strategy on antimicrobial stewardship. Future directions also include evaluations of new molecular AMR assays for high priority targets, such as ceftriaxone (*penA-60*; AusDiagnostics, Mascot, NSW) and azithromycin (A2059G, C2611T; Seegene,

Seol, Republic of Korea) (184). Ultimately however, *N. gonorrhoeae* AMR prediction assays need to progress to the point-of-care (POC) setting and include all the high-priority AMR targets. If such a POC system was validated against laboratory gold standards and showed comparable results, then such a system would allow clinicians to diagnose infection and select the most appropriate therapy on first-time clinical presentation. Depending on the characteristics of the POC system and results, the assay may be part of the primary screening strategy.

Despite laboratory evidence to suggest that supplemental testing may not be required for urogenital samples, supplemental testing for *N. gonorrhoeae* is here to stay at least for oropharyngeal samples. However, predicting AMR may prove more important than *N. gonorrhoeae* supplemental testing to support a positive screening result for a urogenital sample, given that urogenital testing represents the majority of samples tested. New multiplexing technologies would be required for these systems to meet the technical requirements of our stringent supplemental testing guidelines and include AMR markers. A high-throughput sample-to-result dual-target *N. gonorrhoeae* screening assay with an embedded supplemental assay would be ideal. The supplemental result may be accessed for all sample types, or potentially "reveal itself" when triggered by an extragenital sample type. These results could be manually determined or automated in the assay algorithm. Quite simply, the laboratory could customise the handling of the supplementary results according to the guidelines or their own laboratory validation data. Following this, an on-board or off-board reflex assay for multiple AMR markers would complete the diagnostic strategy (Figure 13). Whatever the approach, reliable molecular markers for AMR prediction and POC will play an important role for *N. gonorrhoeae*, given the AMR development and the spread of resistant *N. gonorrhoeae* strains in the future. All laboratories should continue to evaluate new screening and supplemental assays with a high degree of rigor, given the historical context of screening assay non-specificity for *N. gonorrhoeae*.

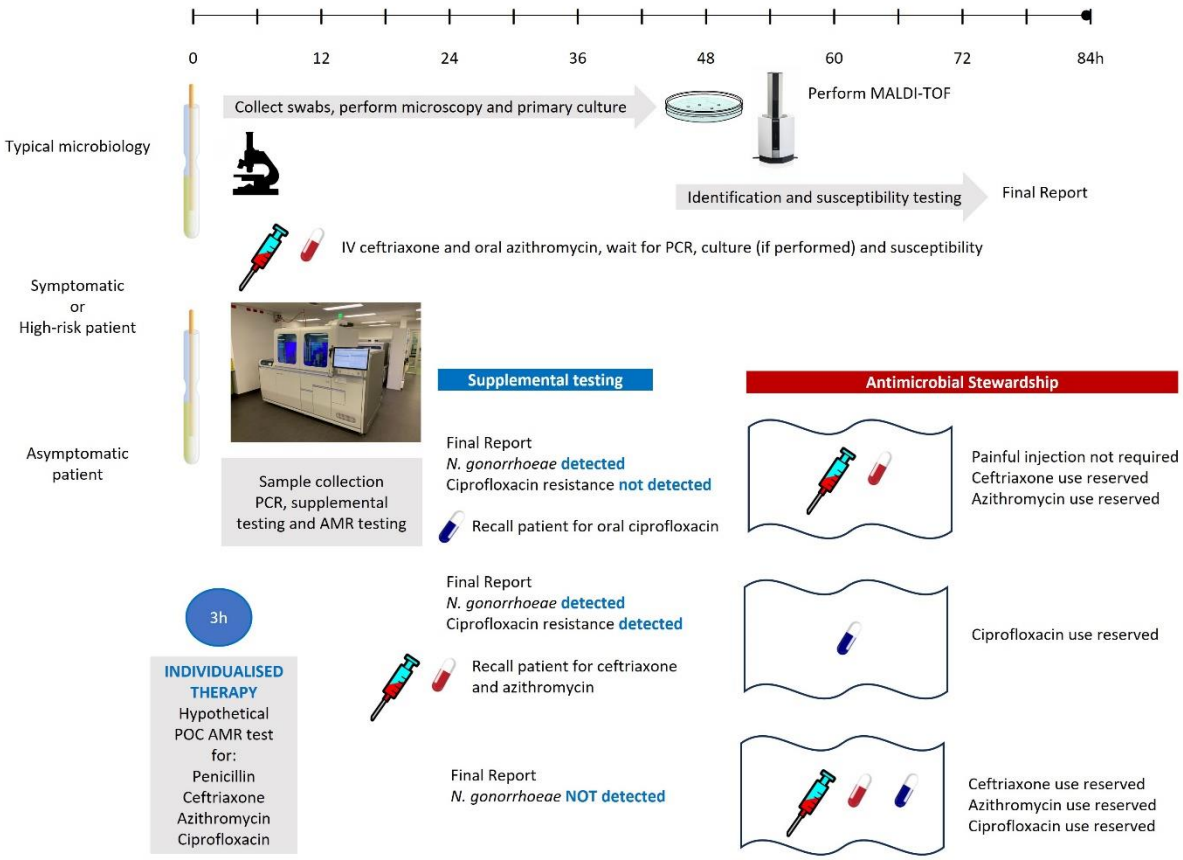


Figure 13. *N. gonorrhoeae* screening, supplemental testing and antimicrobial stewardship. Schematic representation of a supplemental testing that includes a predictor of ciprofloxacin resistance detect and identify *N. gonorrhoeae* cases with a high degree of confidence, and which cases clinicians can effectively limit antibiotic use. A hypothetical 3-hour point-of-care test, with clinically useful molecular predictors of resistance is shown as a comparison. Figure created with PowerPoint using several copyright-free images as detailed in the Appendix. Pryce, TM (2024).

# Chapter 4: Responding to the SARS-CoV-2 pandemic with new molecular methods

## 4.1. Introduction to prior publications

The emergence of the SARS-CoV-2 pandemic, which causes COVID-19, has profoundly affected the global population. Despite extensive efforts to control its spread, SARS-CoV-2 continues to persist as new variants emerge, threatening global public health. Accurate and rapid molecular diagnostic approaches have played a critical role in addressing these needs. However, laboratories have faced significant challenges implementing diagnostic assays, including rapid implementation with little or no control material, lack of reagents, consumables, equipment, laboratory space, adequately trained staff, and concerns around laboratory safety (185). Similarly, clinicians have faced many challenges with COVID-19, such as nonspecific symptoms on presentation, varying clinical manifestations, interpreting non-standardised semi-quantitative results ($C_q$ values), assessing results from various specimen types, and correlations between infectivity and culture positivity (186-190). This chapter documents the challenges encountered chronologically in our laboratory and highlights our responses, impact of new molecular assays and novel approaches to SARS-CoV-2 testing during this pandemic.

### 4.1.1 First SARS-CoV-2 assay and responses in WA

The first cases in WA were reported in February 2020, which resulted in travel restrictions to all other Australian states and territories on March 24. A coordinated approach with a prompt diagnosis, combined with containment and monitoring, was needed to manage the spread of SARS-CoV-2. In response, a PCR test for SARS-CoV-2 needed to be developed and implemented quickly. Two previously published E gene assays were compared (100, 101). The sequence alignments comparing these E gene assays showed different forward primers, a partially shared probe sequence (20 nucleotides) and a partially shared reverse primer (17 nucleotides). At the time, I had to quickly decide whether to implement one of these assays as an in-house test or wait for commercial kit availability. Either way, the time taken to implement an in-house assay or verify a commercial assay was likely to be comparable. Coincidently, our laboratory has collaborated with a member of the European collaborative coauthors for over 20 years (Dr. Olfert Landt, Tib-Molbiol, now owned by Roche), for oligonucleotide primer and probe design and TaqMan/HybProbe LightMix kits for molecular diagnostics (**Chapter 1**). Consequently, we acquired the LightMix Modular E/RdRp assay (LightMix) following a brief communication with Roche confirming test availability. Kits were ordered on

February 10, arrived February 24, and were validated and implemented March 11, 2020—the first laboratory in WA to introduce a commercial SARS-CoV-2 test.

### 4.1.2 Our first inactivation method, reagent shortages and the rapid pivot to alternative SARS-CoV-2 PCR assays

We utilised guanidine hydrochloride (GuHCl) as a pre-treatment step to inactivate SARS-CoV-2, based on its ability to inactivate other highly pathogenic viruses (191-193). Pre-treated samples were extracted using the MP96 system and tested using LightMix. Testing continued with this assay for eight days until we encountered reagent shortages of LightMix and GuHCl due to a lack of supply driven by increased demand. On March 15, we confirmed from Roche Diagnostics Australia that a dual target SARS-CoV-2 test was available for the cobas 6800 instrument as a research-only use (RUO) assay. At the time of launch (March 2020) this assay detected the conserved open reading sequences ORF1a (later revised to ORF1ab June 2020) and E gene, a dual-target assay designed to mitigate false-negative results due to target drop-out. PathWest procured 6,000 tests of the cobas SARS-CoV-2 assay (cobas) on March 18 from the total stock of 100,000 tests received in Sydney the day before (194). On the March 19, I validated the cobas assay against our last aliquots of LightMix. As such, Microbiology at FSH was the first laboratory worldwide to report patient samples using the cobas assay (personal communication, Allison Rossiter, Managing Director, Roche Diagnostics Australia). On March 27, a publication from Slovenia described similar diagnostic switch from LightMix to cobas during a 48-hour period at the height of their COVID-19 pandemic testing (195).

### 4.1.3 Development of the thermal treatment inactivation method

During early implementation of the cobas assay we encountered several challenges with the safe handling of samples—the cobas workflow required samples to be transferred to the instrument in an uncapped tube. Alternative sources and other regents containing GuHCl were considered for inactivation, such as lysis buffers from other diagnostic kits. However, I was concerned about other problems to staff such as the additional pipetting and capping/de-capping workload, potential loss in sensitivity from sample dilution with GuHCl, and potential interference with the cobas assay caused by concentrated GuHCl in the sample. Instead of GuHCl treatment, we transferred these tubes from the class II BSC to the c6800 instrument using PPE. However, after a month of testing, we encountered shortages of PPE due to the demand required for front-line hospital personnel. An alternative method

on inactivation was urgently required as staff were concerned with their personal safety and the possibility of laboratory-acquired infection and transmission to family members. This anxiety added to the intense and stressful working environment that was already affecting staff.

It was clear at the time that a passive method of inactivation was needed to improve safety, with minimal risk to assay sensitivity, specificity and interference. At this point, I turned to heat inactivation as a common and universal inactivation method used in Microbiology. I conducted an extensive literature search of thermal inactivation methods for viruses, focusing on coronaviruses. The challenge was to find the lowest possible temperature for the shortest period of time which led to proven inactivation, which did not affect the PCR assay sensitivity. Several relevant investigations were reported demonstrating the effects of temperature on the viability and copy number of SARS-CoV-2 (196, 197) and other coronaviruses (198-200). As a starting point, 70°C for 5 minutes resulted in undetectable virus using cell culture (196). At the time there was limited information on the quantitative PCR effects of thermal treatment of SARS-CoV-2. Therefore, I needed to assess the effects of thermal treatment on SARS-CoV-2 RNA copy number. The main question was: what impact does thermal treatment have on the sensitivity and/or specificity of the cobas assay for detecting SARS-CoV-2 in nasopharyngeal samples? The scientific enquiry to be investigated was: what is the optimal temperature applied for the shortest period of time which results in the least loss in assay sensitivity, while still inactivating the virus?

To test the effects of thermal treatment on assay performance, I serially diluted a panel of SARS-CoV-2 positive clinical samples and thermally treated them with a range of temperatures and incubation times. The effects on the $C_q$ values for ORF1a, E gene, and the internal control were investigated. Based on these results, 40 consecutive samples were prospectively tested comparing 75°C for 15 minutes to room temperature. This study is presented in **Publication 8** titled "Thermal treatment of nasopharyngeal samples before cobas SARS-CoV-2 testing" (201). The results were published promptly in a letter to the Editor of the Journal of Clinical Microbiology and Infection (IF 10.9). Using the final qualitative outcomes and $C_q$ analysis for the semi-quantitation of SARS-CoV-2, we demonstrated in **Publication 8** that thermal treatment may improve the qualitative detection of SARS-CoV-2, despite marginally higher $C_q$ values for the E gene target from thermally treated samples.

### 4.1.4 Further investigation of the effects of thermal treatment

In my opinion, a further investigation of the thermal treatment method on the qualitative outcomes and the quantitative effects on SARS-CoV-2 copy number was needed using a larger sample set—it was necessary to determine the potential loss or gain in sensitivity in terms of clinical impact and, from a method validation perspective and NATA accreditation, a comprehensive study of the effects of this in-house modification would be desirable. I proposed that improved measurement of thermal treatment effects could be achieved using quantitative PCR as a measurement tool. Based on personal experience with quantitative diagnostic assays, I utilised the principles of quantitative PCR to definitively answer the following questions: a) what are the effects on cobas ORF1ab and E gene target copy number after pre-treating samples at 75°C for 15 minutes? b) what impact does thermal treatment have on the final qualitative outcomes? To investigate, a series of controlled experiments were performed on a large collection of positive and negative samples that had not been pretreated. Here, I describe the first cobas quantitative PCR method for SARS-CoV-2 and present the first study in the literature assessing the quantitative effects of thermal treatment prior to cobas testing. This study is presented in **Publication 9** titled "Qualitative and quantitative effects of thermal treatment of naso-oropharyngeal samples before cobas SARS-CoV-2 testing" (202). We reported no significant differences in the final qualitative outcomes for thermal treatment versus room-temperature (99.8% agreement) despite a statistically significant reduction in target copy number for one of the targets (E-gene) following thermal treatment. Hence, we continued testing with added confidence around the assay sensitivity and improved laboratory safety.

### 4.1.5 Improvements to throughput and testing continuity

From March 2020 until late July 2021, our laboratory performed more than 300,000 tests with a thermal treatment step before cobas testing, at an average of 20,000 per month. However, we continued to face supply issues with cobas reagents and consumables and sought to utilise other instruments in our laboratory for additional SARS-CoV-2 testing capacity and mitigate the risk of a lack of testing continuity. We also aimed to implement a dual-target test to mitigate the risk of target drop-out caused by nucleotide polymorphisms, reported for the cobas SARS-CoV-2 pan-Sarbecovirus E gene target (203). To address these needs, we evaluated the RUO PlexZyme SARS-CoV-2 assay (Plex) (SpeeDx, Sydney, Australia). Plex targets the ORF1ab (as for cobas) and RdRp, giving our laboratory added specificity and target redundancy. Plex utilises the same multiplexing technology as the RP-GC assay (*opa*/*porA*/*gyrA*) described in **Chapter 3 Publication 7** (158). The method also utilises a liquid handler and a 384-well PCR plate. I proposed that Plex could resolve a number of challenges; a) SpeeDx

is an Australian company with local manufacturing capabilities, b) alternative testing strategy to cobas thereby safeguarding testing continuity, c) added specificity and redundancy with dual-target capability and a different target compared to cobas (RdRp), d) able to be performed on existing and underutilised laboratory instrumentation with no additional capital expense, e) significant throughput capability to meet routine testing demand and surge capacity, and f) reduced risk of potential repetitive strain injury (RSI) with reduced pipetting steps. In addition, thermal treatment would not be required with a modified MP96 workflow. This new workflow involved sealing the MP96 input plate in the BSC, transfer to the MP96, then removing the seal inside the instrument. Of particular interest to our clinicians was the correlation of Plex $C_q$ compared to cobas $C_q$. In addition, clinical microbiologists, infectious disease physicians and infection control officers, were requesting $C_q$ results to use as a surrogate for viral load determination to assess the risk of infectivity and to assist with other clinical decisions based on the work of serval groups (186, 190, 204). Therefore, it was also important for our laboratory to know Plex $C_q$ relative to cobas $C_q$ across the dynamic range of viral loads (and the Xpert assay as well).

To evaluate Plex, a validation panel consisting of cobas positive and negative samples was prepared from a routine sample collection using the same sample preparation method as in **Publication 9** (1:10 dilution of original sample in a negative matrix). All samples were retested with cobas and Plex, with one key point of difference: cobas retesting was performed from the original stored patient sample and was performed without thermal treatment using PPE as for **Publication 8,** to minimise potential experimental bias. Again, we tested standards to calculate target copy number and assessed assay performance and commutability of the shared target (ORF1ab). During the write-up of the Plex method for publication, the WA Government announced a lockdown on 31 January 2021, resulting in a surge of COVID-19 testing. This provided a unique opportunity to evaluate our synergistic cobas and Plex testing strategy for high-throughput SARS-CoV-2 testing and add throughput details to the Plex publication. This study presents the first manufacturer-independent evaluation of Plex and examines the throughput capabilities of both methods during a surge of SARS-CoV-2 testing. This study is presented in **Publication 10** titled “Evaluation of a PlexZyme-Based PCR Assay and Assessment of COVID-19 Surge Testing Throughput Compared to Cobas SARS-CoV-2” (205). Based on the work described, Plex was introduced into the diagnostic workflow on the 28 August 2020, and greatly improved our throughput and resolved our continuity of testing risk.

### 4.1.6 Improvements to standardisation of SARS-CoV-2 viral load measurement

In February 2021, the First WHO International Standard (IS) for SARS-CoV-2 RNA (20/146) was released, allowing laboratories and manufacturers to calibrate their assays against a universal standard (206). Prior to our laboratory calibrating to this standard, we used a commercial product which contained SARS-CoV-2 gene targets of known copy number. There had been numerous reports describing quantitative SARS-CoV-2 changes over time, associations with clinical outcomes, associations with various specimen types, correlations with infectivity, and correlations with culture positivity (186, 187, 189, 190, 204). However, most of these reports have studied $C_q$ values which are non-standardised and semi-quantitative, which may not accurately correlate with viral load (207). Secondly, while time from admission to sample collection is sometimes reported, many studies have not described the time from symptom onset to specimen collection which is known to be strongly associated with viral load (189). Given that quantitative standards were not standardised across these investigations, a unique opportunity was presented to be the first in the world to report calibration to an international standard, to study the relationship of SARS-CoV-2 viral load from symptom onset and the clinical associations with disease and outcomes. From 201 cases during our first SARS-CoV-2 wave period in WA during March and May 2020, we retrospectively examined for clinical associations with demographic factors, symptoms and the severity of illness, and describe viral loads at release from isolation. This study was presented in **Publication 11** titled "Clinical associations of SARS-CoV-2 viral load using the first WHO International Standard for SARS-CoV-2 RNA" (208).

### 4.1.7 Response to increased testing demands: extraction-free PCR

From the start of the pandemic to late June 2022, our laboratory tested more than 700,000 samples using a combination of cobas, Plex and Xpert. With the WA borders closed for nearly 2 years, sufficient time had elapsed for the reagent and consumable supply chains to be restored. However, on 13 December 2021, it was announced that WA would fully open its borders to COVID-19 vaccinated people from interstate and overseas on 5 February 2022. Consequently, our laboratory was asked by the PathWest Executive to increase our testing capacity and to consider sample pooling to increase throughput. Despite the advantages of pooling in terms of throughput, reduction in operational costs and conserving reagents, pooling has a few of limitations; a) loss in assay sensitivity, proportional to the number of samples in the pool, b) improvements in turnaround time are lost when the increase in prevalence requires retesting individual samples in the pool (209-211). As an alternative to pooling, I proposed leveraging the high-throughput properties of the Plex and convert it into an extraction-free method as the front-line assay for surge capacity testing, given that the Plex assay exceeds cobas

throughput as shown in **Publication 10**. Moreover, aided by quantitative PCR calculations, I calculated in silico the potential loss in sensitivity for such a method compared to extraction-based pooling and non-pooling methods. Preliminary experiments showed that a suitably sensitive direct method may have similar sensitivity to a four-sample pool.

Extraction-free methods for SARS-CoV-2 have been reported, all reporting a loss in sensitivity compared to extraction-based methods (212-217). Most of these studies are limited by the number of samples tested and report the sensitivity of the extraction-free method compared to the extraction-based method according to a $C_q$ value range or before a $C_q$ value cut-off ($C_q$ stratification of results), rather than an overall sensitivity of the method. These investigations used heat as a pretreatment step for virus inactivation and showed that high temperature and short durations improve (reduce) $C_q$ values compared to lower temperature and longer durations. Additionally, the use of proteinase K in the extraction step can also increase yield by three cycles compared to heat treatment alone (214). However, a thermal pre-treatment step for viral inactivation was not necessary for the Plex extraction-free method I was developing (as for the extraction-based Plex method). Instead, thermal treatment was necessary at the end of the extraction-free method to inactivate proteinase K. Based on the loss in analytical sensitivity from thermal pretreatment prior to cobas testing as demonstrated in **Publication 9**, I experimentally proved the quantitative relationship between increasing temperature, duration and reduced RNA yield in copies/mL. Therefore, an extraction-free method designed to minimise the total time of SARS-CoV-2 exposure to elevated temperatures across the entire procedure is likely to be beneficial.

Similarly, the ideal method would include proteinase K to improve yield, at an optimal concentration with the lowest temperature that does not compromise RNA yield, followed by a heat step of the shortest duration possible for complete proteinase K inactivation. During optimisation experiments with a heat and proteinase K step, we added Chelex-100 ion exchange resin based on our previous experience with Chelex-100 (218, 219) and the work of others for SARS-CoV-2, albeit not peer reviewed at the time (220). Chelex-100 is a chelating resin, composed of styrene-divinylbenzene copolymers with paired iminodiacetate ions (acts as chelating groups). Chelex-100 preserves DNA and RNA in the sample by binding metal ion cofactors needed for deoxyribonucleases and ribonucleases, which degrade DNA and RNA respectively (221). The optimisation of the Chelex-100/proteinase K/heat method for maximum RNA yield was performed experimentally using a simple technique that I conceptualised for similar purposes. This technique is described in the Appendix, Table A1. The challenge was to perform successive extraction-free experiments with the goal to progressively increase yield. Acknowledging the extraction-based method was unlikely to be 100% efficient, what was the experimental difference between these methods and was the loss in sensitivity acceptable? I

primarily focused on sensitivity and yield during assay development, avoiding extended exposure to heat. Combining these strategies, I benchmarked a method which included proteinase K at an optimal concentration, an incubation step of 37°C for 10 min, and then 95°C for 90 s for complete inactivation of proteinase K (Direct). To test the optimised method, a collection of SARS-CoV-2 positive (n = 185) and negative (n = 354) naso-oropharyngeal swabs in transport medium were tested in parallel to compare Plex to the Direct method. I also compared the Direct method to a four-sample pool by combining each positive sample (n = 185) with three SARS-CoV-2-negative samples extracted with MP96 and tested with the PlexPCR SARS-CoV-2 assay (Pool). The investigation analyses the qualitative and quantitative differences using statistical measures. This method was also scaled-up for high-throughput performance and the estimated the 24-hour testing capability was reported. This study is presented in the final publication for this thesis, **Publication 12,** titled "High-Throughput COVID-19 Testing of Naso-Oropharyngeal Swabs Using a Sensitive Extraction-Free Sample Preparation Method" (222).

## 4.2 **Publication 8 (Letter to the Editor)**. Pryce etal., 2021. Thermal treatment of nasopharyngeal samples before cobas SARS-CoV-2 testing.

**Pryce TM**, Boan PA, Kay ID, Flexman JP. 2021. Thermal treatment of nasopharyngeal samples before cobas SARS-CoV-2 testing. Clinical Microbiology and Infection 27:149-150.

DOI link 10.1016/j.cmi.2020.07.042

As corresponding author, I conceptualised the study, methods and procedures. I collated all the samples for the investigation and performed all the laboratory testing and experiments. I wrote the protocol, collected and analysed the results and was the main person drafting the manuscript. All other authors either performed supervisory roles, provided clinical information and/or contributed to the writing and editing. All authors reviewed the manuscript prior to publication.

2024 Clarivate journal impact factor from Journal Citation Reports: 10.9

Clinical Microbiology and Infection 27 (2021) 149–150

Contents lists available at ScienceDirect

Clinical Microbiology and Infection

journal homepage: www.clinicalmicrobiologyandinfection.com

Letter to the Editor

# Thermal treatment of nasopharyngeal samples before cobas SARS-CoV-2 testing

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ARTICLE INFO

*Article history:*
Received 15 June 2020
Received in revised form
28 July 2020
Accepted 30 July 2020
Available online 6 August 2020

Editor: F. Allerberger

Since the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic, we have seen shortages of diagnostic reagents, consumables and personal protective equipment [1,2]. Initially we inactivated samples with guanidine hydrochloride (GuHCl) before SARS-CoV-2 testing [2,3]. Following implementation of cobas SARS-CoV-2 testing (Roche, Basel, Switzerland), we encountered shortages of GuHCl and personal protective equipment. To overcome these issues, we investigated a number of rapid heat treatment steps before cobas testing. Temperatures and durations investigated were based on reports demonstrating the effects of temperature on the viability of SARS-CoV-2 (70°C for 5 minutes) [4] and other coronaviruses [5–7].

To test the effects of thermal treatment on cobas assay performance, we tenfold serially diluted cobas-positive clinical samples collected in Copan UTMRT media (Brescia, Italy) from different patients ($n = 8$) using cobas-negative nasopharyngeal matrix and thermally treated incrementally at 60°C, 65°C and 75°C (UTM internal temperature) for a total time of 15 minutes, 30 minutes and 60 minutes at each temperature point. Aliquots were prepared in cobas omni secondary tubes (ref. 06438776001) and were thermally treated in a Dri-Bath. An aliquot of each dilution remained untreated (room-temperature control), and cobas testing was performed in parallel for all samples ($n = 34$). We also prospectively tested 40 consecutive patient samples comparing 75°C for 15 minutes to room temperature (Table 1 and Supplementary Material).

All samples were heated immediately before extraction and were loaded without delay (<5 minutes). We recorded the cycle threshold ($C_t$) for ORF1a, E-gene and internal control. All $C_t$ values are shown in the Supplementary Material, and the qualitative results are shown in Table 1. Positive $C_t$ values in both ORF1a and E-gene for thermally treated and room-temperature control were compared by the two-tailed paired *t* test ($p < 0.05$). The same statistical approach was applied for all internal control $C_t$ values. All $C_t$ values were normally distributed (D'Agostino-Pearson test). For thermal treated compared to room-temperature control, we found no significant difference in $C_t$ values for ORF1a (22 samples compared, mean difference +0.13 ± 0.89 SD, p 0.502). However, a significant difference was observed for E-gene (21 samples compared, mean difference +0.55 ± 1.15 SD, p 0.040) and internal control (all 74 samples compared, −0.27 ± 0.40 SD, p 0.00001). In summary, the mean ORF1a and E-gene $C_t$ values were 0.13 and 0.55 $C_t$ higher for heat treatment than control respectively.

