CHARACTERISATION OF *STAPHYLOCOCCUS* SPP FROM SOUTH AUSTRALIAN WALLABIES

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Bachelor of Medical Science

Bachelor of Science (Honours)

A thesis submitted for the Degree of Doctor of Philosophy

The School of Biological Sciences
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June 2017

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ABSTRACT

Staphylococcus aureus and coagulase-negative staphylococci are major problems in the hospital environment and are also significant contributors to community-acquired infections in humans and animals. Previous research investigating the prevalence of methicillin-resistant staphylococci has focussed almost exclusively on methicillin-resistant *S. aureus* and/or *S. pseudintermedius* in humans, livestock and companion animals. This is the first study to bring animal health data together with an investigation on the presence, diversity and antibiotic susceptibility of staphylococcal species recovered from apparently healthy captive and free-ranging animals.

Baseline staphylococcal species diversity data is seldom explored with a large portion of studies focussed on the detection and characterisation of *S. aureus, S. epidermidis* and *S. pseudintermedius* from humans and companion animals. Here, we report the presence of 14 staphylococcal species from both captive and free-ranging wallaby populations using standard microbiological culturing and molecular identification methods. Staphylococcal species diversity was greater in captive wallaby populations compared to their free-ranging counterparts. Across the wallabies sampled, common staphylococcal species recovered included *S. delphini, S. succinus*, *S. xylosus and S. warneri.* Single isolates of *S. carnosus, S. cohnii and S. hominis* were also identified.

In tandem with investigating baseline staphylococcal species diversity, antibiotic resistance data is crucial for future surveillance studies. A significant proportion of staphylococcal isolates were found to be resistant to first generation penicillins however resistance against other antimicrobial classes was common. Interestingly, multidrug resistant staphylococci were recovered exclusively from free-ranging wallabies and *S. aureus* appears to be a benign member of the wallaby nasal microbiome with minimal demonstrable ability to resist antibiotic challenges.

The staphylococcal cassette chromosome SCC*mec*, which harbours the methicillin-resistance gene element, is integral in the characterisation of methicillin-resistant staphylococcal isolates. Typing of this element revealed the presence of both hospital and community acquired elements in addition to novel

subtypes. This represents the first report of methicillin-resistant staphylococci in wallabies in Australia and findings suggest SCC*mec* carriage is unrelated to captivity status.

Epidemiological studies have shown global dissemination of successful clones of methicillin-resistant and -sensitive *S. aureus* among a diverse host range. The clonal relationships of seven *S. aureus* isolates were investigated via an interrogation of their genomes by DNA microarray profiling and compared against international typing databases. The majority of the clonal complexes identified were primarily associated either with avian hosts or sporadic human and veterinary cases confined to Western Europe. South Australian wallabies neither harbour unique host-specific *S. aureus* clonal complexes, nor did they carry typical “Australian” clones.

This thesis comprises a body of work aimed at furthering our understanding of the wallaby nasal microbiome with respect to staphylococci carriage and is the first study of its kind to be performed in Australia.

DECLARATION

I certify that this thesis does not incorporate without acknowledgement any material previously submitted for a degree or diploma at any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Michelle Chen

ACKNOWLEDGEMENTS

It would not have been possible to complete this doctoral thesis without the help and support of the people around me.

To my former supervisor Professor Amanda Goodman, I thank her for the enthusiasm and support she provided in the first quarter of this journey. I also extend my gratitude to my current supervisor Professor Melissa Brown for allowing me the opportunity to continue my PhD in her laboratory and proof reading the variety of documents a postgraduate student produces in varying states of readiness (abstracts, manuscripts, posters, presentations, administrative reports and this dissertation). Through this experience I have learnt that we both work well under pressure and finite external deadlines.

This work was made possible through the administrative and financial support offered by Flinders University as a recipient a Flinders Science and Engineering Research Award. I am indebted to the Field Naturalists Society of South Australia's Lirabenda Endowment Fund for the awarding of funds to support wet laboratory consumables and other travel awards which has allowed me to form connections and friendships with domestic and international researchers.

My term as a postgraduate student was greatly enriched by the friendships developed with graduate students, internal and external to Flinders University, Dr John Antiabong, Dr Sylvia Sapula, Dr Mitch Groves, Sarah, Hayden, Jenny, Felise, Mohsen, Jan, David and most of all, Betty for her friendship and lengthy chats. I also take this opportunity to thank Uwe who always had time for morale-boosting chats about experiments.

It's been a long journey and words cannot express my gratitude for the patience and support my partner has shown throughout this time and the sense of relief which will undoubtedly wash over us once this chapter of our lives comes to an end.

CONTRIBUTIONS

I would like to take this opportunity to formally acknowledge the following individuals for their contributions to this doctoral thesis:

Dr Wayne Boardman (University of Adelaide, Adelaide, South Australia) and **Dr Ian Smith** (ZoosSA, Adelaide, South Australia) provided access to captive and free-ranging wallaby nasal samples at Monarto Zoo and the Anangu Pitjantjatjara Yankunytjatjara (APY) Lands. Dr Boardman also provided critical review of manuscripts; Dr Smith permitted me access to the MedARKS animal health database and wallaby health reports (Appendix B and C).

Dr Stefan Monecke (Technische Universität Dresden, Dresden, Germany and Alere Technologies, Jena, Germany) designed, optimised and performed the DNA microarray experiments for determining the *S. aureus* clonal diversity (Appendix E).