Higher $C_t$ values for thermally treated samples may suggest a reduction in detectable virus RNA. A recent study using a commercial qualitative method (BioGerm Medical Biotechnology, Shanghai, China) and quantitative digital PCR (TargetingOne, Beijing, China) demonstrated a drop in SARS-CoV-2 copy number by 50% to 66% after heating at 80°C for 20 minutes [8]. However, internal control test performance was not evaluated (or not included as part of the assay), and correlation with other commercial *in vitro* diagnostic assays or cobas is not known. Despite marginally higher cobas $C_t$ values for thermally treated samples in our study, conflicting findings were observed for the qualitative detection of SARS-CoV-2, specifically detection of ORF1a and E-gene targets at the limit of detection (Table 1). Although we observed $C_t$ shifts in the cobas E-gene target, the cobas assay is dual target, and therefore the delay in one target may not be critical to the qualitative detection of SARS-CoV-2. Of 34 dilutions prepared, SARS-CoV-2 was detected in three thermally treated samples (ORF1a ± E-gene) and three were presumptively positive for SARS-CoV-2 (E-gene only), all of which were negative for the room-temperature control.

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https://doi.org/10.1016/j.cmi.2020.07.042

**Table 1**
Summary of cobas SARS-CoV-2 results in 74 samples

| Result outcome | No. of samples | cobas SARS-CoV-2 result for: | |
|---|---|---|---|
| | | Heat treatment | Room-temperature control |
| 1 | 22 | Detected | Detected |
| 2 | 3 | Detected | Negative |
| 3 | 3 | Presumptive positive | Negative |
| 4 | 1 | Presumptive positive | Presumptive positive |
| 5 | 45 | Negative | Negative |

Detected indicates ORF1a positive, E-gene positive; presumptive positive, ORF1a negative, E-gene positive.
SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

On the basis of these results, heat treatment may improve the qualitative detection of SARS-CoV-2. To confirm our qualitative findings, many replicates at the lower limit of detection combined with probit analysis are required. As a result of safety concerns and suboptimal recovery of SARS-CoV-2 from culture, our laboratory did not confirm the inactivation efficacy of thermal treatment. However, using standard biosafety level 2 (BSL2) laboratory safety procedures, we continue to use the highest temperature assessed with a time that suits the work flow (75°C for 15 minutes), thereby exceeding a previously published temperature and duration of 70°C for 5 minutes for complete SARS-CoV-2 inactivation in virus transport medium [4].

We acknowledge that thermal inactivation of SARS-CoV-2 may not be 100% efficient [4–7]. However, we consider the risk to staff in a well-equipped BSL2 laboratory is greatly reduced with thermal pretreatment. Moreover, thermal treatment negates the need for GuHCl and enables the redirection of personal protective equipment to frontline personnel.

## Transparency declaration

Funding was provided by PathWest. All authors report no conflicts of interest relevant to this article.

## Acknowledgements

The authors are grateful to Adrian Bautista (Acting Chief Executive of PathWest at the commencement of COVID-19 testing) for provisioning the cobas kits.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cmi.2020.07.042.

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## 4.3 **Publication 9**. Pryce et al., 2021. Qualitative and quantitative effects of thermal treatment of naso-oropharyngeal samples before cobas SARS-CoV-2 testing.

**Pryce TM**, Boan PA, Kay ID, Flexman JP. 2021. Qualitative and quantitative effects of thermal treatment of naso-oropharyngeal samples before cobas SARS-CoV-2 testing. Diagnostic Microbiology and Infectious Disease 101:115519.

DOI link 10.1016/j.diagmicrobio.2021.115519

As corresponding author, I conceptualised the study, methods and procedures. I collated all the samples for the investigation and performed all the laboratory testing and experiments. I wrote the protocol, collected and analysed the results and was the main person drafting the manuscript. All other authors either performed supervisory roles, provided clinical information and/or contributed to the writing and editing. All authors reviewed the manuscript prior to publication.

2024 Clarivate journal impact factor from Journal Citation Reports: 2.1

Diagnostic Microbiology and Infectious Disease 101 (2021) 115519

Contents lists available at ScienceDirect

Diagnostic Microbiology and Infectious Disease

journal homepage: www.elsevier.com/locate/diagmicrobio

# Qualitative and quantitative effects of thermal treatment of naso-oropharyngeal samples before cobas SARS-CoV-2 testing

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ARTICLE INFO

*Article history:*
Received 29 March 2021
Revised in revised form 8 August 2021
Accepted 11 August 2021
Available online 24 August 2021

*Keywords:*
Thermal treatment
SARS-CoV-2
Cobas
Qualitative
Quantitative

ABSTRACT

To improve laboratory safety we thermally treated naso-oropharyngeal samples before testing with the cobas SARS-CoV-2 assay. This study aimed to determine if thermal treatment significantly affects the qualitative detection of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and the quantitative measurement of cobas SARS-CoV-2 ORF1a and E-gene target copy number using an in-house quantitative method. A collection of positive ($n$ = 238) and negative samples ($n$ = 196) was tested in parallel comparing thermal treatment (75 °C for 15 minutes) to room-temperature. There were no significant differences in the final qualitative outcomes for thermal treatment versus room-temperature (99.8% agreement) despite a statistically significant reduction ($P < 0.05$) in target copy number following thermal treatment. The median ORF1a and E-gene reduction in target copy number was -0.07 (1.6%) and -0.22 (4.2%) $\log_{10}$ copies/mL respectively. The standard curves for both ORF1a and E-gene targets were highly linear ($r^2$ = 0.99). Good correlation was observed for ORF1a ($r^2$ = 0.96) and E-gene ($r^2$ = 0.98) comparing thermal treatment to room-temperature control.



## 1. Introduction

Since the start of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic, we have seen shortages of laboratory reagents, consumables and personal protective equipment (PPE) [(Tang et al., 2020; World Health Organization (WHO) 2020)]. Our initial testing protocols included guanidine hydrochloride inactivation of SARS-CoV-2 to improve laboratory safety and avoid the use of additional PPE (eye protection, N95 mask, disposable gown) (Corman et al., 2020; Tang et al., 2020). When we transitioned SARS-CoV-2 testing to the cobas 6800 system (Roche Diagnostics, Basel, Switzerland) we were concerned about transferring un-capped samples to the cobas 6800 instrument so returned to using additional PPE as a precaution. We were also concerned about the pipetting workload associated with the addition of guanidine hydrochloride to samples before cobas testing, potential loss of assay sensitivity from sample dilution and assay nonspecific interference. The effects of temperature on the viability of SARS-CoV-2 and other coronaviruses have been reported (Chin et al., 2020; Pagat et al., 2007; Rabenau et al., 2005; Yunoki et al., 2004). Our initial investigations suggested thermal treatment of nasopharyngeal samples resulted in statistically significant higher cycle threshold ($C_t$) values for E-gene ($P$ 0.040), suggesting a reduction in detectable virus RNA (Pryce et al., 2021). Despite marginally higher cobas $C_t$ values for thermally treated samples, conflicting findings were observed for the qualitative detection of SARS-CoV-2, specifically detection of ORF1a and E-gene targets at the limit of detection. The chief aim of this study was to determine if thermal treatment of naso-oropharyngeal samples before cobas SARS-CoV-2 testing affects the qualitative detection of SARS-CoV-2 and the quantitative detection of RNA target copy number. In addition, we developed and present here a quantitative method for cobas SARS-CoV-2 ORF1a and E-gene targets, using a commercially available SARS-CoV-2 standard to assess the effects of thermal treatment on RNA target copy number. We also investigated quantitative and qualitative results of storage at room-temperature for up to 48 hours for thermally treated and non-thermally treated samples in case we encounter testing delays.

## 2. Material and methods

### 2.1. Patient samples

A combined nasopharyngeal and oropharyngeal swab from each patient was inoculated into 3 mL of either Copan UTM-RT media (Brescia, Italy), CITOSWAB (Citotest Scientific Jiangsu, People's Republic of China) or Virus Transport Media (VTM) prepared by PathWest Media (Centers for Disease Control and Prevention, Atlanta GA 2020). All samples were initially tested as part of routine testing

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https://doi.org/10.1016/j.diagmicrobio.2021.115519
0732-8893/

using the cobas SARS-CoV-2 assay (Roche Diagnostics, Basel, Switzerland). This test is performed on either the cobas 6800 or cobas 8800 instrument (Roche) and is a walkaway sample-to-result assay. The cobas SARS-CoV-2 assay targets ORF1a (a nonstructural region that is unique to SARS-CoV-2) and E-gene (a structural protein envelope gene for pan-sarbecovirus detection). According to the manufacturer's instructions, a sample is SARS-CoV-2 positive if ORF1a is detected with or without E-gene detection. In the case of positivity with E-gene alone, the result should be reported as SARS-CoV-2 presumptive positive.

All SARS-CoV-2 positive samples were stored as aliquots at -80 °C. To prepare positive samples for this study ($n$ = 238; positive sample group) a 0.2 mL aliquot from each sample was diluted with 1.8 mL of a nasopharyngeal/throat matrix (1:10 dilution). The matrix consisted of pooled cobas SARS-CoV-2 negative patient samples (oro-nasopharyngeal swabs) in VTM. The pooled matrix tested negative with cobas SARS-CoV-2 and Xpert Xpress SARS-CoV-2 (Cepheid, Sunnyvale, California, USA). All dilutions were prepared in cobas omni secondary tubes (Ref. 06438776001). Cobas SARS-CoV-2 negative samples were stored at 4 °C and were not diluted ($n$ = 196; negative sample group). All samples were tested following protocols issued by the manufacturer (room-temperature control). Un-capped samples in cobas omni secondary tubes were transferred to the cobas 6800 system by laboratory personnel wearing additional PPE (eye protection, N95 mask, disposable gown). Samples were retrieved from the cobas 6800 following sample aspiration with additional PPE, capped and thermally treated for 75 °C for 15 minutes in a QBD4 dry block heater (Grant Instruments, Cambridge, United Kingdom), then retested within 2 hours (thermal treatment) without the use of additional PPE. The qualitative results and $C_t$ values were recorded for ORF1a, E-gene and Internal Control (IC).

### 2.2. Quantitative standards, external control and analysis

Quantitative standards were prepared from a commercially available SARS-CoV-2 standard (Exact Diagnostics, Fort Worth, Texas). Exact Diagnostics SARS-CoV-2 standard contains E-gene and ORF1ab synthetic RNA transcripts quantitated to 200,000 copies/mL using Bio-Rad Digital Droplet PCR (Hercules, California). We pooled multiple vials and 10-fold serially diluted in molecular grade water (G-Biosciences, St. Louis, Missouri, USA) to prepare 6 standards over the range of 0.30 to 5.30 $\log_{10}$ copies/mL. Each standard was tested with cobas SARS-CoV-2 in duplicate (no thermal treatment) on a single run using cobas SARS-CoV-2 kit lot G18524. The mean $C_t$ value at each concentration was used to calculate ORF1a and E-gene standard curves and regression. Both replicates at each dilution were required to be positive to be included in the standard curve and regression analysis. The regression formulas were used to calculate the ORF1a and E-gene copy number for all positive samples and controls over 3 consecutive runs. The testing for all positive samples and the quantitative standards was performed using the same cobas SARS-CoV-2 kit lot (G18524). As part of the NRL QConnect programme (NRL, Victoria, Australia) an external control (EQC) was also performed routinely to monitor reproducibility (Optitrol NAT SARS-CoV-2; DiaMex, Heidelberg, Germany). A single lot number of EQC (DM20119) was tested over 19 runs.

### 2.3. Effects over time

A low-titre positive patient sample in VTM (≈3.00 $\log_{10}$ copies/mL) was used to study effects over time. Two replicates were thermally treated then tested at 2, 4, 6, 10, 24 and 48-hour intervals (kept at room temperature). Another 2 replicates remained at room-temperature and were tested in parallel with the thermally treated samples. The $C_t$ values for ORF1a, E-gene and IC was recorded for each time point and the ORF1a and E-gene copy number was calculated using the standard curves.

### 2.4. Statistical analysis

All results of ORF1a and E-gene ($C_t$ values and $\log_{10}$ copies/mL) for thermal treatment and room-temperature were compared using the Wilcoxon signed-rank test ($P < 0.05$). The same statistical approach was applied for all IC $C_t$ values. The median for each group was calculated and used to determine the percentage reduction or gain in $C_t$ value or $\log_{10}$ target copy number/mL. Differences in the mean and standard deviation were also calculated for comparison. Correlation between comparing thermal treatment to room temperature for ORF1a-positive samples ($n$ = 180) and E-gene-positive samples ($n$ = 201) was also performed. The mean and standard deviation was calculated for the EQC. All statistical analyses were performed by Excel (Microsoft, Redmond, WA, USA) and MedCalc v15.4 (New York, NY, USA).

### 2.5. Ethics statement

Not applicable: the residual samples used in the study were de-identified and results were not used to clinically manage patients [National Statement on Ethical Conduct in Human Research 2007 (May 2015) by the National Health and Medical Research Council, Australian Research Council and Australian Vice-Chancellors' Committee].

## 3. Results

### 3.1. Qualitative analysis

The qualitative results comparing thermal treatment to room-temperature for the positive sample group ($n$ = 238) and negative

**Table 1**
Summary of cobas SARS-CoV-2 results for 434 samples.

| Result outcome | No. of samples | Sample group | cobas SARS-CoV-2 result for: Heat treatment | Room temperature control |
|---|---|---|---|---|
| 1 | 180 | Positive | Detected | Detected |
| 2 | 13 | Positive | Presumptive positive | Presumptive positive |
| 3 | 6 | Positive | Detected | Presumptive positive |
| 4 | 5 | Positive | Presumptive positive | Detected |
| 5 | 6 | Positive | Presumptive positive | Negative |
| 6 | 6 | Positive | Negative | Presumptive positive |
| 7 | 22 | Positive | Negative | Negative |
| 8 | 196 | Negative | Negative | Negative |

Detected indicates ORF1a positive, E-gene positive.
Presumptive positive indicates ORF1a negative, E-gene positive.
SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

sample group ($n$ = 196) are shown in the Supplementary Material and are summarized in Table 1. All samples in the negative sample group were negative ($n$ = 196). Samples in the positive sample group were positive ($n$ = 180; ORF1a positive, E-gene positive or negative), presumptive positive ($n$ = 13; ORF1a negative, E-gene positive), or negative ($n$ = 22; ORF1a and E-gene negative) for both thermal treatment and room-temperature. Discordant results of paired samples comparing thermal treatment and room-temperature were also observed in the positive sample group ($n$ = 23). We expected negative results and discordant results for some samples in the positive sample group as they were diluted 10-fold from archival cobas SARS-CoV-2 positive material already at the lower limit of detection. These discordant results all demonstrate late $C_t$ values as shown in the Supplementary Material.

No significant differences in the qualitative outcomes were observed. The room-temperature group resulted in positive ($n$ = 185), presumptive positive ($n$ = 25) and negative ($n$ = 224) outcomes compared to positive ($n$ = 186), presumptive positive ($n$ = 24) and negative ($n$ = 224) for the thermal treatment group. No samples were inhibited (all had a positive IC).

### 3.2. Standard curves

The $C_t$ values for each target concentration and standard curves for ORF1a and E-gene are shown in the Supplementary Material. The results for both targets were highly linear. ORF1a demonstrated R-squared value of 0.9988 over the range of 1.30 to 5.30 $\log_{10}$ copies/mL (5 standards). ORF1a detection at 0.30 $\log_{10}$ copies/mL was not reproducible and was omitted from the standard curve. E-gene demonstrated R-squared value of 0.9994 over the range of 2.30 to 5.30 $\log_{10}$ copies/mL (4 standards). E-gene detection at 1.30 $\log_{10}$ copies/mL was not reproducible and was omitted from the standard curve. Both replicates were negative at 0.30 $\log_{10}$ copies/mL for E-gene.

### 3.3. Statistical and quantitative analysis

All $C_t$ values for ORF1a, E-gene and internal control for all samples are shown in the Supplementary Material. The quantitative measurement of ORF1a and E-gene targets in copies/mL and $\log_{10}$ copies/mL are also shown, including the ORF1a and E-gene targets above and below the calculated standard curve range; the standard curve for each target was assumed to be linear above and below the calculated standard curve range to simplify statistical analysis. A summary of the Wilcoxon Signed-Ranks statistical analysis for ORF1a, E-gene and IC are summarized in Table 2. The mean differences and standard deviation differences are also shown for comparison. For thermal treatment compared to room-temperature, we found a significant difference ($P < 0.05$) in median $C_t$ and/or quantitative values for ORF1a, E-gene and IC for all samples. The median ORF1a and E-gene reduction in target copy number was -0.07 (1.6%) and -0.22 (4.2%) $\log_{10}$ copies/mL respectively. Good correlation was observed for ORF1a ($r^2$ = 0.96) and E-gene ($r^2$ = 0.98) comparing thermal treatment to room-temperature control (Fig. 1).

The EQC was assessed over 19 runs as shown in the Supplementary Material. ORF1a demonstrated a mean of 3.95 ± 0.20 $\log_{10}$ copies/mL and E-gene 4.77 ± 0.23 $\log_{10}$ copies/mL across 12 reagent lot numbers. ORF1a demonstrated a mean of 3.88 ± 0.12 $\log_{10}$ copies/mL and E-gene 4.63 ± 0.21 $\log_{10}$ copies/mL for the positive sample group tested with the quantitative standards (lot number G18524).

### 3.4. Effects over time

The $C_t$ values for ORF1a, E-gene and internal control and the quantitative results over time for the thermally treated and room-temperature replicates are shown in the Supplementary Material. We observed no evidence of increasing $C_t$ values or decline in target copy number over 2, 4, 6, 10, 24 and 48-hour intervals for either thermally treated or room-temperature replicates. The $C_t$ values and quantitative results remained stable over the 48-hour time period.

## 4. Discussion

We implemented thermal treatment of patient samples to improve laboratory safety and reduce additional PPE use (Pryce et al., 2021). Preliminary results with a limited number of positive samples ($n$ = 34) showed increased $C_t$ values for thermal treatment compared to room-temperature despite a potential improvement in the qualitative detection of SARS-CoV-2 for thermally treated samples. Our aim was to provide clarity using a greater number of positive samples ($n$ = 238) and include more negative control samples to verify that thermal treatment does not cause nonspecific results. We sought to improve this assessment by measuring ORF1a and E-gene target copy number with a commercial quantitative standard. We also assessed thermal treatment compared to room temperature over time to obtain a better understanding of the stability of the ORF1a and E-gene targets with storage at ambient temperature.

A direct comparison of thermal treatment and room-temperature using undiluted original patient material would have been ideal. However, following initial routine testing and our SARS-CoV-2 surveillance testing, the residual sample volume was limited to conduct parallel re-testing with sufficient sample remaining for future research. To overcome this we diluted all positive samples in our collection with a pooled oro-nasopharyngeal matrix derived from SARS-CoV-2-negative patient samples to maximise the number of positive samples from different patients in this study. The closest representation to an original sample was maintained with this approach.

We found no significant differences in the qualitative outcomes (detected, presumptive or negative) for thermally treated samples compared to room-temperature samples in this study. In the previous study (Pryce et al., 2021) we observed additional positive results

**Table 2**
Summary of the Wilcoxon signed-rank test ($P < 0.05$) for thermally treated samples compared to room-temperature control.

| Target | Number compared | Median thermal treatment | Median room-temperature | Median difference | Mean difference | Standard deviation difference | $P$ value |
|---|---|---|---|---|---|---|---|
| ORF1a $C_t$ | 180 | 28.25 | 28.06 | +0.19 | +0.13 | 0.81 | 0.024 |
| ORF1a $\log_{10}$ copies/mL | 180 | 4.42 | 4.49 | -0.07 | -0.04 | 0.28 | 0.029 |
| E-gene $C_t$ | 201 | 29.88 | 29.28 | +0.60 | +0.31 | 0.71 | < 0.01 |
| E-gene $\log_{10}$ copies/mL | 201 | 5.02 | 5.24 | -0.22 | -0.11 | 0.26 | < 0.01 |
| IC (SARS-CoV-2 positive) $C_t$ | 180 | 33.57 | 34.49 | -0.92 | -0.44 | 0.92 | < 0.01 |
| IC (SARS-CoV-2 negative) $C_t$ | 218 | 33.10 | 33.03 | +0.07 | +0.05 | 0.41 | < 0.01 |
| IC (all samples) $C_t$ | 434 | 33.27 | 33.30 | -0.03 | -0.18 | 0.73 | < 0.01 |

IC indicates cobas SARS-CoV-2 internal control.
$C_t$ indicates cycle threshold.
$\log_{10}$ copies/mL indicates the concentration of target quantified using the Exact Diagnostics SARS-CoV-2 standard curves.

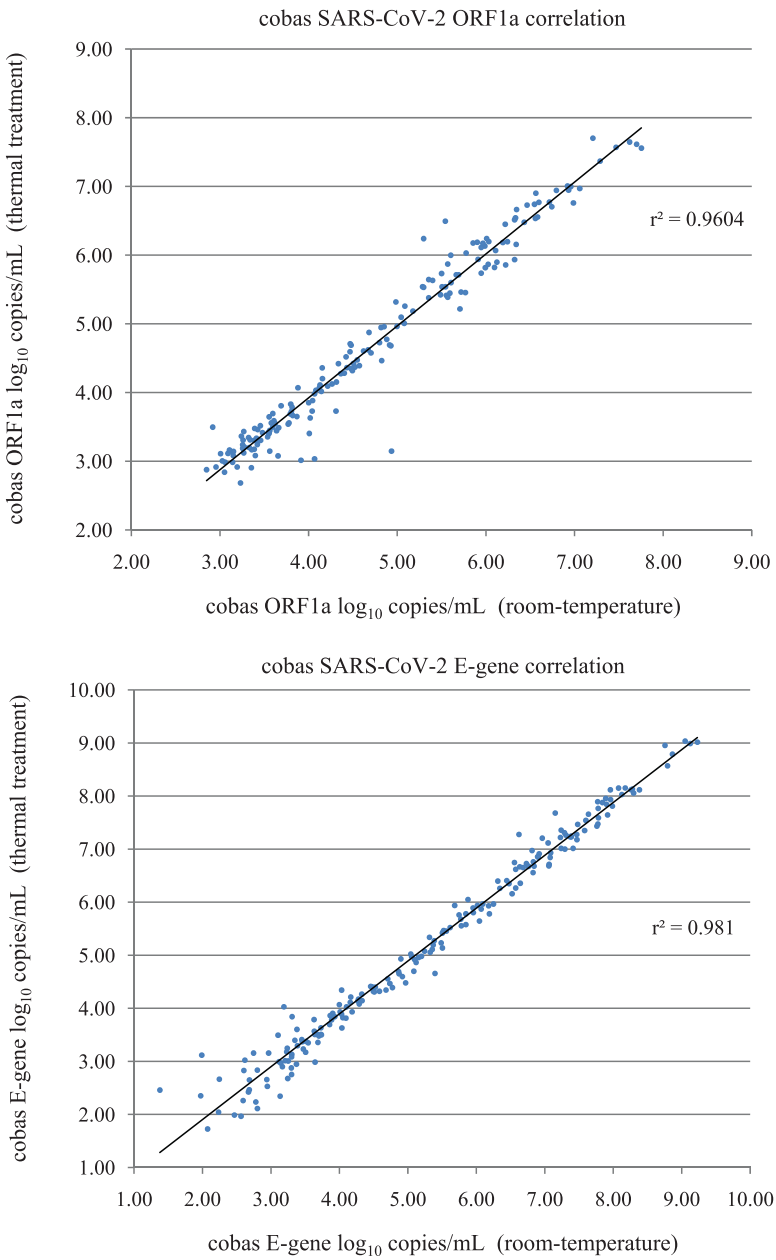


**Fig. 1.** Correlation in $\log_{10}$ copies/mL obtained by cobas SARS-CoV-2 following thermal treatment for ORF1a (target 1) and E-gene (target 2) compared to cobas SARS-CoV-2 room-temperature control. Linear regression was performed using samples positive for SARS-CoV-2 for both thermal treatment and room-temperature control for ORF1a (n = 180) and E-gene (n = 201). The $r^2$ correlation is indicated.

($n$ = 3) and presumptive results ($n$ = 3) for thermal treatment compared to room-temperature (17.6%; 6/34 samples in the positive group), compared to only 1 additional positive result for thermal treatment (0.4%; 1/238 samples in the positive group) in this study. In the previous study we performed 10-fold serial dilutions ($n$ = 34) for each patient sample ($n$ = 8) compared to a single dilution for each patient this study ($n$ = 238). Although assay sensitivity is best shown by testing replicates at the lower limit of detection, we were limited due to costs and reagents to perform similar 10-fold serial dilutions for all positive samples. Nevertheless, the current study included sufficient samples of low concentration where the qualitative results were not different overall. We conclude with this larger study that there are no significant qualitative differences between thermally treated and room-temperature samples. Although not validated by the manufacturer, there was no evidence that thermal treatment leads to non-specificity.

Other investigators using a digital droplet PCR method have demonstrated a median drop in SARS-CoV-2 copy number of 50% to 66% after heating samples ($n$ = 63) at different SARS-CoV-2 concentrations for 80 °C for 20 minutes (Chen et al., 2020). Whilst digital droplet PCR methods are useful for the sensitive detection and quantification of SARS-CoV-2 (de Kock et al., 2021), they are not practical for routine diagnostic SARS-CoV-2 detection as digital droplet PCR machines lack the necessary throughput required for front-line testing (Vasudevan et al., 2021). The cobas 6800/8800 instrument is a sample-to-result platform widely used for molecular diagnostics and SARS-CoV-2 was added in response to the pandemic with recent reports of strong correlation with other assays and utility emerging (Poljak et al., 2020). The exceptional test performance of quantitative cobas 6800/8800 assays (using an internal quantitation standard) for blood-borne virus testing is well known (Roh et al., 2021; Tan et al., 2018; Yao et al., 2018). We sought to utilise the platform as a

quantitative assay using an external standard as a reference. To our knowledge a quantitative cobas SARS-COV-2 method has not been published. To assess the loss of target copy number following thermal treatment we developed standard curves for the quantitation of ORF1a and E-gene copy number for cobas SARS-CoV-2 test. The ORF1a and E-gene standard curves were linear ($r^2 > 0.99$) and the cobas SARS-CoV-2 test was shown to be a reproducible assay in our laboratory using a commercially available quality control. We demonstrated statistically significant reduction in the median target copy number for ORF1a ($P$ 0.03) and E-gene ($P < 0.01$) after heating samples for 75 °C for 15 minutes. However in contrast, the median and mean difference was quantitatively small for both targets (<5%) and is unlikely to be clinically significant. We conclude the negligible loss in ORF1a and E-gene target copy number following thermal treatment is outweighed by a significant improvement of laboratory safety and handling of SARS-CoV-2, particularly with no evidence of detrimental qualitative outcomes.

There are conflicting reports of the effect of thermal treatment of SARS-CoV-2. Hemati et al. (2020) demonstrated that thermal inactivation of patient samples (60 °C for 30 minutes) results significantly lower $C_t$ values for N and ORF1ab gene ($P$ 0.009 and $P$ 0.32 respectively) using an in-house PCR method (Hemati et al., 2021). However, the number of clinical samples tested by PCR ($C_t$ values compared) was low ($n$ = 7). Burton et al. (2021) showed thermal inactivation (56 °C and 60 °C) of a single strain of SARS-CoV-2 did not affect PCR sensitivity using the method of Corman et al. (2020), but showed a minimum increase of 3 $C_t$ values when treated at 80 °C for 30 minutes (Burton et al., 2021). In comparison, we demonstrated mean $C_t$ value increases of +0.13 for ORF1a and +0.31 for E-gene following thermal treatment at 75 °C for 15 minutes when tested with a commercial SARS-CoV-2 assay, using a large number of positive samples (key point of difference). We also assess the effects of thermal treatment of negative controls (nonspecific results were not observed).

We routinely thermally treat samples and leave the aliquots overnight at 4 °C to avoid delays in processing the next day and to maximise throughput. Investigations past 48 hours were not performed as our laboratory has an expected test-turnaround-time of <24 hours from collection. Initially we speculated that thermal treatment may inactivate nucleases and preserve the sample over extended periods of time. To investigate we performed quantitative measurement of ORF1a and E-gene targets comparing thermal treatment and room temperature over time intervals. No reduction in ORF1a or E-gene copy number was observed over a 48-hour duration. This is an important finding for remote collection where refrigeration may not be immediately available and transport to the testing laboratory may be delayed. Delays in testing may also occur due to overwhelming workload. We conclude that SARS-CoV-2 RNA targets remain stable in VTM over the 6-12 hour time delays that may be encountered due to workload. Investigations with other media and manufacturers are ongoing with similar results (data not shown). We recommend other laboratories conduct their own in-house evaluation of the media locally available for cobas or other SARS-CoV-2 testing.

Due to safety concerns at the time and suboptimal recovery of SARS-CoV-2 from culture, our laboratory did not confirm the inactivation efficacy of 75 °C for 15 minutes. However, we implemented a thermal treatment method that exceeds the temperature and time duration from a previously published method of 70 °C for 5 minutes for complete SARS-CoV-2 inactivation in virus transport medium (Chin et al., 2020). Subsequently, a report from the Institut Pasteur (France) has shown that SARS-CoV-2 is relatively sensitive to heat inactivation using a dry heating block and can be inactivated in less than 30 minutes, 15 minutes and 3 minutes at 56 °C, 65 °C and 95 °C respectively (Batéjat et al., 2021) In our laboratory, thermal treatment before cobas SARS-CoV-2 testing is a simple precautionary method to improve safety when transferring un-capped samples to the cobas 6800 instrument, which does not affect the qualitative detection SARS-CoV-2 with this assay. The use of additional PPE has also been reduced.

## Acknowledgments

The authors are grateful to all laboratory personnel that work in the Department of Clinical Microbiology, for their dedication, diligence and perseverance.

## Funding

This study did not receive funding support.

## Declaration of competing Interest

The authors declare that they have no competing interests.

## Authors' contributions

Todd Pryce: Conceptualization, Methodology, Data analysis, Writing- Original draft preparation, Writing – Reviewing and Editing. Peter Boan: Data analysis, Writing – Reviewing and Editing. Ian Kay: Writing – Reviewing and Editing. James Flexman: Writing – Reviewing and Editing.

## Supplementary materials

The authors have provided raw data in the supplementary file submitted.

## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.diagmicrobio.2021.115519.

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## 4.4 **Publication 10**. Pryce et al., 2021. Evaluation of a PlexZyme-Based PCR Assay and Assessment of COVID-19 Surge Testing Throughput Compared to Cobas SARS-CoV-2.

**Pryce TM**, Haygarth EJ, Bordessa J, Boan PA. 2021. Evaluation of a PlexZyme-Based PCR Assay and Assessment of COVID-19 Surge Testing Throughput Compared to Cobas SARS-CoV-2. Pathogens 10(9).

DOI link 10.3390/pathogens10091088

As corresponding first author, I conceptualised the study, methods and procedures. I wrote the protocol, tested samples, analysed the results and was the main person drafting the manuscript. All other authors either assisted with testing, collated testing data and/or contributed to the writing and editing. All authors reviewed the manuscript prior to publication.

Clarivate journal impact factor from Journal Citation Reports: 3.3

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# Evaluation of a *PlexZyme*-Based PCR Assay and Assessment of COVID-19 Surge Testing Throughput Compared to Cobas SARS-CoV-2

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**Abstract:** Reliable high-throughput methods are required for the detection of severe acute respiratory coronavirus 2 (SARS-CoV-2). We evaluated the new research use only (RUO) SpeeDx *PlexZyme* SARS-CoV-2 components (Plex) compared to the Roche cobas SARS-CoV-2 assay (cobas). A collection of positive ($n$ = 214) and negative samples ($n$ = 201) was tested in parallel comparing Plex with cobas. The overall agreement comparing the qualitative outcomes was 96.9%. Using an in-house quantitative PCR method, correlation comparing Plex ORF1ab to cobas ORF1a was $r^2 = 0.95$. The median Plex ORF1ab change in target copy number compared to cobas ORF1a was +0.48 $\log_{10}$ copies/mL respectively. Inter- and intra-assay reproducibility of each assay was compared, including a limit-of-detection study. Reproducibility was comparable; however cobas was more sensitive than Plex by 1-log dilution. Throughput was evaluated during a COVID-19 testing surge of 4324 samples in a 30-h period. Plex demonstrated less hands-on time per reportable result (19% decrease) and increased throughput (155% increase of 102 results/hour) compared to cobas (40 results/hour). Our study demonstrates good qualitative and quantitative correlation of Plex compared to cobas and that Plex is well-suited for high throughput testing.

**Keywords:** SARS-CoV-2; PlexZyme; high-throughput



**Citation:** Pryce, T.M.; Haygarth, E.J.; Bordessa, J.; Boan, P.A. Evaluation of a *PlexZyme*-Based PCR Assay and Assessment of COVID-19 Surge Testing Throughput Compared to Cobas SARS-CoV-2. *Pathogens* **2021**, *10*, 1088. https://doi.org/10.3390/pathogens10091088

Academic Editor: Nicola Coppola

Received: 22 July 2021
Accepted: 24 August 2021
Published: 26 August 2021



## 1. Introduction

As of 1 July 2021, more than 180 million cases of Coronavirus disease 2019 (COVID-19) have been declared worldwide, resulting in 3.9 million deaths [1]. Diagnostic tools are essential to manage the current COVID-19 pandemic and reliable, high-throughput laboratory tests are required [2]. These tools are the strategic cornerstone to mitigate SARS-CoV-2 spread, facilitating the early diagnosis, isolation of infected individuals and clearance of essential personnel to continue to work [3]. Since the 19th of March 2020, our laboratory has performed more than 300,000 tests with the majority of testing performed using the cobas SARS-CoV-2 assay (cobas) (Roche, Basel, Switzerland) [4]. Other essential diagnostic services such as blood-borne virus testing (BBV) on the cobas 6800 instrument (Roche) were maintained despite the additional SARS-CoV-2 workload. As with other laboratories, we implemented SARS-CoV-2 testing in addition to other diagnostic services, placing tremendous strain on laboratory resources. To alleviate the SARS-CoV-2 workload and provide additional routine testing capacity on the cobas 6800 instrument, we sought to utilise other instruments in our laboratory for SARS-CoV-2 testing. These include two MagNAPure 96 instruments (Roche) for nucleic acid extraction combined with two LightCycler 480 thermal cyclers (Roche). We also sought to implement a dual-target test to mitigate the risk of single-nucleotide polymorphisms, which has been reported for the cobas SARS-CoV-2 pan-*Sarbecovirus* E-gene target [5]. A 384-well thermal cycling method

was considered advantageous to maximise testing throughput; hence, a 384-well liquid handler was also a mandatory requirement. To combine these elements, we evaluated the RUO *PlexZyme* CoV-2 (RdRp/ORF1ab) components (Plex) (SpeeDx, Sydney, Australia). Plex targets the conserved open reading sequences (ORF1ab) and the RNA-dependant RNA polymerase gene (RdRp). The *PlexZyme* technology utilises a PlexPCR approach and touch-down PCR for superior specificity and multiplexing capability [6]. The method utilizes the PlexPrep liquid handler (SpeeDx) to prepare and dispense master mix to a 384-well PCR plate then transfer nucleic acids from up to four 96-well MagNAPure 96 output plates. Amplification and detection in the 384-well format takes 82 min irrespective of the number of samples. Since implementation of cobas testing, stored SARS-CoV-2-positive samples (naso-oropharyngeal samples in virus transport medium or universal transport medium) were used to evaluate the *PlexZyme*-based assay and associated RdRp and ORF1ab gene targets to detect SARS-CoV-2. Particular attention to throughput capability of Plex compared to cobas was assessed and significant throughput advantages were noted. As a result of this evaluation the Plex assay was implemented into routine use. On the 31 January 2021, the Western Australian Government announced a lockdown which resulted in a surge of COVID-19 testing. From 31 January 2021 1826 h to 2 February 2021 0050 h (30 h), our laboratory tested and reported 4324 tests combining cobas and Plex methods. We outline the testing performed during this period detailing the cobas and Plex testing strategy for high-throughput COVID-19 testing. We present the first manufacturer-independent evaluation of the *PlexZyme*-based method compared to cobas and examine the throughput capabilities of both methods during a surge of COVID-19 testing.

## 2. Materials and Methods

### *2.1. Routine Sample Testing*

All samples were previously tested using the cobas SARS-CoV-2 assay (Ref. 09175431190) as the primary screening method. All samples were naso-oropharyngeal swabs collected in either Copan UTM-RT media (Brescia, Italy), CITOSWAB (Citotest Scientific Jiangsu, Haimen, China) or Virus Transport Media (VTM) [7]. Thermal pre-treatment of the sample was performed before cobas testing [4]. Briefly, 600 µL of the sample transport media was transferred to a cobas omni secondary tube (Ref. 06438776001) and thermally treated for 75 °C for 15 min in a QBD4 dry block heater (Grant Instruments, Cambridge, UK). Samples were tested on the cobas 6800 instrument without delay. According to the manufacturer's instructions, a sample is reported as SARS-CoV-2 detected if ORF1a is positive with or without a positive E-gene. In the case of positivity with E-gene alone, the result should be reported as SARS-CoV-2 presumptive positive. Our laboratory confirms all single-target positive cobas results with an alternative method. Briefly, samples positive for ORF1a and E-gene were defined as SARS-CoV-2 detected. Samples positive for a single cobas target (either ORF1a or E-gene) were reflexively tested using Xpert Xpress SARS-CoV-2 (Xpert) (Cepheid, Sunnyvale, CA, USA) from the original sample (not thermally treated). Samples positive for at least one different target compared to cobas were defined as SARS-CoV-2 detected (cobas ORF1a positive with Xpert N2 positive and/or Xpert E-gene positive, or cobas E-gene positive and Xpert N2 positive). All other results including Xpert not detected results were considered equivocal for SARS-CoV-2 and repeat collections were performed. All SARS-CoV-2 detected samples were stored at −80 °C as aliquots from the remaining original sample. All negative samples were stored at 4 °C in the original transport media tube.

### *2.2. Validation Panel Characterisation and Preparation*

A validation panel consisting of SARS-CoV-2 detected samples ($n$ = 214; positive sample group) and SARS-CoV-2 not detected samples ($n$ = 201; negative sample group) was prepared from the stored samples above. No equivocal SARS-CoV-2 samples were used in the validation panel as these were not considered true-positives for the purposes of method comparison. To prepare positive samples for this study a 0.2 mL aliquot from each

sample was diluted with 1.8 mL of a naso-oropharyngeal matrix (1:10 dilution). The matrix consisted of pooled cobas negative patient samples (oro-nasopharyngeal swabs) in VTM. The pooled matrix tested negative with cobas and Xpert. All dilutions were prepared in cobas omni secondary tubes and stored at −80 °C until testing. Negative samples were stored at 4 °C and were not diluted.

### *2.3. Cobas and Plex Parallel Testing*

All samples in the validation panel were tested with cobas and *PlexZyme* CoV-2 (RdRp/ORF1ab) components (Ref. 7130010) in a blinded fashion. Five parallel runs were performed. Samples were transferred to MagNAPure 96 deep-well plates for nucleic acid extraction (Plex method: see below) then the remaining sample loaded on the cobas 6800 without delay. Cobas testing was performed following the instructions for use (no thermal treatment). Thermal treatment prior to cobas testing was not compared to Plex as thermal treatment has shown to reduce detectable viral RNA [4,8,9] and not validated by the manufacturer. All samples tested with Plex were extracted using MagNA Pure 96 DNA and Viral NA small volume kit (Roche) using the Pathogens Universal 200 (version 4.0) protocol. The AccuPlex SARS-CoV-2 Reference Material (AccuPlex; Ref. 0505-0126) (SeraCare Life Sciences, Milford, MA, USA) was used as a positive control and VTM was used as a negative control. A sample input volume of 200 µL and an elution volume of 50 µL with on-board Plex internal control addition (20 µL per sample) was used. The internal control consisted of 36 µL of Plex IC RNA in 3564 µL of phosphate buffered saline. The master mix consisted of 5 µL Plex Master Mix (2×), 0.1 µL RTase (100×), 0.2 µL RNase Inhibitor (50×), 0.5 µL CoV-2 Mix and 1.7 µL Nuclease-free water for a total of 7.5 µL per reaction. The PlexPrep liquid handler (SpeeDx) was utilised for distribution of master mix (7.5 µL) and addition of nucleic acid extracts (2.5 µL) to the LightCycler 480 384-well reaction plate (Roche). Amplification and detection were performed using the LightCycler 480 II instrument (Roche). Thermal cycling conditions were 48 °C for 10 min (reverse transcriptase); 95 °C for 2 min (enzyme activation); 10 cycles of a touchdown sequence consisting of 95 °C for 5 sec, 61 °C for 30 sec followed by 0.5 °C reduction per cycle for 30 sec to 56 °C (touchdown); 40 cycles of 95 °C for 5 sec, 52 °C for 50 sec (quantification cycling) with a fluorescence acquisition at 510 nm (ORF1ab), 580 nm (RdRp), 610 nm (internal control); and 40 °C for 30 sec (cooling). Data were analysed on the LightCycler 480 using Abs Quant/Second derivative max method to obtain the cycle of quantification ($C_q$) for Plex. The Plex internal control was used to assess sample extraction validity (<26.0 $C_q$). The AccuPlex positive control (<25 $C_q$) and VTM negative control (negative) was used to assess run validity. The results for cobas were interpreted as detected, presumptively detected and negative as in Section 2.1. Plex results were detected if either target was positive and negative if both targets were negative.

### *2.4. Quantitative Standards and Analysis*

Quantitative standards were prepared from a commercially available SARS-CoV-2 standard (Exact Diagnostics, Fort Worth, TX, USA). Exact Diagnostics SARS-CoV-2 standard (ExactD×) contains ORF1ab, E-gene and RdRp synthetic RNA transcripts quantitated to 200,000 copies/mL using Bio-Rad Digital Droplet PCR (Bio-Rad, Hercules, CA, USA). We pooled multiple ExactDx vials and 10-fold serially diluted in molecular grade water (G-Biosciences, St. Louis, MO, USA) to prepare six standards over the range of 0.30 to 5.30 $\log_{10}$ copies/mL. Each standard was tested in triplicate with cobas (no thermal treatment) and Plex. The mean $C_t$ value at each concentration was used to calculate ORF1a and E-gene standard curves and regression for cobas and similarly for ORF1ab and RdRp for Plex. At least two replicates at each dilution were required to be positive to be included in the standard curve and regression analysis. The regression formulas were used to calculate the respective target copy number for each assay and for all positive samples and controls for the entire study.

### *2.5. Assessment of Intra- and Inter-Assay Reproducibility*

As part of the QConnect programme an external positive control (EQC) was also performed routinely to monitor inter-assay reproducibility (Optitrol NAT SARS-CoV-2; DiaMex, Heidelberg, Germany) [10]. A single lot number of EQC (DM20119) was tested over 20 consecutive runs for cobas and Plex. Intra-assay reproducibility was tested with 10 replicates of the EQC in a single cobas and Plex run. The qualitative outcomes and $C_t/C_q$ values were recorded. The quantitative results were calculated from the quantitative standards as described above.

### *2.6. Assessment of the Lower Limit of Detection*

A high-titre SARS-CoV-2 positive patient sample (alpha variant) was diluted with the naso-oropharyngeal matrix diluent used for the comparative evaluation. The sample was calibrated approximately to 6.00 $\log_{10}$ copies/mL using the cobas ORF1a target from the ExactDx standard curve. Ten-fold serial dilutions were prepared (10 replicates of each) with the naso-oropharyngeal matrix diluent covering the range of 0.00 to 5.00 $\log_{10}$ copies/mL (1 to 1.00 $\times$ $10^5$ copies/mL). Five replicates of each standard were tested in parallel with cobas and Plex. The qualitative outcomes and $C_t/C_q$ values were recorded. The quantitative results were calculated from the quantitative standards as described above. Assessment of the lower limit of detection was performed by log dilution comparison.

### *2.7. Assessment of Hands-on Time and Throughput*

Total hands-on time was assessed for each assay from the sample receipt into the laboratory to reporting the final result. This included the time to register samples in the laboratory information system. Assessment of throughput was performed by retrospective analysis of data captured from the laboratory information system during a period of surge testing. The time period was 1824 min (30.4 h) of testing from 31 January 2021 1826 h to 2 February 2021 0050 h.

### *2.8. Data Analysis*

A contingency table was prepared to assess overall agreement between cobas and Plex with 95% confidence intervals (95% CIs) using Westgard QC 2 $\times$ 2 contingency calculator (Westgard QC, Madison, WI, USA). Linear regression was performed comparing cobas ORF1a and Plex ORF1ab using $\log_{10}$ copies/mL. Quantitative results for cobas ORF1a and Plex ORF1ab ($\log_{10}$ copies/mL) were compared and the median and standard deviation were calculated for comparison. The mean and standard deviation was calculated for the EQC. All statistical analyses were performed by Excel (Microsoft, Redmond, WA, USA) and MedCalc v15.4 (New York City, NY, USA).

## 3. Results

All raw results ($C_t/C_q$ values) including the calculated quantitative results for this study are presented in the Supplementary Material.

### *3.1. Comparative Evaluation*

The qualitative results of the validation panel comparing cobas to Plex are summarised in Table 1. The overall agreement for a Plex detected result (ORF1ab positive $\pm$ RdRp positive or vice versa) or not detected result (ORF1ab and RdRp negative), compared to a cobas detected (ORF1a positive $\pm$ E-gene positive), not detected (ORF1a and E-gene negative) or presumptive result (ORF1a negative and E-gene positive) was 96.9% (402/415; CI 94.7%–98.2%). Five samples (156, 173, 174, 194 and 196) were detected with Plex that were cobas presumptive. From the Supplementary Material these samples showed cobas E-gene $C_t$ values ranging from 35.60 to 38.06 corresponding to 2.81 to 1.79 $\log_{10}$ copies/mL. All of these samples were Plex single-target positive results. Four of these samples were Plex RdRp positive and one sample was Plex ORF1ab positive. The Plex RdRp $C_q$ values for these samples ranged from 24.62 to 28.17 corresponding to 2.94 to 1.85 $\log_{10}$

copies/mL. The Plex ORF1ab $C_q$ value for sample 174 was 25.00 corresponding to 2.93 $\log_{10}$ copies/mL. Plex detected SARS-CoV-2 in sample 201 with an RdRp $C_q$ of 27.17 (ORF1ab negative) corresponding to 2.16 $\log_{10}$ copies/mL (cobas was negative for this sample). Cobas detected 12 samples as presumptive (E-gene only) that were Plex negative. From the Supplementary Material these samples showed cobas E-gene $C_t$ values ranging from 35.09 to 40.70 corresponding to 3.02 to 0.70 $\log_{10}$ copies/mL. All of the $C_t$ values observed for discordant results were considered late PCR amplification and detection of SARS-CoV-2. Following the manufacturer's instructions for reporting results for the positive sample group, 85.0% (182/214) were SARS-CoV-2 detected with cobas (presumptive results excluded) compared to 87.8% (188/214) with Plex. Overall, cobas reported 3.2% more dual-target results than Plex (83.6%; 179/214 for cobas compared to 80.4%; 172/214 for Ple×). All cobas samples with an ORF1a $C_t$ < 31.37 (3.63 $\log_{10}$ copies/mL) and E-gene $C_t$ < 33.58 (3.64 $\log_{10}$ copies/mL) were Plex ORF1a and RdRp positive (dual-target). No unexpected Plex target discordant samples were observed (negative despite low cobas $C_t$ value).

**Table 1.** Results of the comparative evaluation of the cobas compared to Plex [a].

| | No of Samples with the Following Result by Cobas | | | |
|---|---|---|---|---|
| **Plex Result** | **Detected (Presumptive) [b]** | **Not Detected** | **Total** | **SARS-CoV-2 Overall Agreement (%) (95% CI) [d]** |
| Detected [c] | 182 (5) | 1 | 188 | |
| Not detected | 0 (12) | 215 | 227 | 96.9 (94.7–98.2) |
| Total | 199 | 216 | 415 | |

[a] Results of the comparative evaluation for all samples with interpretation of discordant results given in the Results and Discussion. [b] All cobas ORF1a positive results and/or E-gene positive (presumptive). Presumptive results in parentheses. [c] Any Plex ORF1ab positive/RdRp positive result. [d] Indicates overall agreement for Plex detected/not detected results compared to cobas detected/not detected results (including presumptive results).

### *3.2. Quantitative Standards and Assessment of the Lower Limit of Detection*

The $C_t/C_q$ values for each target concentration and standard curves for cobas and Plex are shown in the Supplementary Material. Cobas ORF1a demonstrated R-squared ($r^2$) value of 0.98 over the range of 1.30 to 5.30 $\log_{10}$ copies/mL (5 standards). E-gene demonstrated $r^2$ value of 0.98 over the range of 1.30 to 5.30 $\log_{10}$ copies/mL (5 standards). Plex ORF1ab demonstrated an $r^2$ value of 1.0 over the range of 3.30 to 5.30 $\log_{10}$ copies/mL (3 standards). RdRp demonstrated $r^2$ value of 0.99 over the range of 2.30 to 5.30 $\log_{10}$ copies/mL (4 standards). Cobas limit of detection of the ExactDx ORF1ab and E-gene targets was quantitatively similar. Plex limit of detection of the ExactDx ORF1ab and RdRp targets were different, with RdRp detecting an additional dilution (2.30 $\log_{10}$ copies/mL). For the lower limit of detection study using the clinical sample, cobas E-gene was more sensitive than ORF1a with detection of 4/5 replicates at 1.00 $\log_{10}$ copies/mL compared to 1/5 replicates for ORF1a. Plex RdRp was also more sensitive than ORF1ab with detection of 3/5 replicates at 2.00 $\log_{10}$ copies/mL compared to 1/5 replicates for ORF1ab. Cobas was more sensitive than Plex by at least 1-log dilution overall.

### *3.3. Cobas ORF1a and Plex ORF1ab Correlation*

The correlation between $\log_{10}$ copies/mL values obtained with Plex ORF1ab compared to cobas ORF1a for all ORF positive samples ($n$ = 172) was $r^2$ = 0.95. The median quantitative change for Plex ORF1ab compared to cobas ORF1a was +0.48 $\log_{10}$ copies/mL. The correlation plots and all raw data are shown in the Supplementary Material.

### *3.4. Assessment of Intra- and Inter-Assay Reproducibility*

The $C_t/C_q$ values for each target concentration and standard curves for cobas and Plex are shown in the Supplementary Material, including the results comparing the EQC intra- and inter-assay reproducibility of Plex to cobas. Intra-assay and inter-assay variation for cobas was no more than ±0.38 $\log_{10}$ copies/mL and ±0.25 $\log_{10}$ copies/mL, respectively. Intra-assay and inter-assay variation for Plex was no more than ±0.36 $\log_{10}$ copies/mL and

±0.34 $\log_{10}$ copies/mL, respectively. Overall, the reproducibility of the EQC showed less than ±0.5 $\log_{10}$ copies/mL variation for both assays. Comparing the mean quantitative results for all EQC results (intra- and inter-assay), we observed a mean difference of 0.01 $\log_{10}$ copies/mL between Plex ORF1ab (mean 4.47 $\log_{10}$ copies/mL) and Plex RdRp (4.48 $\log_{10}$ copies/mL). The mean difference for cobas was greater at a mean difference of 0.59 $\log_{10}$ copies/mL between cobas ORF1a (mean 4.27 $\log_{10}$ copies/mL) and cobas-E-gene (4.86 $\log_{10}$ copies/mL).

### *3.5. Assessment of Hands-on Time and Throughput*

Table 2 shows a summary of the surge testing period with cobas and Plex over a 30-h period. Thirteen cobas runs were performed for a total result output of 1222 samples resulting in 40 reportable results per hour. Hands-on-time was 2.1 samples per minute. In contrast, thirty-three MagNAPure 96 extractions were performed, with 15 PlexPrep runs of 188 samples per run and 3 PlexPrep runs of 94. The total result output of Plex was 3102 samples resulting in 102 reportable results per hour. Hands-on time was 1.7 samples per minute. The combined result output was 4324 samples resulting in 142 reportable results per hour with a hands-on time of 3.8 samples per minute. We did not detect any SARS-CoV-2 positive samples during the testing period.

**Table 2.** COVID-19 surge testing comparing cobas to Plex over a 30-h period.

| Assay Workflow | Number of Runs | Number of Samples per Run | Total Number of Samples Tested | Total Result Output | Results per Hour [a] | Hands-on Time (Samples per Min) [b] |
|---|---|---|---|---|---|---|
| cobas | 13 | 94 | 1222 | 1222 | 40 | 2.1 |
| Plex | 15 | 188 | 2820 | 3102 | 102 | 1.7 |
| Plex | 3 | 94 | 282 | | | |
| Combined total | | | | 4324 | 142 | 3.8 |

[a] Based on total time duration commencing 31 January 2021 1826 h to 2 February 2021 0050 h (1824 min). [b] Based on the total result output.

## 4. Discussion

We report the first manufacturer-independent evaluation of the RUO SpeeDx *PlexZyme* SARS-CoV-2 components. Our study aimed to thoroughly evaluate the Plex compared to cobas. We also evaluated the throughput and hands-on time of each assay during a routine COVID-19 testing surge. For the comparative evaluation a direct parallel comparison of Plex with cobas using undiluted original patient material would have been ideal. However, following initial routine testing and our SARS-CoV-2 surveillance testing, the residual sample volume was limited to conduct parallel re-testing with sufficient sample remaining for future research. To overcome this, we diluted all positive samples for this investigation with a pooled oro-nasopharyngeal matrix. The closest representation to an original sample was maintained with this approach. However, the potential disadvantage of this method is that some original samples may be at the lower limit of detection prior to dilution. As such, samples testing negative with both methods are likely as the concentration of SARS-CoV-2 in sample population tested has shifted 1-log closer to (or beyond) the lower limit of detection. The greatest advantage of this approach is sufficient volume to test both methods in parallel, rather than compare the test method results to the retrospective results of a comparative method. Qualitative and quantitative result discrepancies caused by RNA degradation due to storage and freeze-thawing are minimised. A more direct comparison of test performance is possible, with experimental outcomes more likely to be directly attributable to analytical differences. The other advantage is an initial comparative assessment can be made in terms of assay sensitivity of SARS-CoV-2 detection. With our chosen experimental approach, the overall agreement for all samples tested with a positive result for any target in this study was 96.9%. Cobas E-gene was more sensitive than cobas ORF1a (consistent with the manufacturer's claims) and potentially more sensitive than Plex on initial assessment. However, Plex has the added advantage of two SARS-CoV-2 specific targets, whereas cobas has one specific target and a pan-*Sarbecovirus* target. In this case, the overall correlation in our study between both assays for a SARS-CoV-2 detected

result (according to the manufacturer's instructions for reporting results, excluding cobas presumptive detected results) was 99%. Regardless of which target is positive, we confirm all single-target results using Xpert in routine practice. In this study, we observed 20 single-target results (3 ORF1a and 17 E-gene) for cobas (10.0%; 20/199) and 16 single target results (1 ORF1ab and 15 RdRp) for Plex (8.5%; 16/188). Hence, the percentage of single target results was similar. Xpert has two alternative gene targets compared to Plex; therefore, the Plex/Xpert workflow has a greater likelihood of a definitive SARS-CoV-2 detected result, than the cobas/Xpert workflow with E-gene present in both assays. One other advantage of testing as many representative samples as possible is we were able to assess Plex test performance (target detection specificity) across as many infective SARS-CoV-2 strains as possible, albeit not all genotyped. The failure of a PCR target due to mutations within the primer and/or probe binding regions would severely impair efforts to prevent and control community transmission of SARS-CoV-2 [11]. Issues with target detection have been reported in the literature with cobas E-gene [5] and Xpert N2 region [11–13]. We did not observe any unexpected target failures or significantly late $C_q$ values for Plex in the positive sample group when analysing the target regression analysis. Overall, we conclude that Plex performs comparably to cobas for the qualitative detection of SARS-CoV-2.

Our initial observation that cobas was more sensitive than Plex was subsequently confirmed with the lower limit study and testing with the ExactDx standards. However, a key point of difference between patient samples and the standards is the lack of an oro-nasopharyngeal matrix in the standards. We considered diluting the standards in the same oro-nasopharyngeal matrix to control for matrix effects, but we were concerned about nucleases and degradation of the standards which have been calibrated with digital-droplet PCR by the manufacturer. We diluted in nuclease-free water instead and noted a 2-log reduction in the sensitivity of ExactDx ORF1ab detection for Plex ORF1ab compared to cobas ORF1a. However, we observed only a 1-log reduction in sensitivity with Plex ORF1a when multiple replicates of a patient sample were used to assess the lower limit of each assay. We speculate that the nucleic acid matrix of the clinical naso-oropharyngeal specimen may act as an RNA carrier to enhance its recovery during the MagNAPure 96 extraction process. Other reasons for the differences in analytical sensitivity include initial sample volume (400 µL aspirated for cobas compared to 200 µL for Plex), total PCR reaction volume (52 µL for cobas compared to 10 µL for Plex) and template volume (27 µL for cobas, compared to 2.5 µL Plex). We conclude that the Plex approach is a sensitive assay given half the sample volume is extracted and one-tenth of the template used for PCR.

Cycle-threshold values are often used for correlation but should not be used for direct comparisons between assays of different types due to variation in the sensitivity (limit of detection), chemistry of reagents, gene targets, cycle parameters, analytical interpretive methods, sample preparation and extraction techniques [14]. We performed a comparison between cobas and Plex using a quantitative approach. The purpose was to investigate the extent of commutability for the detection of ORF and compare the test performance of target detection, whilst making comparisons with cobas. We found Plex had a smaller difference for the quantitation ORF1ab compared to RdRp than cobas (ORF1a compared to E-gene) for all positive patient samples assessed (n = 172). The smaller difference in Plex target quantitation was also observed for the reproducibility study of the EQC. Overall, Plex demonstrated more consistent results for target quantitation than cobas. We also found a difference in the quantitation of ORF using the ExactDX ORF1ab as a reference (median change +0.48 $\log_{10}$ copies/mL). This difference in commutability should not be inferred as a difference in sensitivity, but rather differences for the quantitation of the ExactDx ORF1ab target. The possible reasons for the difference are broad, but may include assay-specific characteristics such as extractions efficiency of standard material compared to patient samples, primer/probe binding efficiency, variable cycling conditions, PCR product size and fragmentation of the target. We included this type of analysis to determine if there were major quantitative differences between the assays for ORF detection. The median difference of <0.5 log is not unusual, especially when compared to other well-established

quantitative BBV diagnostic assays which are calibrated to International Standards [15]. A quantitative method using the First WHO International Standard for SARS-CoV-2 RNA [16] is currently being developed in our laboratory for cobas and Plex. This approach will be more useful for lower limit of detection comparisons and clinical studies, especially with prospective parallel testing of clinical samples.

Based on the correlation with cobas and excellent analytical test performance we implemented Plex with the understanding that the Plex workflow has higher throughput capabilities than cobas. This was in-part due to the lack of availability of the cobas 6800 instrument allocated to BBV testing, but more importantly due to availability of our MagNAPure 96 instruments and the installation of the PlexPrep 384-well liquid handler. Multiple 96-well nucleic acid output plates (up to 4) can be used to build a 384-well plate (368 samples) for result turnover in less than 3 h (extraction-to-result) for the first plate, then again every 1.5 h. The throughput capabilities were verified during a single surge event lasting 30 h (4324 reportable results) resulting in a 155% increase in result output with Plex compared to cobas. More reportable results per hour was achievable with a 19% decrease in hands-on-time per reportable result due to the larger volume of tests performed, particularly the 188 samples per run workflow. The sample-to-result cobas workflow is a major advantage though with the ability to load three runs (282 samples) and walk-away. However, we did not verify the throughput capabilities of both methods at full utilisation (continuous operation) as the laboratory had a 5-h down-time period for staff and instrument maintenance operations. Finally, we encourage laboratories to perform their own internal cost analysis particularly for surge testing when operating costs are at their peak. Our laboratory identified considerable savings to consumable costs implementing Plex (cost data not shown).

Detection of SARS-CoV-2 with the cobas 6800 assay has demonstrated to be a sensitive and reliable sample-to-result method [17,18]. In conclusion, the results of the first manufacturer-independent evaluation of Plex on a well-characterised panel of 214 positive and 201 negative samples, has shown that *PlexZyme*-based approach is a reliable assay for the qualitative detection of SARS-CoV-2 in oro-nasopharyngeal samples when compared to cobas. Discordant results were related to single target positives at low concentrations. Our study showed that Plex has high-throughput capabilities and when combined with cobas, represents a solid laboratory testing approach to the increasing testing demands brought to Microbiology by the COVID-19 pandemic.

**Supplementary Materials:** The following are available online at https://www.mdpi.com/article/10.3390/pathogens10091088/s1, Table S1: all raw results (Ct/Cq values) including the calculated quantitative results for this study are presented in the Supplementary Material.

**Author Contributions:** Conceptualisation, T.M.P.; methodology, T.M.P. and E.J.H.; investigation, T.M.P., E.J.H. and J.B.; formal analysis, T.M.P., E.J.H. and P.A.B.; writing-original draft preparation, T.M.P.; writing-review and editing, T.M.P., E.J.H., J.B. and P.A.B. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research received no external funding. Funding was provided by PathWest Laboratory Medicine Western Australia.

**Institutional Review Board Statement:** Not applicable: The residual samples used in the study were de-identified and results were not used to clinically manage patients [National Statement on Ethical Conduct in Human Research 2007 (May 2015) by the National Health and Medical Research Council, Australian Research Council and Australian Vice-Chancellors' Committee].

**Informed Consent Statement:** Not applicable.

**Acknowledgments:** The authors acknowledge Ian D. Kay (Principal Scientist, Clinical Microbiology) and James P. Flexman (Head of Department, Clinical Microbiology) for their dedicated scientific, clinical and administrative support.

**Conflicts of Interest:** All authors declare no conflict of interest.

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## 4.5 **Publication 11**. Boan, Jardine and Pryce, 2022. Clinical associations of SARS-CoV-2 viral load using the first WHO International Standard for SARS-CoV-2 RNA.

Boan P, Jardine A, **Pryce TM**. 2022. Clinical associations of SARS-CoV-2 viral load using the first WHO International Standard for SARS-CoV-2 RNA. Pathology 54:344-350.

DOI link 10.1016/j.pathol.2021.11.006

P. Boan was the corresponding author. We both conceptualised the use of viral load to correlate with the clinical associations of disease. P. Boan and A. Jardine collated clinical information from patient records. I conceptualised and developed the viral load assay, procured the 1st International Standard for SARS-CoV-2 RNA (requiring import permits), calibrated the cobas assay in International Units/mL, developed the standard curves, performed and analysed quality control metrics and collected all viral loads for this investigation. I wrote sections of the materials and methods results, performed statistical analysis, prepared Figure 1 and all the supplementary material, and performed writing and editing. P. Boan and I agreed that I contributed equally to this manuscript.

Clarivate journal impact factor from Journal Citation Reports: 3.6

Pathology (April 2022) **54(3)**, pp. 344–350

VIROLOGY

# Clinical associations of SARS-CoV-2 viral load using the first WHO International Standard for SARS-CoV-2 RNA

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## Summary

SARS-CoV-2 viral load declines from the time of symptom onset; in some studies viral load is higher or persists longer in more severe COVID-19 infection, and viral load correlates with culture positivity. This was a retrospective cohort study of inpatients and outpatients during the first wave of COVID-19 infection in Western Australia, March to May 2020, of the relationship of SARS-CoV-2 viral load (using the First WHO International Standard for SARS-CoV-2 RNA) from symptom onset, by clinical subgroups determined from the public health database and hospital records, using regression analysis. We studied 320 samples from 201 COVID-19 cases: 181 mild, seven severe, 11 critical, and four cases who died (two were also critical cases). At symptom onset the mean viral load was 4.34 $\log_{10}$ IU/mL (3.92–4.77 $\log_{10}$ IU/mL 95% CI, cobas SARS-CoV-2 assay ORF1a Ct 28.9 cycles). The mean viral load change was −0.09 $\log_{10}$ IU/mL/day (−0.12 to −0.06 95% CI). $R^2$ was 0.08 and residual standard deviation 2.68 $\log_{10}$ IU/mL. Viral load at symptom onset was higher for those reporting fever compared to those not reporting fever. Viral load kinetics were not different for gender, age, shortness of breath, or those requiring oxygen. Mean viral load at usual release from isolation at 14 days was 2.5 $\log_{10}$ IU/mL or day 20 was 1.8 $\log_{10}$ IU/mL. Variability in respiratory sample SARS-CoV-2 viral load kinetics suggests viral loads will only have a role supporting clinical decision making, and an uncertain role for prognostication.

*Key words:* SARS-CoV-2; COVID-19; viral load; WHO international standard; Ct value.

Received 25 August, revised 12 November, accepted 16 November 2021
Available online 10 February 2022

## INTRODUCTION

Globally SARS-CoV-2 continues to cause morbidity, death and overwhelm healthcare systems. There are many reports describing quantitative SARS-CoV-2 changes over time, associations with clinical outcomes, associations with various specimen types, correlations with infectivity, and correlations with culture positivity.[1–5] Most of these reports have studied cycle threshold (Ct) values which are semi-quantitative results and do not always accurately correlate with viral load,[6] and no studies to date have used an international standard to determine quantitative RNA in respiratory specimens. In 2021, the First WHO International Standard for SARS-CoV-2 RNA (First WHO IS SARS-COV-2 RNA) was released.[7] While time from admission to sample collection is sometimes reported, many studies have not described the time from symptom onset to specimen which is known to be strongly associated with viral load.[4] Some studies have pooled results from lower and upper respiratory tract specimens,[2,5] when viral load is higher in the lower respiratory tract.[4]

Western Australia had a single small wave of COVID-19 infection between March and May 2020 and has had sporadic cases thereafter. Taking into account the time from symptom onset, using viral loads determined retrospectively against the First WHO IS SARS-CoV-2 RNA, we examined for associations with demographic factors, symptoms and the severity of illness, and describe viral loads at release from isolation.

## MATERIALS AND METHODS

### Demographic and clinical data

The PathWest Laboratory Medicine Molecular Microbiology Department at Fiona Stanley Hospital is one of two public laboratories in Western Australia performing SARS-CoV-2 molecular testing. All routine testing for SARS-CoV-2 was performed in our laboratory with the cobas SARS-CoV-2 assay (cobas; Roche, Switzerland) on the cobas 6800 instrument according to the manufacturer's instructions. Positive COVID-19 cases were identified as those with positive (ORF1a positive with/without E-gene positive) or presumptive positive (E-gene only positive) cobas SARS-CoV-2 results in our laboratory. For hospitalised patients, the clinical records were examined for supplemental oxygen use, invasive ventilation and death. For all cases including non-hospitalised cases, the public health database was examined for the date of symptom onset and specific symptoms of the cases which had been captured by a standardised case report form (fields of arthralgia, chills/rigors, conjunctivitis, cough, diarrhoea, fatigue, fever, malaise, nausea, rhinorrhoea, shortness of breath, sore throat, vomiting). There were no asymptomatic cases. We chose to examine fever and shortness of breath as possible manifestations of more severe illness.

Severity was based on WHO categories.[8] Respiratory rate and oxygen saturations were not always reported, so supplemental oxygen was used to define severe COVID-19 disease, and invasive ventilation defined critical COVID-19 disease. All cases admitted to the intensive care unit had invasive ventilation. Non-invasive ventilation was not used for any patients. In all cases of death no other cause was evident apart from COVID-19, and all deaths were within 30 days of COVID-19 diagnosis. Mild cases were those not requiring oxygen, not requiring invasive ventilation, and not dying from COVID-19.

Conditions for release from isolation were taken from the most recent Australian COVID-19 series of national guidelines, where COVID-19 cases may be released from isolation 14 days after symptom onset in those with


DOI: https://doi.org/10.1016/j.pathol.2021.11.006

resolution of fever and respiratory symptoms, and at 20 days after symptom onset in those without complete resolution of fever and acute respiratory symptoms.[9] As we could not determine when fever or respiratory symptoms resolved in our cases, we reported viral load data at day 14 and 20 of the whole cohort.

At the time of the cases in this study, PCR testing for release from isolation was performed at least 7 days after symptom onset in the group of patients who planned to visit high risk settings such as aged care facilities, healthcare facilities, childcare centres and correctional facilities.[10]

The study was approved by the Governance, Evidence, Knowledge, Outcomes system of the Western Australia Department of Health, GEKO activity 35397. The study was exempt from informed participant consent as it was a retrospective observational study with negligible risk to participants, de-identification of data, presentation as composite rather than individual data, and due to the impracticality of contacting participants.

### Patient samples

We included all positive and presumptive positive samples run in our laboratory from March to May 2020 inclusive. Where positive/presumptive positive cases had a follow-up negative sample, the first negative sample ('not detected' by the cobas assay) was included with an assigned viral load of zero international units/mL (IU/mL). A combined deep nasal/throat swab from each patient was inoculated into 3 mL of either Copan UTM-RT media (Brescia, Italy), CITOSWAB (Citotest Scientific Jiangsu, People's Republic of China) or Virus Transport Media (VTM) prepared by PathWest Media.[11] All positive samples were stored as aliquots at −80°C. Samples were thermally treated for 75°C for 15 min in a Dri-bath, then tested with cobas SARS-CoV-2 assay within 2 hours.[12] The Ct values were recorded for ORF1a and E-gene.

### Quantitative standards, external control and analysis

Quantitative standards were prepared from the First WHO International Standard for SARS-CoV-2 (NISBSC code 20/146), supplied as 7.7 $\log_{10}$ IU/mL (National Institute for Biological Standards and Control, UK). The standard was reconstituted with 0.5 mL of phosphate buffered saline following the manufacturer's instructions. Once reconstituted, the standard was 10-fold serially diluted in a naso-oropharyngeal matrix. This matrix consisted of pooled naso-oropharyngeal samples from samples previously tested as SARS-CoV-2 negative using cobas. Seven standards were prepared over the range of 0.7–6.7 $\log_{10}$ IU/mL. Each standard was tested in triplicate with cobas. The mean Ct value at each concentration was used to calculate ORF1a and E-gene standard curves and regression. The regression formulas were used to calculate the ORF1a and E-gene IU/mL for all positive samples retrospectively. Given the strong correlation of IU/mL for both targets (Fig. 1), for simplicity the results from a single target (ORF1a) were used as the reported viral load or Ct value unless otherwise stated. An external control (EQC) was also performed routinely to monitor reproducibility (Optitrol NAT SARS-CoV-2; DiaMex, Germany).[13]

### Statistical analysis

Viral loads in $\log_{10}$ IU/mL were plotted against time from symptom onset. Comparison of regression lines (viral load at symptom onset and change of viral load over time) was used to compare subgroups of gender, age, shortness of breath, fever, supplemental oxygen requirement, invasive ventilation, or death. Regression analysis was also used to examine the relationship of Ct values for ORF1a or E-gene targets to viral load dilutions of the First WHO IS SARS-CoV-2 RNA. For the complete data set, in addition to simple linear regression a moving average trend line with window width of five samples was performed, with locally weighted scatterplot smoothing (LOESS) at 80% smoothing span. Comparison of medians was performed with the Mann–Whitney test for non-parametric data. A significance $p$ value of $<0.05$ was used. Analysis was performed in MedCalc version 15.4 (MedCalc Software, Belgium).

## RESULTS

### Demographic and clinical results

There were 201 COVID-19 cases between 15 March and 11 May 2020 diagnosed by our laboratory. Median age was 53 years [interquartile range (IQR) 33–68 years, range 8–85 years] and 50.2% were male. There were 320 samples (249 positive, five presumptive positive, and 66 negative samples), median one positive sample per patient (164 cases with one sample, 29 cases with two positive samples, three cases with three positive samples, one case with four positive samples, four cases with five positive samples), and 66 (32.5%) cases had a follow up negative test. There were 162 cases not admitted to hospital (235 samples), 39 cases admitted to hospital (85 samples); 181 were mild cases (272 samples), seven were severe cases (16 samples), 11 were critical cases (28 samples), and four patients died (two were also critical

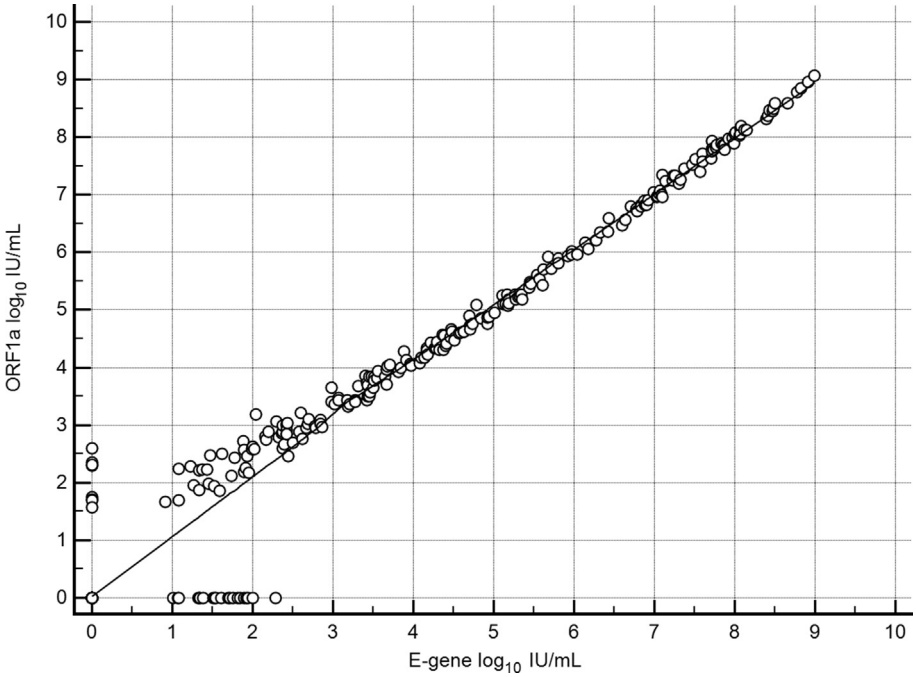


**Fig. 1** Correlation of viral load in $\log_{10}$ IU/mL in each sample for the ORF1a and E-gene targets of the Cobas SARS-CoV-2 PCR assay.

cases, one was a severe case, one was a mild case; total six samples). The mild case who died was an elderly patient with comorbidities admitted with respiratory complaints. The patient tested SARS-CoV-2 PCR positive on combined nose/throat swab, also had myocardial infarction, did not receive oxygen and was palliated.

### Viral load associations with demographic factors

At symptom onset the mean viral load was 4.34 $\log_{10}$ IU/mL [3.92 to 4.77 $\log_{10}$ IU/mL 95% confidence interval (CI), Ct 29.0]. The mean viral load change was −0.09 $\log_{10}$ IU/mL/day (−0.12 to −0.06 95% CI). The highest viral load was 9.1 $\log_{10}$ IU/mL (Ct 15.7) at day 1.5 and lowest viral load was 1.57 $\log_{10}$ IU/mL (Ct 36.8) at day 36. The earliest and latest positive results were at day −4 (1.33 $\log_{10}$ IU/mL for E-gene, negative for ORF1a so not seen in Fig. 2) and day 49 (2.82 $\log_{10}$ IU/mL), respectively. $R^2$ was 0.08 and residual standard deviation 2.68 $\log_{10}$ IU/mL. See Fig. 2 for simple linear regression with 95% CIs and Fig. 3 for moving average trend line with LOESS.

Subgroup regression analysis suggested those patients reporting fever had a higher viral load at symptom onset (Fig. 4). The finding of higher viral load at symptom onset in those who died and lower viral load of those who required invasive ventilation should be interpreted cautiously due to the small numbers in these subgroups. Additionally, samples were taken later from symptom onset in those who required invasive ventilation compared to those who did not require invasive ventilation (median 14.5 days, IQR 8.4–20 days, compared to median 6.4 days, IQR 3.3–10.6 days, $p<0.001$), and for those who required oxygen compared to those who did not require oxygen, nor ventilation, and did not die (median 12.2 days, IQR 8.5–33 days, compared to median 6 days, IQR 2.6–9.8 days, $p<0.001$). There was no significant difference when samples were taken from symptom onset for those who died compared to those who did not die (median 9.5 days, IQR 0.4–14.4 days, compared to 6.5 days, IQR 3.4–11.6 days, $p=0.97$). Subgroup regression analysis did not demonstrate significant differences in viral load at symptom onset or change in viral load over time for gender, age >60 vs ≤60 years, age >70 vs ≤70 years (data not shown), age >80 vs ≤80 years (data not shown), shortness of breath, or oxygen requirement (Table 1).

### Serial viral load results for individual cases

Figure 5 demonstrates significant heterogeneity in viral load changes with time for those eight cases with more than two positive PCR results during the course of their infection.

### Viral load at release from isolation

At 14 days from symptom onset, viral load by moving average trend with LOESS was 2.5 $\log_{10}$ IU/mL, and +/− 0.3 $\log_{10}$ IU/mL 95% CI of the regression line. At 20 days from symptom onset, viral load by moving average trend with LOESS was 1.8 $\log_{10}$ IU/mL, and +/− 0.5 $\log_{10}$ IU/mL 95% CI of the regression line.

### First WHO IS SARS-CoV-2 RNA standard curves

Detection of ORF1a was log-linear over the range of 2.70–6.70 $\log_{10}$ IU/mL with an $R^2$ value of 0.99 (regression equation y = −0.3557x + 14.649, where y=viral load in $\log_{10}$ IU/mL and x=Ct value). Detection of E-gene was log-linear over the range of 1.70–6.70 $\log_{10}$ IU/mL with an $R^2$ value of 0.99 (regression equation y = −0.3451x + 14.502) (Supplementary Data, Appendix A). The EQC inter-assay reproducibility analysed retrospectively over 20 consecutive runs for ORF1a was 4.09 $\log_{10}$ ± 0.21 IU/mL and E-gene was 3.93 $\log_{10}$ ± 0.21 IU/mL. There was a strong correlation of IU/mL for both targets, $R^2$=0.98 (Fig. 1).

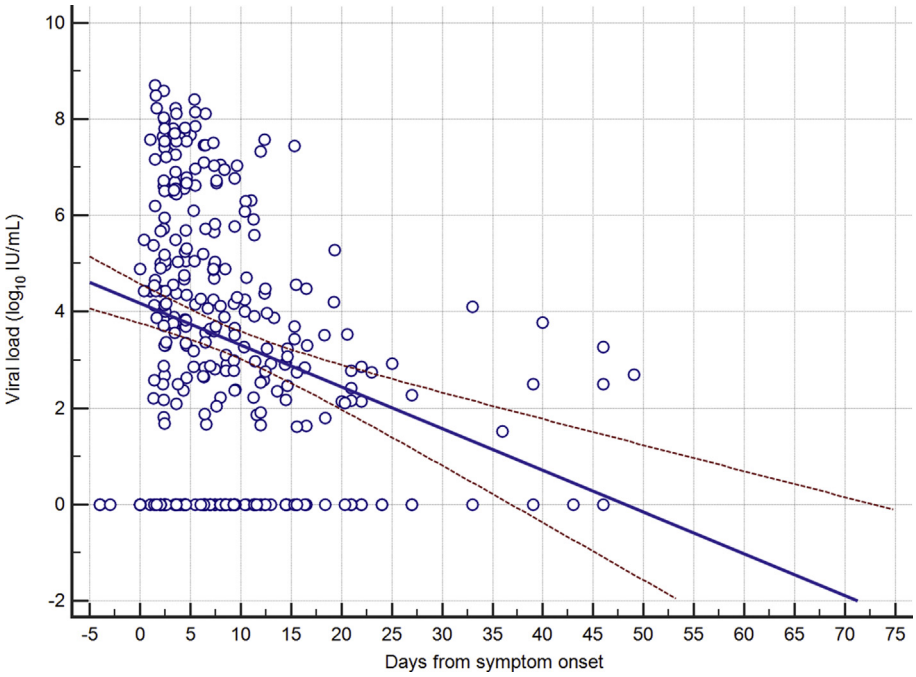


**Fig. 2** Viral load in $\log_{10}$ IU/mL against time from symptom onset, with 95% CI of the regression line intervals shown by dashed lines.

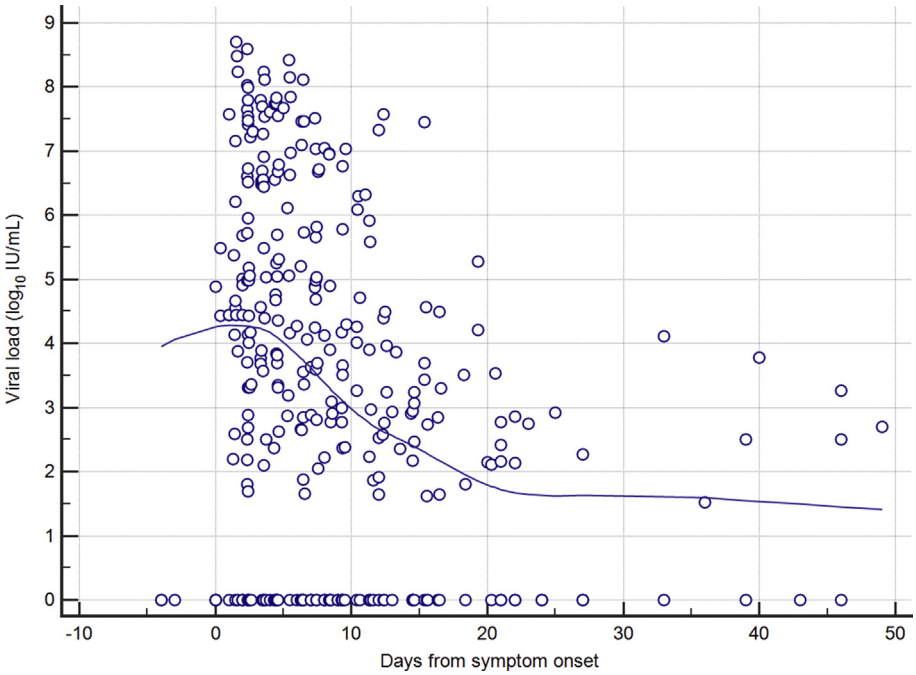


**Fig. 3** Viral load in $log_{10}$ IU/mL against time from symptom onset with a moving average trend line and locally weighted scatterplot smoothing (LOESS).

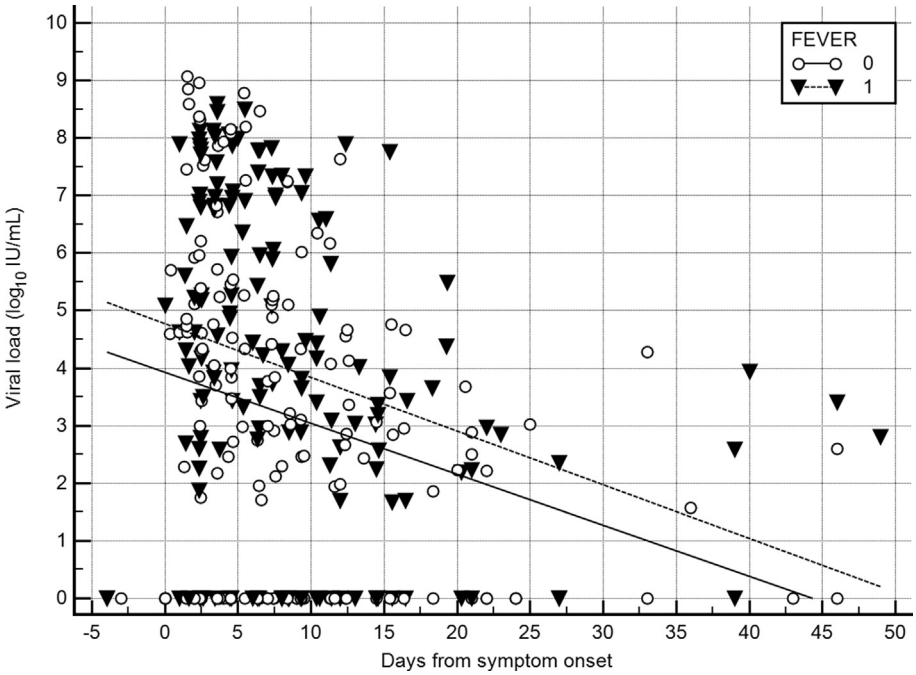


**Fig. 4** Viral load in $log_{10}$ IU/mL against time from symptom onset for patient reporting fever compared to those not reporting fever. Fever=1, no fever=0.

## DISCUSSION

We examined SARS-CoV-2 viral load changes according to the time from symptom onset for subgroups of demographic factors, symptoms, and severity of illness. We showed a higher viral load at symptom onset in those who reported fever, a finding of uncertain significance which should be confirmed with further data. Our findings of higher viral load at symptom onset in those who died and a lower viral load at symptom onset in those who had invasive ventilation are to be taken with caution due to small numbers, and the potential bias introduced by the later sampling in those requiring invasive ventilation.

We have controlled a number of factors which are lacking in other studies such as including only one sample type (combined deep nasal/throat swab), controlling for the time of sample collection from symptom onset, using a single PCR assay, using viral load as a more accurate quantitative assessment than Ct values, for the first time using the First WHO IS SARS-CoV-2 RNA, and providing statistical assessment by regression analysis. It is difficult to directly compare with other studies due to the variable methods they have employed, including pooling of lower and upper respiratory tract samples,[2,5] and using Ct values rather than a formal

**Table 1** Extrapolated viral load ($log_{10}$ IU/mL) at the time of symptom onset ($VL_0$), and slope of viral load change (change in $log_{10}$ IU/mL/day) according to subgroups

| Variable | $VL_0$ | 95% CI | *p* value | Slope | 95% CI | *p* value |
|---|---|---|---|---|---|---|
| Sex | | | | | | |
| Male (*n*=152) | 4.35 | 3.74 to 4.95 | | −0.08 | −0.13 to −0.03 | |
| Female (*n*=168) | 4.36 | 3.75 to 4.97 | 0.47 | −0.10 | −0.16 to −0.05 | 0.46 |
| Age, years | | | | | | |
| >60 (*n*=126) | 4.66 | 3.99 to 5.32 | | −0.09 | −0.13 to −0.05 | |
| ≤60 (*n*=194) | 4.30 | 3.69 to 4.91 | 0.07 | −0.11 | −0.18 to −0.05 | 0.55 |
| SOB | | | | | | |
| Yes (*n*=50) | 4.62 | 3.16 to 6.08 | | −0.12 | −0.29 to 0.05 | |
| No (*n*=270) | 4.32 | 3.86 to 4.77 | 0.85 | −0.09 | −0.12 to −0.05 | 0.71 |
| Fever | | | | | | |
| Yes (*n*=160) | 4.76 | 4.16 to 5.37 | | −0.09 | −0.14 to −0.04 | |
| No (*n*=160) | 3.93 | 3.33 to 4.53 | 0.008 | −0.09 | −0.14 to −0.03 | 0.91 |
| Death | | | | | | |
| Yes (*n*=6) | 5.73 | 2.08 to 9.38 | | 0.02 | −0.33 to 0.38 | |
| No (*n*=314) | 4.31 | 3.88 to 4.74 | 0.03 | −0.09 | −0.13 to −0.06 | 0.52 |
| Invasive ventilation | | | | | | |
| Yes (*n*=28) | 3.38 | 1.76 to 4.99 | | −0.09 | −0.19 to 0.01 | |
| No (*n*=292) | 4.38 | 3.93 to 4.82 | 0.047 | −0.08 | −0.12 to −0.05 | 0.95 |
| Oxygen | | | | | | |
| Yes (*n*=16) | 2.37 | 0.48 to 4.27 | 0.15 | −0.03 | −0.11 to 0.05 | 0.23 |
| No (and not ventilation and not death) (*n*=272) | 4.42 | 3.95 to 4.89 | | −0.09 | −0.13 to −0.04 | |
| Total (*n*=320) | 4.34 | 3.92 to 4.77 | | −0.09 | −0.12 to −0.06 | |

*n*, number of samples; SOB, shortness of breath.

viral load.[1] Many studies did not take into account the sample collection time with respect to the onset of symptoms, instead reporting an average Ct or viral load for a subgroup of patients.[1] Many studies have recorded the time from admission to sample collection but this may be biased by a variable time from onset of symptoms to presentation to hospital for specific subgroups.[1,2,4] Indeed we found cases requiring oxygen or invasive ventilation had sampling later from symptom onset compared to those cases which did not require these interventions. Some studies of larger numbers of severe and critical cases have shown lower Ct values (higher viral loads) in these subgroups while others have not.[1,4] One study demonstrated a higher peak viral load and longer duration of viral shedding in 71 ventilated compared to 90 non-ventilated hospitalised patients, though initial viral loads were similar.[14] Like our findings, a large study of 3712 positive samples showed no association of viral load with age, and there has not been clear association of viral load with gender.[15] While not the focus of our study, quantitative SARS-CoV-2 results have been examined for associations with infectivity, most recently for the Delta SARS-CoV-2 variant for which Ct values on the day of first detection were lower compared to clade 19A/19B

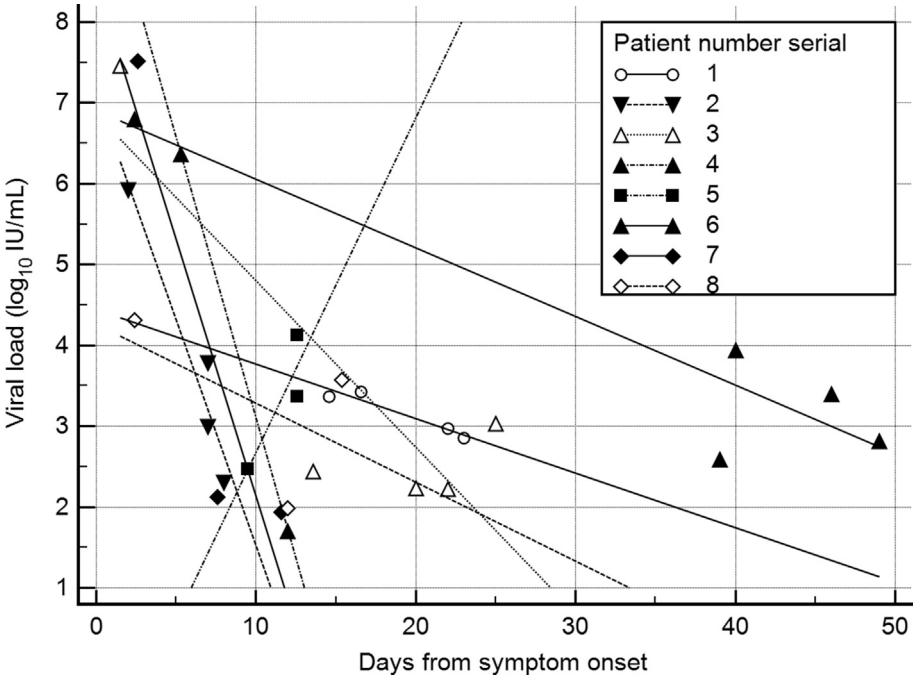


**Fig. 5** Viral load in $log_{10}$ IU/mL against time from symptom onset, showing serial viral loads in eight patients with more than two positive results.

viruses (average Ct 24 versus 34 for an in-house ORF1ab gene assay).[16]

At the usual times of release from isolation, the viral load was 2.5 $\log_{10}$ IU/mL at 14 days and 1.8 $\log_{10}$ IU/mL at 20 days from symptom onset, with +/− 0.3−0.5 $\log_{10}$ IU/mL 95% confidence intervals of the linear regression line. Overall, there was wide variation in viral loads compared to time from symptom onset with a residual standard deviation of 2.68 $\log_{10}$ IU/mL, and heterogenous individual kinetics for the small number of cases with serial PCR positive results. These findings are consistent with a report from New Zealand where Ct values ranged from <20 to >35 more than 10 days from symptom onset in non-hospitalised patients.[17] Apart from differences in the virus behaviour between individuals, the heterogeneity of respiratory samples and variability in collection method likely contribute to these poorly reproducible quantitative results which is not a problem encountered with quantitative testing of blood, serum or plasma. Nucleic acid extraction, amplification and detection are not the cause of quantitative variability, as we found excellent performance of serial dilution of the First WHO IS SARS-CoV-2 RNA, and very strong correlation of the two gene targets of the cobas SARS-CoV-2 PCR assay. Generally, previous studies have shown high Ct values or low viral loads to be associated with culture negativity,[3] but culture may not be sensitive enough to correlate with infectivity, requires increased laboratory precautions, is not routinely available, and is laborious.[18]

A strength of our study is reporting viral loads at times of interest in a moderate sized data set with the First WHO IS SARS-CoV-2 RNA, which allows comparison to other data examined with this standard. However, the designation of 'zero' IU/mL when nucleic acid targets are not detected is dependent on the limit of detection of the assay, so there may be a different threshold for 'zero' IU/mL with other assays. Our data only include upper respiratory tract samples so we cannot comment on viral load kinetics in lower respiratory tract samples and includes a limited number of cases with severe and critical COVID-19 illness, and no cases of asymptomatic infection (during the period of the study, Western Australian state policy restricted testing to symptomatic people). Our study of the first wave of COVID-19 infection may not represent viral load kinetics of variants of concern which have since evolved. Follow-up testing for positive cases was not routine or protocolised which leads to an incomplete picture of viral load dynamics in all patients and the potential for bias. We have determined viral loads at usual times of release from isolation, though it is acknowledged there are additional conditions to be met for release from isolation in public health guidelines and the time frames represent the earliest possible time of release.

We analysed SARS-CoV-2 viral load from symptom onset in mild, severe and critical COVID-19 cases. We found higher viral load at symptom onset for those reporting fever, a finding which needs further validation. Findings of higher viral load at symptom onset in those who died and lower viral load at symptom onset in those who required invasive ventilation are interpreted cautiously due to small numbers. SARS-CoV-2 quantitative measurement should evolve with routine viral load assessment rather than Ct values, and to reporting in IU/mL as assays are calibrated to the international quantitative standard. However, the variability of viral load kinetics between individuals and the poor quantitative reproducibility inherent in respiratory samples suggests that viral load assessment will only ever be able to support clinical decision making rather than be determinative in prognostication or infection control.

**Acknowledgements:** Dr Donna Mak and Dr Paul Effler, Public Health Department of Western Australia, for facilitating data from the public health COVID-19 database.

**Conflicts of interest and sources of funding:** The authors state that there are no conflicts of interest or funding sources to disclose.

## APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pathol.2021.11.006.

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## 4.6 **Publication 12**. Pryce et al., 2022. High-Throughput COVID-19 Testing of Naso-Oropharyngeal Swabs Using a Sensitive Extraction-Free Sample Preparation Method.

**Pryce TM**, Haygarth EJ, Bordessa J, Jeffery CT, Kay ID, Flexman JP, Boan PA. 2022. High-Throughput COVID-19 Testing of Naso-Oropharyngeal Swabs Using a Sensitive Extraction-Free Sample Preparation Method. Microbiology Spectrum 10:e0135822.

DOI link 10.1128/spectrum.01358-22

As corresponding author, I conceptualised the study, methods and procedures. I collated all the samples for the investigation and performed all the laboratory testing and experiments. I wrote the protocol, collected and analysed the results and was the main person drafting the manuscript. All other authors either assisted with testing, performed supervisory roles, provided clinical information and/or contributed to the writing and editing. All authors reviewed the manuscript prior to publication.

Clarivate journal impact factor from Journal Citation Reports: 3.7

AMERICAN SOCIETY FOR MICROBIOLOGY | Microbiology Spectrum

RESEARCH ARTICLE

# High-Throughput COVID-19 Testing of Naso-Oropharyngeal Swabs Using a Sensitive Extraction-Free Sample Preparation Method

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**ABSTRACT** High-throughput diagnostic assays are required for large-scale population testing for severe acute respiratory coronavirus 2 (SARS-CoV-2). The gold standard technique for SARS-CoV-2 detection in nasopharyngeal swab specimens is nucleic acid extraction followed by real-time reverse transcription-PCR. Two high-throughput commercial extraction and detection systems are used routinely in our laboratory: the Roche cobas SARS-CoV-2 assay (cobas) and the Roche MagNA Pure 96 system combined with the SpeeDx PlexPCR SARS-CoV-2 assay (Plex). As an alternative to more costly instrumentation, or tedious sample pooling to increase throughput, we developed a high-throughput extraction-free sample preparation method for naso-oropharyngeal swabs using the PlexPCR SARS-CoV-2 assay (Direct). A collection of SARS-CoV-2-positive ($n$ = 185) and -negative ($n$ = 354) naso-oropharyngeal swabs in transport medium were tested in parallel to compare Plex to Direct. The overall agreement comparing the qualitative outcomes was 99.3%. The mean cycle of quantification ($C_q$) increase and corresponding mean reduction in viral load for Direct ORF1ab and RdRp compared to Plex was 3.11 $C_q$ (−0.91 $\log_{10}$ IU/mL) and 4.78 $C_q$ (−1.35 $\log_{10}$ IU/mL), respectively. We also compared Direct to a four-sample pool by combining each positive sample ($n$ = 185) with three SARS-CoV-2-negative samples extracted with MagNA Pure 96 and tested with the PlexPCR SARS-CoV-2 assay (Pool). Although less sensitive than Plex or Pool, the Direct method is a sufficiently sensitive and viable approach to increase our throughput by 12,032 results per day. Combining cobas, Plex, and Direct, an overall throughput of 19,364 results can be achieved in a 24-h period.

**IMPORTANCE** Laboratories have experienced extraordinary demand globally for reagents, consumables, and instrumentation, while facing unprecedented testing demand needed for the diagnosis of SARS-CoV-2 infection. A major bottleneck in testing throughput is the purification of viral RNA. Extraction-based methods provide the greatest yield and purity of RNA for downstream PCR. However, these techniques are expensive, time-consuming, and depend on commercial availability of consumables. Extraction-free methods offer an accessible and cost-effective alternative for sample preparation. However, extraction-free methods often lack sensitivity compared to extraction-based methods. We describe a sensitive extraction-free protocol based on a simple purification step using a chelating resin, combined with proteinase K and thermal treatment. We compare the sensitivity qualitatively and quantitatively to a well-known commercial extraction-based system, using a PCR assay calibrated to the 1st WHO international standard for SARS-CoV-2 RNA. This method entails high throughput and is suitable for all laboratories, particularly in jurisdictions where access to instrumentation and reagents is problematic.

**KEYWORDS** PlexPCR, cobas, SARS-CoV-2, extraction-free, high-throughput

Diagnostic tools are essential to manage the current coronavirus disease 2019 (COVID-19) pandemic, and reliable, high-throughput laboratory tests are required (1). These tools are the strategic cornerstone to mitigate severe acute respiratory

**Editor** Salika M. Shakir, University of Utah and ARUP Laboratories



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The authors declare no conflict of interest.

**Received** 20 April 2022
**Accepted** 26 July 2022
**Published** 11 August 2022

*[This article was published on 11 August 2022 with the wrong supplemental material file. The file was replaced in the current version, posted on 18 August 2022.]*

syndrome coronavirus 2 (SARS-CoV-2) spread, facilitating early diagnosis, isolation of infected individuals, and clearance of essential personnel to continue to work (2). Since 20 March 2020, we have performed more than 700,000 tests using two commercial extraction and detection systems: the cobas SARS-CoV-2 assay (Roche Molecular Systems, Branchburg, NJ, USA), and the PlexPCR SARS-CoV-2 assay (SpeeDx, Eveleigh, NSW, Australia) (3–6). The PlexPCR SARS-CoV-2 assay workflow in our laboratory utilizes a maximum of four MagNA Pure 96 instruments (Roche) for RNA extraction, two PlexPrep liquid handlers (SpeeDx) for 384-well PCR plate preparation, and four LightCycler 480 thermal cyclers (Roche) for amplification and detection. Using both commercial systems, we performed 4,324 tests over a 30-h period (24-h hands-on) in a recent surge testing event, with the PlexPCR SARS-CoV-2 workflow demonstrating 155% higher throughput than cobas SARS-CoV-2 (5). That surge testing event and others since have shown that we have additional PlexPrep and thermal cycling capacity; our testing throughput is limited by sample handling and the capacity of the extraction-based systems. Our 24-h extraction-based testing capacity is estimated to be 1,316 results for cobas SARS-CoV-2 (14 runs with 94 samples per run) and 6,016 results for PlexPCR SARS-CoV-2 (64 MagNA Pure 96 runs with 94 samples per run for 16 PlexPCR runs with 376 samples per run). If fully utilized, the additional thermal cycler capacity could add more than 12,000 results in a 24-h period. Sample pooling is considered a viable strategy for increased testing capacity while in a low-prevalence setting (7). However, with the inevitability of testing surges and with testing capacity gains from pooling diminishing at high disease prevalence, alternative strategies were investigated. These included additional instrumentation—requiring significant expenditure and laboratory space—or rapid extraction-free methods of sample preparation. Extraction-free methods result in a loss in sensitivity compared to extraction-based methods (8–12); however, pooled testing strategies also demonstrate loss in sensitivity compared to single-specimen testing, depending on the sample pool size (7, 13). The key differences between single-specimen testing methods compared to pooled-specimen approaches are that single-specimen testing methods are not affected by high disease prevalence, do not require laborious positive-pool retesting, and allow test turnaround time to be maintained (7). After considering our options, we sought to increase the single-specimen testing capacity by developing a rapid extraction-free sample preparation method for the PlexPCR SARS-CoV-2 workflow. A pre-implementation study was performed using a panel of stored positive and negative samples. We assessed the relative sensitivity of the optimized extraction-free method and compared it to that of the MagNA Pure 96 extraction method for undiluted samples and a four-sample pool by using the PlexPCR SARS-CoV-2 assay. To standardize cycle of quantification ($C_q$) analysis, we developed an in-house quantitative method using standards prepared from the 1st WHO international standard (IS) for SARS-CoV-2 RNA. Further analysis was performed to assess the mean change in the $C_q$ or reduction of viral load of the extraction-free method compared to that with the MagNA Pure 96 extraction method.

## RESULTS

All raw results ($C_q$ values), including the calculated quantitative results and the SARS-CoV-2 IS curves, are presented in the supplemental material.

**Quantitative standards.** The $C_q$ values for each SARS-CoV-2 IS concentration and Plex ORF1ab and RdRp standard curves are provided in the supplemental material. ORF1ab demonstrated an $r^2$ value of 1.0 over the range of 2.70 to 6.70 $\log_{10}$ IU/mL. ORF1ab detection at 0.70 and 1.70 $\log_{10}$ IU/mL was not reproducible and was omitted from the standard curve. RdRp demonstrated an $r^2$ value of 0.998 over the range of 2.70 to 6.70 $\log_{10}$ IU/mL. RdRp detection at 0.70 and 1.70 $\log_{10}$ IU/mL was not reproducible and was omitted from the standard curve. The Plex PCR targets standard curves demonstrated a high degree of linearity and were commutable within 0.23 $\log_{10}$ IU/mL over the range tested.

**TABLE 1** Results obtained with the Direct and Pool methods in comparison to results with Plex[a]

| Method | Detected | Not detected | % PPA (95% CI) | % PNA (95% CI) | % POA (95% CI) | Total |
|---|---|---|---|---|---|---|
| Direct | 181 | 358 | 97.8 (94.6–99.2) | 100 (98.9–100) | 99.3 (98.1–99.7) | 539 |
| Pool | 185 | 354 | 100 (98.0–100) | 100 (98.9–100) | 100 (99.3–100) | 539 |

[a]Detected and not detected columns report the number of positive and negative results, respectively. PPA, percent positive agreement; PNA, percent negative agreement; POA, percent overall agreement.

**Qualitative comparison.** The qualitative results comparing the Direct and Plex methods are summarized in Table 1. The overall agreement comparing Direct to Plex was 99.3% (535/539; confidence interval [CI], 98.1% to 99.7%). As shown in the supplemental material, four samples were negative with Direct (samples 72, 76, 106, and 147). Corresponding Plex $C_q$ values ranged between 20.77 and 23.12 $C_q$ for ORF1ab (<3.77 $\log_{10}$ IU/mL) and between 20.01 and 23.25 $C_q$ for RdRp (<3.60 $\log_{10}$ IU/mL). Four samples were positive for a single target when tested with Direct. Three were RdRP positive only (samples 65, 78, and 145) and one was ORF1ab positive only (sample 120). The qualitative results for the positive samples comparing Pool to Plex demonstrated 100% concordance. However, two samples (72 and 78) were positive for a single target (ORF1ab) when tested with Pool.

**Quantitative comparison.** The mean change in $C_q$ and $\log_{10}$ IU/mL for Pool and Direct compared to Plex are shown in Table 2. The mean $C_q$ and $\log_{10}$ IU/mL change for Pool ORF1ab and RrRp compared to Plex was 1.64 $C_q$ (−0.48 $\log_{10}$ IU/mL) and 1.74 $C_q$ (−0.49 $\log_{10}$ IU/mL), respectively. The mean $\log_{10}$ IU/mL change was quantitatively very similar for each target, differing by 0.10 $\log_{10}$ IU/mL. Pool ORF1ab detected two more samples than Pool RdRp (185 compared to 183). The mean $C_q$ and $\log_{10}$ IU/mL change for Direct ORF1ab and RdRp compared to Plex was 3.11 $C_q$ (−0.91 $\log_{10}$ IU/mL) and 4.78 $C_q$ (−1.35 $\log_{10}$ IU/mL), respectively. A larger difference, 0.44 $\log_{10}$ IU/mL, was observed for each target, with Direct RdRp showing the greatest change in $C_q$. However, despite the greater change in $C_q$, Direct RdRp qualitatively detected more samples than Direct ORF1ab (180 compared to 178).

## DISCUSSION

Real-time reverse transcription-PCR combined with purified RNA from samples is the gold standard method for SARS-CoV-2 detection. The development of extraction-free methods during the early days of the COVID-19 pandemic was largely driven by the lack of reagents for RNA extraction, with the added benefit of reduced cost and speed. In contrast, we developed the Direct method to improve throughput as our primary goal. With the Western Australia borders closed for nearly 2 years, sufficient time has elapsed for the reagent and consumable supply chains to be restored. We sought to utilize additional testing capacity of the liquid handlers and the thermal cyclers while overcoming the RNA extraction bottleneck. If testing demand increased above our extraction-based capacity, we could divert samples to the Direct method if required.

Extraction-free methods for use with nasopharyngeal specimens have been investigated in a number of pilot studies (8, 10, 11, 14–16). The overall sensitivity comparing

**TABLE 2** Mean change in $C_q$ and reduction in viral load detected with the Pool and Direct methods compared to results with Plex[a]

| Method | PlexPCR target | No. compared | Mean $\Delta C_q$ (95% CI) | Mean $\Delta\log_{10}$ IU/mL (95% CI) | *P* value |
|---|---|---|---|---|---|
| Pool | ORF1ab | 185 | 1.64 (1.42 to 1.87) | −0.48 (−0.55 to −0.42) | <0.05 |
| Pool | RdRp | 183 | 1.74 (1.56 to 1.92) | −0.49 (−0.54 to −0.44) | <0.05 |
| Direct | ORF1ab | 178 | 3.11 (2.83 to 3.39) | −0.91 (−0.99 to −0.83) | <0.05 |
| Direct | RdRp | 180 | 4.78 (4.52 to 5.04) | −1.35 (−1.42 to −1.27) | <0.05 |

[a]Summary data and two-tailed paired *t* test results ($P < 0.05$), showing the mean change in $C_q$ or $\log_{10}$ IU/mL for the Pool and Direct methods, compared to Plex.

extraction-free methods to extraction-based methods vary from 55 to 99% according to the PCR assays evaluated. These studies are limited by the low number of samples tested (both positive and negative) and reported different sensitivities when stratifying $C_q$ values obtained with the reference method. Investigations with a larger number of samples have reported sensitivities of 96% ($n$ = 597, no $C_q$ stratification) (9) and 95% ($n$ = 155 positive samples with $C_q$ values of $<$33) (17). These investigations used heat as a sample pretreatment step for virus inactivation and operator safety (95°C for 5 to 10 min), followed by the addition of lysate directly into the PCR mixture. Optimization experiments have shown that high temperature and short durations (95 to 98°C for 5 to 15 min) improve (reduce) $C_q$ values compared to lower temperature and longer durations (60°C for 30 min) (9). Heat inactivation prior to testing with extraction-based methods also reduces the sensitivity of SARS-CoV-2 RNA detection (4, 18, 19). Other investigators have shown the addition of proteinase K (55°C for 15 min) followed by heat treatment also improves $C_q$ values up to three cycles compared to heat treatment alone (11). Based on the correlation between heat and reduced yield, a method designed to minimize the total time of SARS-CoV-2 exposure to elevated temperatures across the entire procedure would be advantageous. The extraction-free method we developed incorporates an optimal proteinase K concentration, a proteinase K incubation step at the lowest temperature that does not compromise yield, and a heat step at the highest temperature and shortest duration possible for proteinase K inactivation. We also included an optimized concentration (8 to 9% final concentration) of Chelex-100 ion exchange resin in a low concentration of Tris-HCl buffer (10 mM Tris-HCl), which has been shown to dramatically improve sensitivity (12). The inclusion of Chelex-100 resin is particularly important, since the chemical and biological constituents of the viral transport media (such as salts and denatured proteins) are directly transferred into the PCR mix; Chelex-100 removes free ions and positively charged contaminants in solution that may affect polymerase activity or specificity and preserves SARS-CoV-2 RNA in the sample by binding cofactors required for nucleases (12). As extraction-free approaches are competing with extraction-based methods in terms of purity and yield, the final goal is to achieve the highest RNA yield possible compared to the reference method. During assay optimization, RNA yield was measured with quantitative PCR. Hence, we primarily focused on sensitivity and yield during assay development, avoiding extended exposure to heat; SARS-CoV-2 exposure for laboratory personnel can be mitigated with adherence to strict laboratory procedures and personal protective equipment. Combining these elements, we benchmarked a method which included proteinase K at a final concentration of 0.58 mg/mL, an incubation step of 37°C for 10 min, and then 95°C for 90 s for heat inactivation of proteinase K. The duration of 90 s was the minimum time required to completely inactivate the proteinase K concentration used (which is essential).

Following optimization of the Direct method, we benchmarked the method against the routine Plex method, which uses MagNA Pure for extraction. As expected, we observed a loss in sensitivity of the Direct method compared to Plex both qualitatively and quantitatively. Despite the loss in analytical sensitivity, the overall agreement was 99.3% combined without $C_q$ stratification of results. We note that other studies have focused on positive sample comparisons and the number of negative samples tested with extraction-free approaches has been limited or absent (8, 9, 11, 18). Diagnostic PCR assays are optimized for purified nucleic acids in terms of primer-probe stringency and test performance. Compared to extraction-based methods, crude lysates may contain more interfering substances, including salts carried over from the transport media. PCR assays need to be thoroughly tested for nonspecific primer-probe interactions when modifying the method of template preparation. We included a large number of positive and negative samples in this evaluation. We did not observe any nonspecificity or PCR inhibition for any of the samples tested. Furthermore, given the high positive prevalence of the population tested, the extraction-free method must exhibit a high degree of specificity and not rely on extraction-based approaches for SARS-CoV-2

confirmation; the extraction-free method must be robust, high-throughput and stand alone as a single diagnostic test. During this study and the subsequent training of staff, we found the Direct method to be robust and reliable.

To further investigate the loss in sensitivity for the Direct method compared to Plex and Pool, we performed quantitative analysis of the $C_q$ values using the 1st WHO international standard for SARS-CoV-2 RNA. The mean reduction in viral load for Direct ORF1ab and RdRp compared to Plex was $-0.91$ and $-1.35$ $\log_{10}$ IU/mL, respectively. Compared to a mathematical model, a sample with a SARS-CoV-2 concentration of 5.00 $\log_{10}$ IU/mL extracted with MagNA Pure (200 $\mu$L), eluted in 50 $\mu$L, with 2.5 $\mu$L then used as template for PCR, would contain 3.00 $\log_{10}$ IU per reaction mixture (assuming 100% efficiency of extraction). The same sample tested with Direct (100 $\mu$L), added to 40 $\mu$L of the 30% Chelex-100–proteinase K–internal control mixture, with 2.5 $\mu$L then used as template for PCR, would contain 2.25 $\log_{10}$ IU per reaction mixture (assuming 100% efficiency of lysis). Therefore, a loss of 0.75 $\log_{10}$ IU per reaction mixture (25%) based on template volume would be expected. Nonetheless, the Direct method performed well qualitatively against 185 positive samples, with a mean viral load of 6.00 $\log_{10}$ IU/mL when tested with Plex. Eleven Plex samples contained $<$3.76 $\log_{10}$ IU/mL ($\approx$20.8 $C_q$), and the Direct method failed to detect four samples below this value. The PlexPCR 20.8 $C_q$ value was approximately equivalent to a cobas $C_q$ of 31.5, based on previous work (5). We acknowledge that caution should be taken when comparing $C_q$ values with different assays for quantifying SARS-CoV-2 RNA (20). However, SARS-CoV-2 is unlikely to be recovered from culture at late cycle threshold values, which carries less importance for viral transmission (21). The loss of sensitivity at this level may be an acceptable trade-off for higher throughput. Where sensitivity is a primary clinical concern (when screening solid organ donors and candidates or on an immunocompromised host ward), one may elect to continue with extraction-based methods.

In conclusion, the Direct method resolves throughput bottlenecks and adds an additional 12,032 results per day, comprising 128 Direct plates of 94 samples per plate, for an additional 32 PlexPCR runs with 376 samples per run. This complements our existing maximum of 7,332 per day with the extraction-based methods, to achieve a total of 19,364 results in a 24-h period. This method is useful to the wider scientific community as an alternative to RNA extraction, particularly for jurisdictions with issues with reagent supply or a lack of instrumentation (22). Finally, while less sensitive than the gold standard extraction-based method, this extraction-free method represents a viable, high-throughput diagnostic approach, with a degree of sensitivity that is suitable for COVID-19 testing.

## MATERIALS AND METHODS

**Study setting.** The Department of Clinical Microbiology Laboratory (PathWest, Fiona Stanley Hospital, Murdoch) is a reference laboratory located in Perth, Western Australia. During the early stages of the pandemic, samples were tested using the cobas SARS-CoV-2 assay (Roche catalog number 09175431190) as the primary testing method. Thermal pretreatment of the sample was performed before cobas testing (3, 4). We implemented the PlexPCR SARS-CoV-2 assay (SpeeDx catalog number 1301384) in September 2020 as an additional testing method to increase testing capacity (5). All samples were naso-oropharyngeal swabs collected according to a combined nasopharyngeal and oropharyngeal swab procedure (23). Swabs were placed in Copan UTM-RT medium (Copan, Brescia, Italy), CITOSWAB (Citotest Scientific Jiangsu, People's Republic of China), or PathWest virus transport medium (VTM) (24). In anticipation of increasing demand for testing, a head-to-head evaluation was performed comparing the MagNA Pure 96 extraction-based method (Plex) with the optimized extraction-free method (Direct). We also compared the results to a four-sample pool also tested with PlexPCR using the MagNA Pure 96 method (Pool). The testing was performed on 8 February 2022 and 9 February 2022. All samples were tested in parallel.

**Samples tested.** Positive samples were collected from 30 January 2021 to 8 February 2022. All SARS-CoV-2-positive samples were stored at $-80$°C as aliquots from the remaining original sample. Negative samples were collected 3 February 2022. Due to $-80$°C storage constraints, all negative samples were stored at 4°C in the original transport media tube. Samples positive for cobas ORF1ab and E-gene were defined as SARS-CoV-2 detected. Similarly, samples positive for Plex ORF1ab and RdRp were defined as SARS-CoV-2 detected. Samples positive for a single target were reflexively tested using Xpert Xpress SARS-CoV-2 (Xpert; Cepheid, Sunnyvale, CA, USA) from the original sample (not thermally treated). Samples positive for at least one different target compared to cobas or Plex were defined as

SARS-CoV-2 detected. All other results including negative Xpert results were considered equivocal for SARS-CoV-2, and repeat collections were performed. For the preimplementation study, we selected 185 consecutive positive samples and 354 consecutive negative samples. No equivocal SARS-CoV-2 samples were used in the preimplementation study, as these were not considered true positives for the purposes of method comparison.

**Plex method.** All positive and negative samples tested with Plex were extracted using MagNA Pure 96 DNA and viral nucleic acids small-volume kit (Roche catalog number 06543588001) using the Pathogens Universal 200 protocol (version 4.0). Twenty microliters of the PlexPCR internal control (SpeeDx catalog number 1301384) was added to each sample using the MagNA Pure 96 internal control tube (Roche catalog number 06374905001). A sample input volume of 200 $\mu$L and an elution volume of 50 $\mu$L were used. PlexPrep processing, including PlexPCR amplification and detection and result analysis, was performed following the protocols issued by the manufacturer.

**Four-sample pool preparation method.** For the Pool method, 50 $\mu$L of each positive sample ($n$ = 185) was combined with three separate 50-$\mu$L aliquots from different SARS-CoV-2-negative patient samples ($n$ = 555 samples in total) previously tested with cobas. All samples in the four-sample pool method were extracted and tested as described above for Plex.

**Direct method.** We optimized the Direct method prior to the preimplementation study in terms of analytical sensitivity, efficient reagent use, and throughput. A sensitive extraction-free method using a chelating resin was optimized for sample preparation based on the work of others and our own experience (12, 25, 26). A 10 mM Tris-HCl solution was prepared from a 1 M Tris-HCl solution (catalog number T2663; Sigma-Aldrich, Saint Louis, MO, USA) with molecular biology-grade water (catalog number W4502; Sigma-Aldrich). A 30% (wt/wt) suspension of Chelex-100 resin (catalog number 142-1253; Bio-Rad, Hercules, CA, USA) was prepared with 10 mM Tris-HCl. For 94 samples, 4.8 mL of the Chelex suspension was transferred to a secondary tube (Greiner Bio-One; catalog number 459000; Kremsmünster, Austria). To this suspension, 144 $\mu$L of a 20-mg/mL proteinase K solution (catalog number A5051; Promega, Madison, WI, USA) and 100 $\mu$L of PlexPCR internal control RNA (catalog number 1301384; SpeeDx) was added and mixed. Forty microliters of the Chelex reagent was added to each well of an Axygen PCR microplate (PCR-96-AB-C; Corning, New York, NY, USA) using a multichannel pipette and 200-gauge wide-bore pipette tips (2069G; Molecular BioProducts, San Diego, CA, USA). A 100-$\mu$L aliquot of each patient sample or control was added to each well without mixing. The Optitrol NAT SARS-CoV-2 reference material (NT04032; DiaMex, Heidelberg, Germany) was used as a positive control and VTM was used as a negative control. The plate was sealed with ThermalSeal film (100THERPLT; Excel Scientific, Victorville, CA, USA) and placed into a GeneAmp PCR system 9700 thermocycler (Applied Biosystems, Waltham, MA, USA) in a pre-PCR area. The incubation program consisted of 37°C for 10 min, 95°C for 90 s, and 4°C for 1 min. The lid was preheated to 103°C. Following the incubation program, the plate was removed and sealed in a zip-lock bag, transferred to a sealed centrifuge plate holder, and centrifuged at 4,000 rpm for 5 min using a Sigma 4-15 centrifuge (Sigma-Aldrich). The sealed centrifuge plate holders were opened in a class 2 biological safety cabinet, and the ThermalSeal film was removed. Processed plates (up to 4 at a time) were loaded into the PlexPrep for 384-well PCR plate preparation using appropriate personal protective equipment.

**Preimplementation study.** All PlexPrep runs were prepared using PlexPCR master mix of a single lot number. The master mix consisted of 5 $\mu$L Plex master mix (2×), 0.1 $\mu$L reverse transcriptase (100×), 0.2 $\mu$L RNase inhibitor (50×), 0.5 $\mu$L CoV-2 mix, and 1.7 $\mu$L nuclease-free water for a total of 7.5 $\mu$L per reaction mixture. The PlexPrep liquid handler was utilized for master mix dispensing (7.5 $\mu$L) to the LightCycler 480 384-well reaction plate. The nucleic acid extracts for Plex and Pool, including the lysates for Direct, were all added to each well (2.5 $\mu$L), also using the PlexPrep. PlexPCR amplification and detection and result analysis were performed following the protocols issued by the manufacturer. The PlexPCR amplification protocol includes a 10-cycle touchdown. No fluorescent acquisitions are performed during the touchdown cycles; hence, $C_q$ values are reported approximately 10 cycles earlier than with conventional real-time PCR (5). The $C_q$ values were recorded for ORF1ab, RdRp, and the internal control.

**Quantitative standards, external control, and analysis.** Quantitative standards were prepared from the 1st WHO international standard for SARS-CoV-2 (NISBSC code 20/146), supplied as 7.70 $\log_{10}$ IU/mL (National Institute for Biological Standards and Control, Hertfordshire, UK). The standard was reconstituted with 0.5 mL of phosphate-buffered saline following the manufacturer's instructions. Once reconstituted, the standard was 10-fold serially diluted in a naso-oropharyngeal matrix. This matrix consisted of pooled naso-oropharyngeal samples from samples that previously tested negative for SARS-CoV-2 using cobas. Seven standards were prepared over the range of 0.70 to 6.70 $\log_{10}$ IU/mL. Each standard was tested in triplicate with Plex using the same amplification and detection lot number used for patient samples. The mean $C_q$ value at each concentration was used to calculate ORF1ab and RdRp standard curves and regression. At least two positive replicates at each dilution were required to be included in the standard curve. The regression formulas were used to calculate the ORF1ab and RdRp $\log_{10}$ IU per milliliter for all positive samples.

**Data analysis.** A contingency table was prepared to assess overall agreement between Plex and Direct-Plex with 95% CIs using a Westgard QC 2 × 2 contingency calculator (Westgard QC, Madison, WI, USA). ORF1ab and RdRp $C_q$ values were compared with a two-tailed paired $t$ test ($P < 0.05$). Similarly, the quantitative differences in IU per milliliter were also calculated. All statistical analyses were performed in Excel (Microsoft, Redmond, WA, USA) and MedCalc v15.4 (New York City, NY, USA).

**Institutional review board statement.** The residual samples used in the study were deidentified and results were not used to clinically manage patients (National Statement on Ethical Conduct in

Human Research 2007 [May 2015], National Health and Medical Research Council, Australian Research Council, and Australian Vice-Chancellors' Committee).

## SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

**SUPPLEMENTAL FILE 1**, XLSX file, 0.09 MB.

## ACKNOWLEDGMENTS

We thank the following dedicated staff currently working in the Department of Clinical Microbiology for their hands-on contributions to the COVID-19 pandemic in Western Australia (at the time of this study): C. Aylward, K. Bachynsky, J. Bayliss, J. Beverwijk, S. Bowley, K. Carvalho, J. Christie, N. Chung, V. Chung, T. Costello, E. D'Orazio, A. Davis, C. Dunbar, O. Foti, A. Hill, L. Holyoak-Roberts, A. Ienco, E. Juanillo, C. Kaltenrieder, K. Kusse, A. Lacsina, D. Lee, L. Lim, R. Malik, R. Meng, J. Milan, P. Mitchell, S. Ng, A. Niescierowicz, R. Oorschot, G. Panahi, S. Patel, G. Penman, N. Pindoria, M. Porter, A. Powell, D. Price, N. Pring, M-L. Read, M. Rhodes, J. Robertson, A. Rogers, T. Short, E. Sloan, N. Steven, J. Strickland, K. Suckling, S. Wescombe, E. Whisson, and C. Yan. We also thank all the Microbiology Clinical staff, PathWest Media Manufacturing, Adrian Bautista (Chief Executive of PathWest), PathWest Executive staff (including Procurement and Purchasing, PathWest ICT, COVID-19 Command), and Fiona Stanley Hospital Facilities Management (Serco) for their continual support throughout the COVID-19 pandemic.

This work was funded by PathWest Laboratory Medicine Western Australia.

We declare no competing interests.

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## 4.7 Conclusion

The SARS CoV2 pandemic led to the rapid development of various detection technologies with different applications, such as for laboratory use, POC testing, and home testing. Challenges included testing capacity, variations in sensitivity and specificity, and changes to assay sensitivities due to virus variants over time. All this development and testing occurred in real time during the pandemic, impacting all jurisdictions globally, all competing for labour, consumables and hospital resources. In WA, our introduction to SARS-CoV-2 in the first few weeks of the pandemic focussed on implementing any suitable sensitive PCR test available that would be compatible with our existing laboratory equipment. Following implementation of the LightMix method, our next challenge was to test throughput and testing continuity in the face of global shortages of reagents and consumables. Our rapid adoption of cobas testing (with reagent and consumable allocation) enabled continuity of testing and allowed our laboratory to evaluate new methods of sample preparation for the safe handling of SARS-CoV-2, improving operator safety and reducing the dependency on PPE. We then investigated new methods of SARS-CoV-2 detection with the aim of increasing testing capacity and throughput. Finally, with the expected increase in SARS CoV-2 testing with the WA borders opening, we sought to re-define laboratory throughput with a very high throughput extraction-free method, at least equivalent to the sensitivity achieved by sample pooling.

### 4.7.1 Thermal treatment

We developed and piloted a thermal treatment method for nasopharyngeal samples before cobas testing (**Publication 8**) and found no significant difference in $C_q$ values for ORF1a. However, a statistically significant difference was observed for the E gene target and internal control. Small delays in $C_q$ values were observed, most likely arising from the heat-induced hydrolysis of RNA or additional interfering substances from heating the sample, causing some level of PCR inhibition (223). The surprise in our investigation was the assessment of the qualitative differences, with some evidence that thermal treatment may improve the qualitative detection of SARS-CoV-2 using cobas. The reasons for this were not clear. Based on the finding that thermal treatment was not detrimental to the qualitive outcomes, this study led to routine implementation of thermal treatment before cobas testing. This method negated the need for PPE, which was redirected to front-line personnel, and improved operator safety whilst reducing the level of staff anxiety in the laboratory (established through personal communication).

**Publication 9** achieved a deeper understanding of the effects of thermal treatment using quantitative PCR as the tool, with target copy number as the measurement. The median ORF1a and E gene reduction in target copy number was -0.07 (1.6%) and -0.22 (4.2%) $\log_{10}$ copies/mL respectively. In terms of $C_q$ we demonstrated a maximum mean increase in $C_q$ value of +0.31 using 75 °C for 15 minutes, while others have shown a minimum increase of +3 $C_q$ using 80 °C for 30 minutes (224). In the same study the authors demonstrated no loss in assay sensitivity at 56 °C and 60 °C. Similarly, we demonstrated no loss in the qualitative outcomes at 75 °C for 15 minutes, using a commercial assay and a larger number of positive samples (key points of difference). There are also conflicting reports of thermal treatment significantly lowering $C_q$ values for N and ORF1ab gene targets, improving RNA quality and quantity, using an in-house PCR method (225). However, the study design had severe limitations and lacked statistical evidence, with only 7 positive samples included to reach this conclusion. Moreover, the authors measured the total RNA concentration in ng/µl of RNA using 260/280 nm absorbance spectroscopy. The authors concluded that the concentration of RNA in thermally treated samples was higher than non-treated samples and tied this argument to the reduction in $C_q$ values for SARS-CoV-2 treated samples. However, an oversight was that the investigators were measuring total RNA yield, which includes patient RNA, so a sound correlation of improved SARS-CoV-2 RNA yield cannot be determined from this measurement process. Following these publications, heat inactivation on SARS-CoV-2 survival and virion protein and structure has been thoroughly investigated, with a 15 minute incubation at 65 °C completely inactivating (226). Based on this, our thermal treatment method at 75 °C for 15 minutes, is likely to be more effective at inactivation due to the higher temperature.

The work in **Publication 9** contributes significantly to the understanding of the negligible effects of thermal treatment on the test performance of cobas SARS-CoV-2 and RNA stability, which can be directly applied to other tests and other laboratories. For example, thermal treatment is a simple technique that requires minimal equipment or reagents, making it well-suited for resource-challenged jurisdictions or laboratories that have restricted access to safety equipment such as biological safety cabinets or PPE. For this reason, we were asked by the Editor In-Chief of another journal to share our initial work regarding thermal treatment with the WHO ahead of acceptance. A relevant example was the use of heat inactivation for SARS-CoV-2 in a reference virology unit in Cambodia, for deployment to remote areas which have basic facilities (227). Conversely, thermal treatment using similar temperature and time has been applied to samples on an industrial scale for mass testing of the population, as shown in the UK in 2022 (228). Here, the authors used industrial catering ovens for bulk heat inactivation of swab samples in their original collection devices. Our work in **Publication 9** also determined the RNA stability long-term following thermal treatment. Experimental data

published in the supplemental file showed that SARS-CoV-2 RNA remained stable for 48 hours post thermal treatment. This also has implications for low-resource settings and remote areas where thermal treatment can occur near the site of collection and then be transported to the laboratory for testing in a reasonable amount of time (227). The impact in our laboratory was improvement to workflow and the assurance of RNA integrity, as some heat-treated samples were being tested the next day. This was useful as the samples were ready to be tested with cobas early the following morning to ensure the 24-hour test turnaround time was kept. Moreover, this work in **Publication 9** is a reminder that although thermal treatment or other pre-analytical inactivation methods may show a reduction in viral RNA, the qualitative outcomes may not be significantly affected, and local validation should be performed to assess.

### 4.7.2 Improving testing throughput and reducing the dependency on a single high-volume assay

Despite the testing throughput of cobas, our laboratory was allocated cobas kits by the manufacturer based on sample testing numbers, with no guarantee of supply. Alternative and higher throughout testing methods were required. As such, **Publication 10** describes Plex compared to cobas in a large evaluation. We show superior throughput with Plex and how both assays performed in our hands during a surge testing event. Both assays worked well in synergy with one another. The sample-to-result cobas method workflow enabled the laboratory to load three runs (282 samples) and walk-away at the end of the shift, with results available the following morning. Meanwhile, the Plex method enabled higher throughput during daytime hours when staffing levels were higher. Additionally, during peak testing times, the laboratory could pivot from one testing workflow to another, depending on staffing levels, instrument down-time, or reagent/consumable resources. Plex added guaranteed redundancy to our single cobas 6800 instrument as we had multiple MP96 extractors and LC480 thermocyclers. Furthermore, SpeeDx guaranteed supply with local manufacturing in Sydney, with a significant stock holding of approximately 1 million units of partzyme A and B reagents (personal communication with Colin Denver, CEO SpeeDx). Overall, our study demonstrated the capability of two high-throughput systems, with Plex being described for the first time in the literature, to highlight the throughput capability of Plex and the importance of strong Australian biotechnology manufacturing capability for future preparedness. The Plex evaluation was presented at the 37th NRL Workshop on Infectious Disease Testing 2021 titled “PlexZyme SARS-CoV-2: a validation and throughput evaluation” (229).

### 4.7.3 Calibration

Throughout the pandemic, $C_q$ values have been used as an indicator for viral load. They have been proposed to predict disease severity, assess transmissibility as a proxy for using viral culture, and to help with decisions on removing patients from isolation. The paper "The dangers of using $C_q$ to quantify nucleic acid in biological samples: a lesson learnt from COVID-19" (230), summarises the flaws of using $C_q$ as a diagnostic threshold or cutoff to quantify and stratify risk, aid patient management and assess infectivity. A classic example is Plex compared to cobas, whereby $C_q$ values from one assay can be correlated with another, but the relationship is assay-dependent. These cautions have also been reaffirmed by others (231). The situation is further complicated by numerous testing platforms and the variety of PCR methods used. By using quantitative PCR as the alternative, SARS-CoV-2 viral load has been shown to be useful for following treatment response and guiding duration of antiviral treatment in immunocompromised hosts (232). Secondly, it is also useful with clinical parameters to assign non-infectious status in immunocompromised hosts who can remain infectious for a long and variable period of time (232). However, without a standard measure of viral load, relating these studies to other studies or our own data is difficult. Therefore, assay calibration to a single standard, particularly one that is curated and internationally available would be ideal. Another limitation on the quantification and interpretation of viral load results in respiratory samples, is the potential for variability in the quality of sample collection, and not having a defined space or volume to test, compared to viral loads in blood plasma or whole blood.

**Publication 11** describes the first assay (in-house or commercial) published in the literature, calibrated to the 1$^{st}$ IS for SARS-CoV-2 RNA. This clinical study was performed as it is difficult to directly compare results with other studies due to the different methods that they have employed, including pooling of lower and upper respiratory tract samples and using $C_q$ values rather than an accurate measurement of viral load (186, 188, 190). We report the clinical associations compared to viral load and found some consistent with other studies, such as no clear association of viral load with age or gender (233), and a wide variation in viral load compared to time from symptom onset (234), which may be due to variable viral kinetics between individuals, heterogeneity of respiratory samples and variability in collection. Despite the difficulties of making comparisons with other studies that measure $C_q$, the strength of our study was the use of the 1$^{st}$ IS SARS-CoV-2 RNA as a calibrator. Using our published cobas 1$^{st}$ IS SARS-CoV-2 RNA regression formula, other cobas users are able to approximate SARS-CoV-2 RNA concentration in IU/mL Consequently, our laboratory calibrated (harmonised) all our routine assays (cobas, Plex and Xpert) to the IS, thereby describing the accurate relationship of individual assay

$C_q$ with one another, with the intention to standardise reporting for clinical and infection control purposes, such as response to treatment and assessing infectivity. This work was presented in the Standardisation and Accuracy of Testing session at the 38th NRL Workshop on Infectious Disease Testing 2022 titled "Quantification of SARS-CoV-2 RNA" (235). Other reports started to emerge in the literature whereby several commercial assays including cobas, Hologic Aptima SARS-CoV-2/Flu, Hologic Panther Fusion SARS-CoV-2 Assay, PerkinElmer SARS-CoV-2 and Cepheid Xpert Xpress SARS-CoV-2, were calibrated to the IS and resolved issues where analysis of $C_q$ values may have led to erroneous quantitation (236, 237). We note that other investigators have followed suit with POC tests, calibrating assays such as the cobas Liat ("Lab in a tube") (238).

Three months after our publication, Roche released the cobas SARS-CoV-2 Duo assay. This assay was the first commercial assay quantified by a manufacturer to the 1st IS SARS-CoV-2 RNA. The cobas SARS-CoV-2 Duo test targets ORF1a and ORF1ab. At the time I had already determined Plex ORF1ab and cobas ORF1ab to be highly commutable to one another, differing by 0.01 $\log_{10}$ at 4.0 $\log_{10}$ IU/mL (235). A comparison of cobas SARS-CoV-2 and cobas SARS-CoV-2 Duo was subsequently published in 2023, for the aforementioned reasons of standardisation of viral load to guide patient treatment, as well as to determine infection control measures and policies (238). As such, I can assess commutability of our calibration compared the Roche calibration used for cobas SARS-CoV-2 Duo by comparing regression curves. Based on my calculations, a cobas SARS-CoV-2 $C_q$ of 27 in our study measures 5.06 $\log_{10}$ IU/mL compared to 4.87 $\log_{10}$ IU/mL in the cobas SARS-CoV-2 Duo study. The difference of less than 0.2 $\log_{10}$ IU/mL is small, highlighting how similar standardisation can be achieved with commercial assays calibrated by the manufacturer, independently compared to in-house calibration with the same standard.

### 4.7.4 Increasing throughput with an extraction-free PCR method

**Chapter 3** concludes by removing the bottleneck of nucleic acid extraction to dramatically increase the throughput of SARS-CoV-2 testing. While the overall goal in **Publication 12** concerned throughput, the underlying science behind the extraction-free method was concerned with impact to sensitivity and specificity, with throughput a matter of scaling the methodology to achieve the output required. The development of the extraction-free test and the laboratory workflow required for its implementation is a good example of where my prior experience has significantly influenced the testing direction. For example, my experience with simple extraction methods, Chelex-100, using quantitative PCR as a tool for experimental work-up, optimising automation and assay implementation, and mitigating potential sources of failure such as contamination. Our study's

strength, compared to other investigations, is we demonstrate an overall agreement of 99.3% for all samples tested. This contrasts to other studies that performed $C_q$ stratification, reporting overall agreement for samples flagging positive before a given $C_q$ value. We utilised quantitative PCR calibrated to the 1st IS to investigate the loss in sensitivity of the extraction-free method compared to nucleic acid extraction and compared that to a four-sample pool. As such we observe experimental losses in sensitivity that align with losses calculated in silico and concluded that this loss in sensitivity was an acceptable trade-off to achieve a significant increase in throughput from 7,000 samples to nearly 20,000 samples in a 24-hour period. I presented the scientific work-up and results of this work at the 38th NRL Workshop on Infectious Disease Testing 2022 (239).

At a similar time, the UK group which reported the heat inactivation of samples in industrial ovens prior to nucleic acid extraction (228), published a further study which modelled a test throughput of up to 20,000 samples per day using an extraction-free method. The authors reported a loss in sensitivity of less than 1 $C_q$ for heat treatment compared to samples that were not heat treated using an extraction-based method (228). There was a loss in sensitivity of 2-4 $C_q$ comparing the extraction-free method to an extraction-based gold standard. From our perspective using quantitative PCR, we developed a better understanding of the loss in sensitivity with thermal treatment using quantitative PCR (**Publication 9**), and likewise for the extraction-free approach compared to the gold standard (**Publication 12**). Given our confidence with our methods of quantitative investigation, observation and analysis, we would not hesitate to consider the implementation of an extraction-free method for other respiratory viral pandemics in the future, especially if assay throughput was the primary goal. For example, during the influenza A H1N1 2009 pandemic, testing capacity for our organisation was exceeded, so this new approach to high-throughput testing developed for the COVID-19 pandemic could be helpful in the future. For our method, proteinase K is readily accessible in high concentrations and Chelex-100 is an industrial reagent that has an exceedingly long shelf-life. Finally, we report our sensitive extraction-free method for SARS-CoV-2 and potentially any other virus in transport medium, to the wider scientific community, which uses affordable and accessible reagents and consumables. This method can be adopted in jurisdictions where access to instrumentation and reagents is problematic, or if cost is the primary concern. An example of the latter would be in large-scale screening in farming and agriculture, such as the highly pathogenic avian influenza (HPAI) H5N1, an imminent threat (240).

# Chapter 5: Conclusion

This thesis concludes with a summation of the chapters, current testing and future challenges to be addressed.

## 5.1. Detection, identification and characterisation of fungi

As described in **Chapter 2**, species-specific fungal detection and identification have been challenging tasks for clinical microbiology laboratories. While molecular methods address many of the shortcomings of traditional fungal identification, MALDT-TOF is routinely used front-line for the identification of yeasts and moulds due to its accuracy, ease of use, rapidity and cost. MALDI-TOF plays a central role in many laboratories for identifying of bacteria and fungi, with CLSI and CDC guidelines and methods established for yeasts and moulds to assist and improve the level of standardisation (241-243). MALDI-TOF databases are continuously updated and improved by the manufacturers and users, with on-line and user-developed databases. For example, the latest Bruker library for yeast reports 1,276 spectral profiles from 220 yeast species across 68 genus groups (244). Consequently, MALDI-TOF only has a few limitations for yeast identification, and most common yeasts are easily resolved to the species level (245). However, there are special cases where ITS sequencing is used to confirm successful MALDI-TOF yeast identification. For instance, we reported to the CDC in 2019 one of our first cases of invasive infections caused by *C. auris*, where definitive ITS identification is required due to global significance of this important pathogen (246). In our laboratory and others, yeasts not identified by MALDI-TOF are further identified using Vitek, with ITS sequencing occasionally performed for further identification (247). If MALDI-TOF discrepancies occur, they usually involve delineations within the species complexes such as the *C. glabrata* complex (*C. glabrata*, *C. nivariensis*, *C. bracarensis*) and the *C. parapsilosis* complex (*C. parapsilosis*, *C. metapsilosis*, *C. orthopsilosis*) (248).

Similarly, the latest Bruker library for filamentous fungi reports 1,021 spectral profiles from 225 fungal species across 70 genus groups (249). However, identifying multicellular filamentous fungi is a challenge for MALDI-TOF due to the diverse effects of fungal culture conditions, which result in varying mass spectra due to different cellular growth and cellular composition. Consequently, MALDI-TOF sample preparation for filamentous fungi is more challenging than for yeasts and requires the careful transfer of mycelium and thorough homogenisation. Additionally, the culture process and extraction method for filamentous fungi have been areas of high variability in the literature, making it difficult to

report with accuracy. Given the variable performance of filamentous fungi identification reported, multicentre studies have become the key to develop standardised processes. For instance, one study demonstrated a wide range in performance (33–77%) across eight different testing centres analysing 80 identical isolates (250). Therefore, refining standardised techniques for the filamentous fungi will be critical for improved performance in the future. However, when compared to traditional morphological identification, MALDI-TOF performs adequately (77-94%) for a wide range of filamentous fungi, including *Aspergillus* (86%), Mucorales (90%), and correct species-complex level for *Fusarium* (94%) (248, 251). MALDI-TOF can also identify most non-dermatophyte moulds after 1-2 days of growth, but some fungi may require longer culture or subculture, particularly if the culture is mixed, thereby delaying results (252). Additionally, identification can be heavily influenced based on the culture media, age of the culture and library used (252). This may be a problem in clinical practice where a short turnaround time is needed, especially for invasive mycoses. For filamentous fungi that do not readily identify with MALDI-TOF, a combination of phenotypic tests and ITS sequencing can resolve identification issues.

Unsuccessful MALDT-TOF identification for yeasts and filamentous fungi can also be caused by the absence of the species in the database. Therefore, rigorous database representation is a key factor for MALDI-TOF MS success, and user-developed MALDI-TOF databases play a key role in improving fungal identification. Many MALDI-TOF performance studies for fungi use rRNA gene sequencing methods (mostly ITS) as the gold standard comparator for definitive identification (245, 247, 253-255). When there is failure to meet an expected MALDI-TOF score for species identification, additional phenotypic, genotypic testing, or both are recommended (241-243). In our laboratory, definitive ITS identification has been useful to improve our user-developed MALDT-TOF database for the filamentous fungi, resulting in improved fungal identification for uncommon species. These mostly include cryptic species of the genus *Aspergillus* (256), *Fusarium* and some species of *Penicillium*, including *Talaromyces marneffei* (formerly called *Penicillium marneffei*, which is an important opportunistic dimorphic fungal pathogen).

Given that MALDI-TOF has taken the front-line position in fungal identification, the laboratory workflow for ITS sequencing has transitioned to a reflexive assay for difficult-to-identify moulds or as a rapid fungal pathogen detection or exclusion tool. In 2021, our laboratory published MALDI-TOF and ITS sequencing workflow for sterile specimens, using mass spectrometry scores of > 1.7 for genus-level identification, > 2.0 for species-level, and ITS sequencing for further identification when required (257). In our laboratory, approximately 5-10 fungi are submitted for sequencing every week, which accounts for approximately 10% of all fungal identifications performed. These requests are largely driven by unsuccessful MALDI-TOF identification and the clinical need for rapid identification of young,

non-sporulating cultures. For example, in 2019, our laboratory published the first case of *Tintelnotia destructans*-associated keratitis in a contact lens wearer, where the isolate was unable to be identified by MALDI-TOF and was rapidly identified by ITS sequencing (258). Our laboratory is also encountering a greater proportion of fungi not represented in our curated in-house ITS sequencing database, as highlighted by the example above, requiring submission to the broader GenBank nucleotide database. The reasons for this are not clear but may be associated with the increase in the number of immunosuppressed patients, the diversity of the fungi that colonise them, and exposure to antifungal agents that may select for cryptic species. While common pathogens can be excluded by this process, the compounding problem of whether to add a new species to our in-house ITS sequencing database is an ongoing issue, largely driven by the clinical uncertainly of infection with the identified species and the unlikelihood of recovering the same species from other patients in the future. Regardless, our 2003 published ITS sequencing method has stood the test of time and was cited in the latest 13$^{th}$ edition of the *Manual of Clinical Microbiology: Molecular Techniques* (259).

There have been advances in laboratory molecular methods that are impacting the need for ITS sequencing in some circumstances. Examples include the direct identification of dermatophytes and other fungi from skin, hair, and nail samples using qPCR (140). These qPCR methods are being increasingly utilised due to speed (same day testing) and a sensitivity exceeding culture (260, 261). These assays have progressed from in-house PCR assays (262) to commercial assays, such as those from AusDiagnostics (140), Seegene (263), and PathoNostics (261). All these tests have been shown to outperform the sensitivity of microscopy and culture and are scalable to meet the laboratory needs. Additionally, some of these assays can also resolve species identification that MALDI-TOF cannot reliably differentiate (244). Direct PCR identification of the *T. rubrum* complex, which accounts for more than 70% of the dermatophytes recovered from culture (260), usually requires no further work. Similarly, for *Microsporum canis* and *Epidermophyton floccosum.* However, identification to the genus level of *Microsporum* or *Trichophyton*, may require reflexive culture and ITS sequencing if an isolate is recovered. PCR detection of the *T. mentagrophytes* species complex may also require reflexive culture and ITS sequencing to differentiate *T. interdigitale*, *T. mentagrophytes, Arthroderma benhamiae and A. simii.* (264). Recently, *T. indotineae* has also been added to this list of the *T. mentagrophytes* species complex. Identification is important as *T. indotineae* is causing an epidemic across the Indian subcontinent associated with severe, recalcitrant dermatophytoses (265). *T. indotineae* can only be reliably distinguished from *T. mentagrophytes*/*interdigitale* using molecular methods, usually ITS sequencing (266-268). Infections can cause extensive, highly inflammatory plaques of tinea corporis (body), cruris (groin), and faciei (face) (269). Furthermore, *T. indotineae* isolates are often terbinafine-resistant, and infections may require months of treatment, with second-line therapies such as

itraconazole or other antifungals typically reserved for invasive fungal infections (269). It is therefore necessary to identify this species from other members of the complex for clinical management of patients and surveillance (267).

A useful advantage of these dermatophyte qPCR assays is the ability to detect and identify dermatophytes in these challenging samples where normal flora and environmental contaminants are present and may overgrow. Although culture, MALDI-TOF, and ITS sequencing remain the gold standard for identification, as we have shown in this thesis, contaminants and normal flora are challenging for the ITS sequencing approach due to the broad-range nature of the ITS domain (262). ITS PCR assays may also cross-react with human DNA, requiring alternative extraction techniques (129). A major disadvantage of the Sanger sequencing approach is poor sequencing depth and the inability to resolve heterogeneous mixtures of PCR products, especially those in low copy numbers. Hence, the use of ITS Sanger sequencing has been restricted to tissues and fluids that are not readily colonised with fungi (sterile sites). To address these issues, a revision of our ITS amplicon sequencing method using long-read NGS, such as the cost-effective Oxford Nanopore Technology (ONT), may resolve dermatophytes from other colonising fungi present in skin, hair and nail samples. The same technique could also be applied to deep respiratory samples such as lung biopsies or bronchoalveolar lavages. As of November 2024, our laboratory has transitioned the ITS amplicon Sanger sequencing as described in **Publication 1 and 2**, to a high-throughput ONT method with an automated analysis pipeline capable of resolving mixed infections. This presents an opportunity to reestablish the utility of a new direct ITS PCR and ONT sequencing method for these challenging sample types. For example, we have routinely implemented the AusDiagnostics dermatophyte assay for the front-line detection and identification of dermatophytes in skin hair and nail samples. We previously compared this assay to microscopy, culture and ITS sequencing and found it to be highly sensitive and specific for dermatophyte detection (Pryce, 2024, unpublished data). Using the same nucleic acid extracts for AusDiagnostics, we can potentially compare and validate the direct ITS PCR and ONT sequencing approach for dermatophyte detection and identification in these challenging samples. The assay could also be multiplexed with the squalene epoxidase gene, identifying mutations associated with high-level terbinafine resistance in *T. indotineae* (267). These types of targeted, multiplex PCR assays with high-throughput NGS amplicon sequencing, are common sequencing applications for microbial ecology and fungal identification (270, 271). As for **Publication 1**, which was developed based on the availability of ITS sequences used for fungal taxonomy, a target-gene sequencing approach combining multiple identification targets in a single PCR reaction (such as ITS, 18S, D1/D2 domain of the 28S rRNA gene, calmodulin, and beta-tubulin), followed by ONT amplicon sequencing, may resolve the limitations of identification using ITS sequencing alone, beyond the capability of Sanger sequencing.

Furthermore, other genes could be added, such as AMRs for echinocandins and azoles in *Candida* and *Aspergillus* (272, 273).

NGS-based methodologies offer a variety of solutions to modern-day problems and challenges in clinical microbiology. Although whole genome sequencing (WGS) can speciate, epidemiologically type, and predict AMR, relatively few clinical laboratories have implemented them into routine workflows. Laboratories need to establish new testing infrastructure, develop new laboratory methods, and connect these with data processing, interpretation, and clinical reporting. However, for a successful transition to routine use, it is necessary to ensure the WGS data generated meets defined quality standards for pathogen identification, typing, antimicrobial resistance detection, surveillance, and has clinical oversight. As such, quality assurance programs and clinical governance training for WGS sequencing approaches have been implemented to ensure laboratories can generate and analyse high-quality data, either meeting or exceeding the minimum requirements (274). These approaches are moving from the research arena to clinical laboratories, and the capabilities of a frontline infectious disease diagnostic tool are now being recognised (275). For example, in 2020, our laboratory investigated a cluster of cases of *Lomentospora prolificans* (*Scedosporium prolificans*) from hospitalised haematology patients over an 8-month period using whole genome sequencing and showed a high number of mutational differences with no single causative strain (276). The fact that these cases were not linked was reassuring from infection prevention and management perspective, as the mortality rate of *L. prolificans* infection is universally high at 47-88% (277). We also recently described the use of WGS to prove transmission of the Mucorales *Apophysomyces variabilis* from an organ donor with undiagnosed systemic disease to two lung and liver transplant recipients, which led to fatal outcomes (278).

The detection of fungaemia is still a challenge despite all these advances. Blood cultures remain the best-standardised test for the diagnosis of fungaemia as no method has been shown to be the ultimate standard for the detection of fungi in blood (279). Direct blood culture testing with MALDI-TOF has proved successful for yeast species identification with 90% reliability (280). This has reduced the frequency of urgent ITS sequencing directly from blood cultures in our laboratory, other than inconclusive MALDI-TOF results. Rapid molecular detection of yeasts using the BioFire FilmArray Blood Culture Panel has shown improved specificity compared to direct MALDI-TOF (281). However, the BioFire FilmArray Blood Culture Panel has considerable costs associated with testing for the identification of only seven yeast species (*C. albicans*, *C. auris*, *C. glabrata*, *C. krusei*, C. *parapsilosis*, C. *tropicalis*, *C. neoformans*/*C. gattii*). Methods of direct detection and identification of fungi in whole blood have not been widely reported. There are some reports of molecular assays for fungal detection

in blood, including WGS (282-285). However, the number of positive sample numbers are low, are proof of concept using laboratory-prepared samples, or are likely due to contamination (286). Other new technologies of amplicon detection and characterisation have also been reported for blood. For example, the T2 Magnetic Resonance (T2MR) assay has shown the capability to detect and speciate *C. albicans*, *C. glabrata*, *C. krusei*, C. *parapsilosis* and C*. tropicalis* in whole blood in approximately 5 hours (285). This technology combines the nuclear magnetic resonance and ITS PCR in a fully automated system. Specifically, whole blood is extracted, ITS PCR is performed, followed by hybridisation capture using species-specific probes hybridised to magnetic particles, which causes changes in the nuclear magnetic resonance signal of the sample. In a multicentre clinical trial that included both a prospective and a contrived arm to represent the full range of clinically relevant concentrations of *Candida* species., T2MR demonstrated a sensitivity and specificity of 91.1% and 98.1%, respectively (287). T2MR was recently compared for the detection of bacterial and fungal pathogens to blood cultures and was found to be more sensitive than blood culture, with T2MR detecting an additional nine cases of candidemia compared to one from blood culture from a cohort of 60 patients (288). The authors conclude that although a more rapid result can be achieved, the cost implications of parallel T2MR and blood culture testing for other organisms not represented in the T2MR panel need to be taken into consideration.

As far as the impact of qPCR for the management of IA and other causes of invasive fungal disease, qPCR has been more useful for patients with suspected disease from material collected from the infected site, rather than blood samples alone. For example, *Aspergillus* qPCR testing of bronchoalveolar lavage fluid and blood has been recently accepted as a mycological criterion for probable IA in consensus guidelines for research studies (289, 290). Commercial and in-house assays have been reported for *Aspergillus* and demonstrate good negative predictive value for excluding disease, but the low prevalence of disease limits the ability to rule in a diagnosis (291). Although potentially nonspecific, the co-detection of the fungal biomarker galactomannan in blood using a galactomannan-enzyme immunoassay has been more clinically useful in combination with PCR (292).

There is also the problem that an invasive fungal disease may not result in sufficient fungal cells or nucleic acids entering the bloodstream to be detected by PCR. As such, the challenges for direct detection of fungal cells or nucleic acids which may be present in blood, return to hypothetical example described in **Publication 4**, of the likelihood of fungal nucleic acid being recovered from nucleic acid extraction and its subsequent presence in the PCR reaction, at a given fungal load and volume of blood. While it is well established that PCR is sufficiently sensitive and can detect a single copy of a target gene, the remaining question is how do we guarantee the presence of a single copy of target in the volume of template used for the PCR reaction? This is especially challenging in the

presence of abundant human DNA in whole blood samples. Some form of selective target enrichment may resolve this challenge in the future. For example, new non-amplification enrichment technologies such as single molecule tethering, whereby magnetic beads tethered to DNA probes are used to capture nucleic acids, show promise for the detection of bacteria and fungi in blood, but are still under development (284).

Given the current improvements in automated molecular methods, qPCR methods may still be up to the challenge. For example, detection of pathogen cell-free DNA (cfDNA) using qPCR in the acellular fraction of plasma, also known as "liquid biopsy," has emerged in recent years and shown promise for the detection of a range of fungi (293). In this investigation, the authors used five separate multiplex PCR reactions covering 12 genera and species most commonly causing IFI and tested nucleic acid recovered from DNA extraction of plasma. The assay showed an overall sensitivity of 56.5% and specificity of 99.5% in this study when compared to laboratory confirmed and suspected IFI (293). Another recent investigation by the same group showed 88.5% concordance of the cfDNA PCR with laboratory confirmed IFI (294). The authors used 4 mL of plasma for extraction, eluted in 100 μL and used 10 μL for PCR, enhancing the sensitivity of detection. However, the workflow was a multi-step hands-on process which may be prone to environmental laboratory contamination. This is where sample-to-result or closed systems may prove beneficial. To elaborate, we recently developed and validated an in-house assay for the direct detection of *C. auris* from groin and axilla swabs, using the cobas omni utility channel (laboratory-developed channel for in-house tests) on the cobas 6800 instrument (Pryce et al., 2025, manuscript in preparation) (295). This in-house assay leverages the sample-to-result capability of the cobas 6800 instrument and performing a high-volume extraction (850 μL) and a high volume of template (25 μL), thereby increasing the likelihood of capturing low copy number targets in the sample and subsequent presence in the template. Using this approach, we can increase the likelihood of detection 20-fold to 100%, when compared to the example given in **Publication 4** of only 10%. The assay contains dUTP to control for carryover, which is important given the high sensitivity of this test we have developed (2 CFU/mL). Again, I used quantitative PCR to optimise the yield to an analytical sensitivity for *C. auris* detection in clinical samples. Based on this approach, it is possible to adapt this assay to detect other *Candida* species, or other fungi as described in the cfDNA study (293), and conduct a clinical trial assessing the utility of this third-generation qPCR platform with a new level of sensitivity for the detection of fungi in whole blood or plasma. Therefore, the application of new technologies with the current state of improved instrumentation may be the key to resolving the challenge of reliably detecting fungaemia and IFI in the future.

## 5.2. Molecular testing for *N. gonorrhoeae*

Summarising future state of molecular diagnostics for *N. gonorrhoeae* is challenging due to the complex biology of the genus *Neisseria* and this species. The evolution of commercial assays has largely been driven by workload and improving the laboratory efficiency of sample handling, increased throughput and reduced operational costs. Commercial assays have achieved significant improvements with laboratory automation in this regard. However, despite the improvements to assist at the laboratory level, there are still shortfalls with assay specificity, despite two decades following the first implementation of *N. gonorrhoeae* NAAT's. My published work with supplemental testing has shown that non-specificity issues still exist. This contribution and the science behind supplemental testing is useful to the wider scientific community and to helps inform new guidelines and policies for the PHLN. For example, I presented to the NNN and at CliniCon (Australian Society for Microbiology Annual Scientific Meeting 2024), our laboratory's diagnostic testing strategy detailed in **Publication 7,** describing the high *N. gonorrhoeae* confirmatory rate that can be achieved with a separate extraction for RP-GC, with selected samples tested with Xpert (296, 297). Here, reasonable attempts to improve assay specificity have been achieved with dual-target commercial screening and supplemental assays, but it appears we are still challenged with the biology of the organism. The propensity for genetic exchange between the *Neisseria* species is still causing diagnostic errors. For example, a few NNN group members, including myself, are currently investigating a number of cases of false positive results or misidentification of *N. gonorrhoeae* using modern screening assays (298). These include:

a) Two independent occurrences of a non-gonococcal *Neisseria* species from a urine and a throat swab, both cross-reacting with a commercial screening assay
b) A cultured *N. meningitidis* isolate from a urine which harbours a *N. gonorrhoeae opa* gene, which was detected with a commercial screening assay
c) A cultured *N. meningitidis* isolate from a urine which harbours a *N. gonorrhoeae* 16S rRNA gene, which was detected with a commercial screening assay

These cases highlight several important ongoing challenges with the *Neisseria* species group for modern *N. gonorrhoeae* screening assays. The first example confirms the issue of screening assay non-specificity leading to false-positive diagnoses. We highlighted and predicted the potential of these false positive results for cobas NG in **Publication 5 and 7**, based on the high *N. gonorrhoeae* load in some samples that were negative with supplemental tests. The other examples confirm screening assay non-specificity for *N. gonorrhoeae* due to genetic exchange between *N. gonorrhoeae* and *N. meningitidis*. As such, molecular diagnostic assays that detect more than one genetic target for

*N. gonorrhoeae* are favourable to reduce the likelihood of issuing a false positive result caused by gene transfer, or a false negative result caused by target dropout. In the case of cobas NG, a dual target assay targeting a single gene may not be sufficient if this single gene is shared with non-gonococcal *Neisseria* species. To mitigate this, an on-board cobas reflexive supplemental test targeting a separate gene specific for *N. gonorrhoeae* may resolve most screening positive samples, but not all. For example, nonspecific detection with an early $C_q$ is likely to be resolved as a false-positive with a suitably sensitive supplemental test, but nonspecific detection with a late $C_q$ may still go unresolved. To combat this, a dual target screening assay which simultaneously detects two different gene targets at equal analytical sensitivity would be ideal. Next, a second supplemental test (third target) could be used when the initial screening result was only positive for a single target. This could be inbuilt in the assay algorithm and performed automatically. However, for this reflexive testing claim to be more accepted by the scientific community, manufacturers need to disclose all screening and supplemental testing results for each target and not supress them from scrutiny. Therefore, whilst the third-generation instrumentation may stand the test of time regarding throughput and operational efficiency, the "fourth generation" assays are likely to arise from assay re-design on existing instrumentation.

As we have described in this thesis, genetic exchange within the *Neisseria* is one of the biggest challenges for screening assays. However, recent examples of non-specificity with *N. meningitidis* discussed at the NNN meeting highlighted a more pressing and concerning issue: the potential emergence and spread of *N. meningitidis* causing meningococcal urethritis, and potentially other urogenital or extragenital infections, which have previously been reported (299). In addition, there is increasing evidence to support that diverse strains of *N. meningitidis* cause sporadic cases of urogenital and anorectal infection transmission among people who have heterosexual sex and, more so, among MSM (300). A recent review highlighted a particular *N. meningitidis* urethritis clade (US_NmUC), which has unique genotypic and phenotypic features that may increase its fitness in the male urethra (301). In all cases, urethritis caused by *N. meningitidis* is clinically indistinguishable from gonococcal urethritis. However, whilst treatment is universally guaranteed for symptomatic infections, a significant diagnostic challenge remains detecting and treating asymptomatic infection, should it occur. Our local examples of *N. meningitidis* harbouring *N. gonorrhoeae* gene targets were only detected because of negative supplemental test results with recovery and identification of *N. meningitidis* by culture. In other words, unless all these *N. meningitidis* strains harbour *N. gonorrhoeae* gene targets, or when culture is performed, the detection of *N. meningitidis* causing urogenital infections with these commercial assays is futile and may go undiagnosed. As such, what is the diagnostic testing strategy for these transmissible *N. meningitidis* strains in extragenital sites, such as

the pharynx, where *N. meningitidis* inhabits the nasopharynx commensally in about 10% of the population? It may be necessary to include *N. meningitidis* specific targets for these strains in future. For example, a qPCR assay has been shown to detect *N. meningitidis* in urine samples and differentiate US_NmUC from *N. gonorrhoeae* by targeting a single nucleotide polymorphism of the *norB* allele (302). No routine diagnostic approach (other than culture) is currently available for the clinical detection of urethritis caused by *N. meningitidis* and future opportunities exist to fulfil this need. For example, new multiplexing technologies are emerging to improve the capability of high-throughput testing platforms, with the progression towards multi-target syndromic testing (described further below for respiratory testing). Here, these multi-marker assays may be able to simultaneously detect multiple targets for *N. gonorrhoeae* and strains *N. meningitidis* that cause urogenital and extragenital infections. For now, we may have to rely on culture and supporting clinical evidence of infection with *N. meningitidis* and the current molecular screening assays developed for *N. gonorrhoeae.*

As highlighted in **Chapter 3**, future screening assays for *N. gonorrhoeae* could also include AMRs. Our decision to transition from NG-Duplex to RP-GC was driven in-part by the clinical need for ciprofloxacin AMR prediction and targeted use, resulting in improved antimicrobial stewardship. However, although prediction of ciprofloxacin resistance by PCR is reasonably reliable and uncomplicated targeting a single mutation, extended spectrum cephalosporin AMR (cefixime and ceftriaxone) in *N. gonorrhoeae* is complex, with up to nine verified mutations in *penA* contributing to resistance and several additional mutations enhancing cephalosporin minimum inhibitory concentrations (84). This complexity has so far prevented the development of rapid and reliable PCR assays for extended spectrum cephalosporin AMR prediction. There have been numerous in-house molecular and NGS assays published for many *N. gonorrhoeae* AMR determinants. For example, Golparian and Unemo (2022) have presented an expert review of the current status and future prospects of antimicrobial resistance prediction in *N. gonorrhoeae* (176). Here, the authors provide a comprehensive review of published NAATs for AMR prediction in *N. gonorrhoeae* for ciprofloxacin, azithromycin, cefixime, ceftriaxone, penicillin and spectinomycin. While a detailed overview is not be presented in this thesis, it is worth mentioning that the short test turnaround time offered by qPCR may lead the way in routine AMR prediction. As these molecular assays become increasingly implemented in clinical laboratories and used for AMR surveillance, they will inform treatment guidelines and guide individualised gonorrhoea treatment for patients. These qPCR assays may progress to rapid POC systems. A very promising rapid, low-cost and portable magnetofluidic platform (PROMPT) was evaluated in 2021, which included *N. gonorrhoeae* detection (*opa*) and *gyrA* for ciprofloxacin prediction (303). This platform was tested on penile swab samples from sexual health clinics in the USA (*n* = 66) and Africa (*n* = 151) with an overall sensitivity and specificity of 97.7% and 97.6%, respectively, for *N. gonorrhoeae* detection and 100% concordance

with culture results for ciprofloxacin resistance. However, no further reports of this assay have been published. For now, it appears that traditional qPCR tests without AMR prediction are likely to lead the way with POC detection of *N. gonorrhoeae* in the first instance, then may progress to include AMR markers in the future. For example, the Xpert CT/NG test was the first genetic POC test and was first reported in 2013 by Australian collaborators and was investigated for analytical sensitivity and specificity for *N. gonorrhoeae* isolates and a nongonococcal *Neisseria* species cross-reactivity panel (73). Over time, this collaborative group expanded and piloted this test for Indigenous Australians in remote primary health services in the Test Treat and Go (TTANGO) trial, in a collaboration with a number of health services and research facilities, including Flinders University International Centre for Point-of-Care Testing (304, 305). Today, Australia has a total of 72 operational sites with 24 in WA (306).

In **Publications 5-7,** we have also shown a high-degree of sensitivity and specificity of Xpert CT/NG and demonstrated its usefulness as supplemental test. Our laboratory plans to trial Xpert CT/NG against newer molecular POC tests in a metropolitan sexual health clinic. Currently, there are several POC tests available for *N. gonorrhoeae* detection and other STI's (307, 308). It is interesting to note that SpeeDx developed the ResistancePlus MG assay for detection of *Mycoplasma genitalium* (MG) and macrolide resistance, then partnered with Cepheid to develop this assay in a GeneXpert cartridge form called ResistancePlus MG FleXible (309). Our laboratory has been using this assay not as a POC test but as a supplemental test for MG-positive samples from cobas MG screening. It will be interesting to see whether the same partnership continues combining Xpert CT/NG and SpeeDx's *gyrA* for ciprofloxacin, including other AMR's such as azithromycin and/or ceftriaxone. If so, such an assay would have significant advantage over the latest rapid POC assays that lack AMR prediction. For example, the cobas Liat for CT/NG/MG was recently cleared by the Food and Drug Administration in the United States of America with a 510(k) clearance and Clinical Laboratory Improvement Amendments of 1988 (CLIA) waiver (the equivalent of the Therapeutic Goods Administration POC test registration in Australia) (310). This test meets many of the criteria of a POC test that were first described in 2004 with the ASSURED criteria (Affordable, Sensitive, Specific, User-friendly, Rapid/Robust, Equipment-free, Delivered or accessible to end-users) (311). This POC test is capable of results in 20 minutes but does not include AMR prediction. However, in a recent abstract, the cobas Liat CT/NG/MG assay has demonstrated good clinical performance in a clinical evaluation for urogenital samples with >97% specificity and ≥95% sensitivity, except in female urine (CT 87.0%, NG 93.1%, MG 78.9%) (312). I envisage a prospective head-to-head POC comparison of Xpert and Liat, including an analytical comparison assessing sensitivity, specificity and the limit of detection would be a worthy study.

As described in my recent review, the diagnostic challenges for *N. gonorrhoeae* detection or detection of other *Neisseria* species causing STI infections, including AMR, remain a complex challenge for the future (80). Given my prior published work setting the benchmark for the measurement of *N. gonorrhoeae* assay specificity and our *N. gonorrhoeae* AMR surveillance (79, 158, 161), our laboratory is well-placed to compare new screening assays, supplemental tests and molecular predictors of AMR. Combined, the work presented in this thesis provides renewed insight into the diagnostic challenges for *N. gonorrhoeae* detection and detection of other *Neisseria* causing STI infections, including AMR, and will help inform future development to enhance patient management.

## 5.3. SARS-CoV-2

This final discussion concludes with SARS-CoV-2, five years after the beginning of the COVID-19 pandemic. It is important to reflect on the events that have occurred during that time, the lessons learnt and the knowledge that has been gained. For the most part, our laboratory implementation of molecular SARS-COV-2 testing was relatively straight forward. As with many other laboratories, our laboratory was only performing rapid viral respiratory testing at the time (Xpert), and we were relatively unencumbered in terms of adding a new viral target into our existing high-throughput PCR workflows. In this regard, I viewed SARS-CoV-2 as another new standalone viral target to be validated and implemented, using a combination of one or more of our high-throughput extraction and PCR platforms. However, the speed in which our SARS-CoV-2 tests were implemented and the pressures that were met were profoundly different. For example, personal safety was the primary concern during method validation and routine testing and many new laboratory safety practices were implemented. In addition to PPE, staff were segregated into separate working groups to reduce contact and prevent transmission, and other aspects such as social distancing, limitations on the number of people using common facilities were implemented. I consider these laboratory aspects of responding to a new infectious disease of unknown safety risk fundamental to our training and good laboratory practice in an infectious disease testing laboratory.

The first lesson learnt was the unforeseen and unprecedented demand for all laboratory consumables and our reliance on supply chains. The impact of the lack of PPE had a dramatic effect, as our personal frontline defence was under threat and there were growing concerns among staff about personal safety. I admit that the thermal treatment of samples prior to SARS-CoV-2 testing was unorthodox, but not in a way that is contrary to what was already known about the thermal inactivation for other coronaviruses. Nor was thermal treatment contrary to the historical use of heat to inactivate microorganisms or facilitate lysis prior to nucleic acid extraction. It was unorthodox because the viral

inactivating effects of our chosen temperature were not experimentally verified and the effects of thermal treatment on assay sensitivity were not known to a high degree of certainty. As such, both were open to criticism. We could have tested the effectiveness with cell culture in the physical containment level-3 laboratory at QEII, however this would have been impractical due the amount of work required, and the time required to complete. Culture has limitations as well in terms of sensitivity and variability of culture, especially at low vial titres, the matrix evaluated, the duration of cell culture incubation, and the different experimental methods of viral recovery (196-200). At the time it was inconceivable that SARS-CoV-2 would behave differently compared to other related coronaviruses regarding thermal protective mechanisms, based on the premise of the efficacy of thermal inactivation for SARS-CoV-2 and other coronaviruses by others. Reports of thermal treatment applied to clinical samples prior to laboratory testing have since been reported (224, 313, 314). Thermal treatment has been used to effectively decontaminate PPE (315, 316) and samples for SARS-CoV-2 quality assurance programs (317). However, since 2023 there has been a lack of reports of thermal treatment for SARS-CoV-2 prior to diagnostic testing, with only a few reports optimising thermal treatment methods (317, 318). The lessons learnt with thermal inactivation and the impact for future preparedness, is that potentially any pandemic respiratory virus in virus transport media, exposed to the same temperature and duration, is likely to demonstrate similar results qualitatively and quantitatively, compared to SARS-CoV-2. In this regard, our laboratory and others can rapidly respond and quickly confirm the utility of such a method for a new viral etiologic agent, using our published experimental templates, methodology and statistical analysis as for SARS-CoV-2. We no longer perform thermal treatment due to cessation of cobas SARS-CoV-2 testing in November 2022 and now perform testing with Plex, which is a more cost-effective assay which we have combined with other viral respiratory targets. However, my publications regarding thermal treatment for SARS-CoV-2 serve as a reminder the effectiveness of this simple technique and the necessary steps required to validate and measure the effects of thermal treatment on assay test performance.

All laboratories should be more prepared for future pandemics given our collective experience with COVID-19. Diversification of testing solutions and being able to pivot from one platform to another is essential to spread the risk of supply issues with a particular test. This was emphasised in **Publication 8** as we rapidly moved from LightMix to cobas, then progressed to synergistic testing using cobas and Plex (**Publication 10**). Whilst it was unnecessary for our laboratory to pool samples, we need to strongly acknowledge the method of sample pooling in response to the shortage of reagents and to cope with the sheer number of tests laboratories were faced with. However, there were new challenges faced with pooling. Some laboratories lacked instrumentation to automate pooling, requiring manual pooling and testing stages with various sizes of pools (3-48 samples) studied (319).

In addition, these methods had to respond quickly to the change in the pool size. For example, Daniel et al. proposed the use of pooled testing strategy of ≤10 could be used when the expected prevalence was <1%, and a pool of ≤5 when the expected prevalence was <5%, according to data from validation studies (319). For future preparedness it is essential that we develop nimble automated sample pooling and molecular diagnostics systems, as developed and evaluated recently for SARS-CoV-2 (320). Depending on prevalence, required testing capacity and turnaround time, the appropriate testing platform and appropriate pool size could be quickly modified. Such systems will have the up-front capability and will respond well to future pandemics. Regardless, we add to the literature the impact of our direct PCR approach on throughput and sensitivity, which may enable laboratories with limited access to consumables or the inability to efficiently pool samples, an adequately sensitive approach for future pandemics.

In response to the testing demand in WA, the cobas and Plex methods performed well for sustained and surge testing events. The extraction-free method was developed for additional testing capacity after the WA borders opened, albeit at reduced sensitivity. The sensitivity difference is due to the concentration effect of extraction-based methods. Some form of enrichment or selective concentration prior to an extraction-free method would balance this difference. I am unaware of a method that has successfully achieved this for SARS-CoV-2 in a laboratory-based PCR for clinical diagnosis with comparable or better sensitivity than extraction-based methods. If such a selective concentration method existed, it could be applied to extraction-based methods, setting new benchmarks for analytical sensitivity. Despite extraction-free methods not exceeding the sensitivity of extraction-based methods, many in-house and commercial extraction-free methods have been published for respiratory samples (including saliva), using various qPCR and non-amplification technologies, promoting rapid results at reduced operating costs, with some adding high-throughput capability (321-328).

Most of these methods compare their extraction-free approach to extraction-based methods, focusing on $C_q$ comparisons, are not standardised (230). Five years after the pandemic, the focus has shifted to simple, ultrafast, non-invasive saliva testing for POC or resourced-challenged jurisdictions (321, 322, 325, 326, 328). A recent technological advance in the saliva POC space is a 4-parameter clinical assay using Electric Field Induced Release and Measurement (EFIRM) technology to simultaneously assess SARS-CoV-2 infection (RNA detection), nucleocapsid antigen, binding antibody and neutralizing antibody levels, from a drop of saliva, with performance that equals or surpasses other direct methods of saliva PCR or serology (328). This one-stop-shop approach combining Reverse Transcription Loop-Mediated Isothermal Amplification (RT-LAMP) and serology, could be used for mass screening to simultaneously address SARS-CoV-2 infection and immunity and be used for

potential future pandemics. The system utilises a desk-top EFIRM reader and costs $5.00 USD for the RT-LAMP consumables and $25.00 USD for the antigen, antibody and neutralising antibody components (328). The sensitivity of this test for SARS-CoV-2 RNA is around 50 copies/reaction, which is comparable to cobas and Plex as described in **Publication 10**. This assay would have been useful addition at the PathWest Murdoch drive-through clinic for mass screening, as we were evaluating a rapid non-molecular POC test at this clinic for saliva at the time. Here, I was a study investigator and coauthored a publication evaluating the Virulizer system (Alcolizer Technology, Balcatta, WA), comprising of a rapid antigen test lateral flow strip with a programmable test-specific electronic chip, read by the hand-held Virulizer instrument (329). We compared the Virulizer results to routine cobas SARS-CoV-2 testing, including quantitative PCR as described in **Publications 11 and 12**. When compared to cobas, the Virulizer produced some false-positive results and a false-negative result at 4.7 $\log_{10}$ IU/mL. These examples highlight where a sensitive molecular component to POC testing is advantageous. Although this study was small, deploying a rapid POC screening test in a drive-through clinic was a valuable experience for the future. The drive-through clinic, coupled with rapid testing, offers a scalable and efficient mass testing methodology adaptable for other infectious disease outbreaks. Our experience underscores the importance of this type of POC testing strategy in public health emergencies, enabling a faster response to future pandemics.

As noted throughout this thesis, quantitative PCR is a valuable tool for method comparisons, assay development and standardisation. The ongoing demand for in-house and commercial SARS-CoV-2 PCR testing assays means standardisation challenges will persist. Furthermore, $C_q$ values and their interpretation has extended beyond the laboratory into clinical settings. The significant $C_q$ difference between Plex and cobas for a given sample highlights the issue of misinterpretation and the need for assay calibration and education for healthcare professionals interpreting $C_q$ values. The ultimate solution is to use a single standard calibrator across all assays and implement a standard procedure for each test to ensure consistent analysis. This could involve harmonising the interpretation of $C_q$ values from one assay to another or reporting viral load results calibrated to a standard. This single standard approach was performed in **Publication 10**, harmonising cobas and Plex using a widely available commercial standard, and progressed to an international standard as demonstrated in **Publication 11 and 12**. A recent multisite study assessed $C_q$ values for SARS-CoV-2 generated across several commercial PCR assays, reaffirming the need standardised control material (330). The authors demonstrate the inappropriateness of using $C_q$ values without established quantitative standards. It is important to note that this study was performed prior to the establishment of the IS and used control material quantified using droplet-based digital PCR (ddPCR). This same technique was used by the manufacturer to calibrate the copy number of the SARS-CoV-2 targets in the ExactDx standard we

used in **Publication 10**. Therefore, standards that are calibrated by ddPCR can be used with confidence in inter-laboratory comparisons if the same standard is used.

However, standardised control materials face issues concerning calibration and supply. The regulatory landscape and the importance of diagnosing COVID-19 mean that demand for the IS will continue, necessitating continuity in both unitage and availability. With commercial NAAT-based platforms on the market, there has been high demand for the 1st IS, with stocks nearing depletion. Therefore, new calibrated materials are periodically required, which require a significant work-effort to recalibrate. Consequently, the 2nd WHO IS for SARS-CoV-2 RNA (22/252) (2nd IS) has been prepared as like-for-like replacement of the 1st IS. It is composed of an inactivated pre-variant of concern (VOC) isolate of SARS-CoV-2 (BetaCoV/Australia/VIC01/2020) (331). The collaborative study included a panel of five inactivated SARS-CoV-2 VOC, to demonstrate harmonisation across variants and recruited a broader range of participants to capture the wide range of molecular technologies now commercially available. Although the dominant circulating variants have been under the Omicron lineage since early 2022, the authors emphasise the importance of maintaining continuity in IU with the level of genetic diversity within circulating strains. This, along with the requirement for molecular diagnostics to detect all sequences, did not support transitioning to a more recent strain. Performance was assessed alongside the 1st IS with the five variants, with only a 0.01 $Log_{10}$ IU/mL difference in the mean potency for the candidate 2nd WHO IS relative to the 1st IS (331). Therefore, users calibrated to the 1st IS can continue to use their own calibrator to reduce the demand on the 2nd IS. Regardless, our laboratory continues to use IU/mL to the 1st IS where clinically necessary to better define parameters such as analytical sensitivity/limits of detection of our evolving assays.

This thesis concludes with exciting developments in new multiplex technologies. SARS-CoV-2 RNA testing is now embedded into routine multiplex assays with other respiratory viruses. As with STI testing, new multiplex technologies are being developed for high-throughput testing assays and platforms, enabling large-scale respiratory virus testing at reduced operational costs. Examples include Temperature-Activated Generation of Signal (TAGS) by Roche Diagnostics (332, 333) (video link) and PlexPlus by SpeeDx (334, 335) (video link). Both technologies double the multiplexing capability for each optical channel in a thermal cycler compared to standard qPCR. For instance, the SpeeDx RespiV PlexPlus (RespiV) assay detects 14 viral respiratory pathogens in a single PCR reaction. We have evaluated RespiV against 2000 consecutive respiratory samples that were prospectively tested with BioFire Respiratory Panel 2.1 *plus* (BioFire-RP) (Pryce et al., 2025, manuscript in preparation) (336). We confirmed a high degree of concordance between the two assays and demonstrated a considerable reduction in consumable costs compared to the BioFire-RP, albeit with an increased test turnaround time. Alternatively, the Roche cobas Respiratory Flex TAGS assay (R-Flex)

(332) can detect the same viral pathogens as BioFire-RP. This test can be performed on the cobas 6800 instrument with the ability to perform more than 1400 tests in 24 hours with a sample-to-result workflow. These new technologies offer high-throughput multi-marker capacity to molecular microbiology, not just for respiratory PCR, but may include meningitis, encephalitis, gastroenteritis, joint infections, septicaemia and blood cultures in the future. Such testing panels have already been developed for the laboratory and near-POC using the sample-to-result BioFire system (337). In this regard, cobas TAGS and PlexPlus technologies are catching up to BioFire in terms of the number of pathogens and closing the gap on test turnaround with reduced operational costs. However, the BioFire-RP panel includes other respiratory targets that RespiV and R-Flex does not, such as *Bordetella pertussis*, *B. parapertussis*, *Chlamydia pneumoniae* and *Mycoplasma pneumoniae.* As such, BioFire-RP is a more comprehensive panel for syndromic testing. The success of the BioFire system has led to the further development by the manufacturer. The SPOTFIRE POC system is small, portable, and has the remarkable ability to detect the same pathogens in 15 minutes compared to 50 minutes for BioFire-RP (337). SPOTFIRE uses similar cartridges and same nested PCR approach, but with faster thermal cycling ramp rates and improved instrumentation. The SPOTFIRE Respiratory panel was shown in a recent study to be highly concordant to the BioFire-RP assay (338). In the future, high-throughput and POC assays will continue to compete in terms of targets and cost. However, laboratories that can effectively use both synergistically will offer the greatest benefit to patients, clinicians and hospitals they serve.

In conclusion, the science of microbiology as a discipline has similar challenges to the forensic sciences. Both fields involve ruling-in and ruling-out potential suspects to identify causative agents. Both focus on qualitative detection (sensitivity) and distinguishing one entity from another (specificity), often in environments filled with bystanders (commensals) and potential contaminants (environmental organisms). Microbiology has other challenges such as quantifying the target (quantitation) and determining the necessary chemicals to selectively kill the pathogens present (antimicrobial therapy). Historically, PCR assays were low throughput and used selectively to detect a limited number of individual pathogens. Today, PCR technologies are high throughput and multiplexed, with targets bundled together in syndromic tests to detect a wide variety of pathogens. The most promising automated PCR systems offer a sample-to-result workflow at a reasonable price, with the greatest number of targets, either separately or multiplexed, whilst maintaining a high degree of clinical sensitivity and specificity. These assays will always include pathogens that are detected qualitatively and quantitatively. However, future assays that include AMR markers will be more clinically advantageous compared to those focusing solely on pathogen detection. These new PCR assays require the capacity to respond to new disease associations, the changing biology of organisms,

including progressive AMR development, and the emergence of novel pathogens. As we advance in microbiology, the development and integration of cutting-edge technologies such as NGS, with new innovative methods will be crucial. The future of molecular microbiology lies in its ability to adapt and evolve, much like the organisms it seeks to identify, understand and control. Through these advancements, we can enhance our diagnostic capabilities in the laboratory, at points of care, or even in the home, leading to improved patient outcomes and contributing to the broader goal of improving public health and safety.

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## Appendix 1

Table A1. Table showing the quantitative bucket concept. This table was prepared from original experimental data used to optimise the extraction-free method as presented in **Publication 12 (222)**. Bucket 1 (standards treated with proteinase K) are compared to Bucket 2 (standards not treated with proteinase K). The total yield can be measured for each bucket and the lower limit compared. Paired samples that are qualitatively positive can also be compared statistically using a non-parametric method of statistical analysis (e.g., Friedman test for repeated measures). An estimate of reproducibility at each dilution can also be assessed. The same method was performed for a) proteinase K compared to no proteinase K, b) different suppliers of proteinase K, c) different proteinase K inactivation temperatures, d) different concentrations of Chelex-100 (data not shown).

| **Standard tested** | **Bucket 1 Proteinase K $Log_{10}$ IU/mL** | **Bucket 2 NO proteinase K $Log_{10}$ IU/mL** | **Difference IU/mL** | **Observation** |
|---|---|---|---|---|
| COV19 STD | 7.24 | 6.81 | 10,921,466 | **Higher yield with Proteinase K** |
| COV19 STD | 7.40 | 7.02 | 14,647,579 | |
| COV19 STD | 7.49 | 6.89 | 23,140,483 | |
| COV19 STD | 7.46 | 6.97 | 19,507,772 | |
| COV19 STD 10-1 | 7.43 | 6.81 | 20,458,806 | **Higher yield with Proteinase K** |
| COV19 STD 10-1 | 7.40 | 6.97 | 15,786,321 | |
| COV19 STD 10-1 | 7.40 | 6.93 | 16,607,484 | |
| COV19 STD 10-1 | 7.34 | 7.06 | 10,396,080 | |
| COV19 STD 10-2 | 6.50 | 5.85 | 2,454,332 | **Higher yield with Proteinase K** |
| COV19 STD 10-2 | 6.44 | 5.76 | 2,178,789 | |
| COV19 STD 10-2 | 6.55 | 6.03 | 2,476,615 | |
| COV19 STD 10-2 | 6.55 | 5.73 | 3,011,102 | |
| COV19 STD 10-3 | 5.29 | 4.44 | 167,442 | **Higher yield with Proteinase K** |
| COV19 STD 10-3 | 5.51 | 4.70 | 273,475 | |
| COV19 STD 10-3 | 5.55 | 4.23 | 337,831 | |
| COV19 STD 10-3 | 5.34 | 4.75 | 162,542 | |
| COV19 STD 10-4 | 4.19 | Negative | 15,488 | **Higher yield with Proteinase K** **Lower limit of detection with no Proteinase K** |
| COV19 STD 10-4 | 4.28 | Negative | 19,055 | |
| COV19 STD 10-4 | 3.75 | Negative | 5,623 | |
| COV19 STD 10-4 | 4.03 | Negative | 10,715 | |
| **Bucket sum** | 215,426,658 | 72,847,658 | 142,579,000 | |
| **Repeated measures** | ***p*-value < 0.00001 (significant *p* < 0.05)** | | | |

## Appendix 2

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Figure 5.

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https://commons.wikimedia.org/wiki/File:Blue_computer_icon.svg

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Figure 6, 7, and 8.

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Figure 9.

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Figure 13.

Cotton swab | Free SVG
https://images.app.goo.gl/rncc8VrEgQQh34Tg7

http://openclipart.org/people/pitr/pitr_Syringe_icon.svg

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