Professor Mary Barton (University of South Australia, Adelaide, Australia) for the provision of staphylococcal stains used in SCC*mec* typing.

Miss Amanda Kidsley (Flinders University, Adelaide, Australia) for the initial staphylococcal species isolation from wallabies sampled in the APY Lands.

PUBLICATIONS

Published work arising from data compiled in this thesis

1. **Chen MMS, Boardman WSJ, Smith I, Goodman AE and Brown MH** (2014) "Nasal colonization of *Staphylococcus spp* among captive and free-ranging wallabies in South Australia." *Journal of Veterinary Science and Medical Diagnosis* 3, 1-9 DOI: 10.4172/2325-9590.1000136. (Appendix F)

2. **Chen MMS, Boardman WSJ, Smith I, Goodman AE and Brown MH** (2015) "Characterisation of β-lactam resistance mediated by *blaZ* in staphylococci recovered from captive and free-ranging wallabies." *Journal of Global Antimicrobial Resistance* 3(3) 184-189. (Appendix G)

3. **Chen MMS, Monecke S and Brown MH** (2016) "Clonal diversity of methicillin-sensitive *Staphylococcus aureus* from South Australian wallabies." *One Health* 2 31-32. (Appendix H)

4. **Chen MMS, Boardman WSJ and Brown MH** (2016) "Methicillin resistance gene diversity in staphylococci isolated from captive and free-ranging wallabies." *Infection Ecology & Epidemiology* DOI: 10.3402/iee.v5.31507. (Appendix I)

Abstracts arising from data compiled in this thesis

1. [ORAL] **Chen, MMS** "Antibiotic resistance in native Australian fauna" *Flinders University School of Biological Sciences Postgraduate Conference*, Flinders University, Adelaide, Australia, June 28 - 30 2011

2. [ORAL] **Chen MMS, Kidsley A, Smith I, Boardman W, Goodman AE, Brown MH** "Antimicrobial resistance in South Australian wallabies: captive vs wild". *Becton-Dickson Student Awards Night*, University of South Australia, South Australia, Australia, November 21 2012

3. [ORAL] **Chen, MMS** "Beta-lactam resistance in staphylococci isolated from captive and wild wallabies in South Australia" *Flinders University School of Biological Sciences Postgraduate Conference*, Flinders University, Adelaide, Australia, July 3 - 5 2013

4. [POSTER] **Chen MMS, Kidsley A, Smith, I. Boardman W, Brown MH, Goodman AE** "Antimicrobial resistance in staphylococci from South Australian wallabies: captive vs wild". *ASM 2013*, Adelaide Convention Centre, South Australia, Australia, July 7 - 10 2013.

5. [ORAL] **Chen MMS, Goodman AE, Brown MH** "Antibiotic resistant staphylococci in native Australian wallabies". *Field Naturalists Society of South Australia Annual General Meeting 2013*, University of Adelaide, South Australia, August 14 2013

6. [POSTER] **Chen MMS, Boardman W, Smith I, Goodman AE, Brown MH** "Genetic characterization of methicillin-resistant staphylococci in wallabies". *3rd ASM-ESCMID conference on Methicillin-resistant Staphylococci in Animals: Veterinary and Public Health Implications*, University of Copenhagen, Copenhagen, Denmark, November 4 - 7 2013.

7. [ORAL] **<u>Chen MMS</u>, Boardman W, Smith I, Goodman AE, Brown MH** “Genetic characterization of methicillin-resistant staphylococci in wallabies”. *Becton-Dickson Student Awards Night*, University of South Australia, South Australia, Australia, November 12 2013

8. [POSTER] **<u>Chen MMS</u>, Boardman W, Smith I, Goodman AE, Brown MH** "Prevalence and diversity of antibiotic resistant staphylococci in wallabies”. *Zoonoses in a Changing World: Two Professions, One Health*, Brisbane Convention & Exhibition Centre, Queensland, Australia, July 25 - 26 2014.

9. [ORAL] **<u>Chen, MMS</u>** “Characterisation of antibiotic resistant staphylococci from Australian wallabies”. *Flinders University School of Biological Sciences Postgraduate Conference*, Flinders University, Adelaide, Australia, November 12 – 14 2014

10. [POSTER] **<u>Chen MMS</u>, Boardman W, Smith I, Goodman AE, Brown MH** "Prevalence and diversity of antibiotic resistant staphylococci in wallabies”. *Australian Centre for Antimicrobial Resistance Ecology One-Day Symposium*, The Science Exchange, Adelaide, Australia, June 24 2016.

The presenting author has been underlined

CHAPTER 1

GENERAL INTRODUCTION

1.1 Introduction

Over the last two decades there has been a dramatic surge in the number of multi-drug resistant bacteria, yet paradoxically the number of pharmaceutical companies developing new antibiotics has dwindled during this same period with only four new classes of antibiotics being launched in the last 40 years (Figure 1.1). Cost has been a significant factor contributing to the lack of progress in discovering or creating new classes of antimicrobial agents. It has been estimated that US$70 million is required to conduct phase III clinical trials for a single drug (Cooper and Shlaes 2011).

International agencies such as the World Health Organisation (WHO), European Commission and the World Economic Forum have identified antibiotic resistance as one of the major challenges of the 21st century (Carlet et al. 2014). A global action plan has been endorsed by WHO to tackle the spread and increase in antibiotic resistance (Cooper et al. 2011). In 2014, the USA's Centers for Disease Control (CDC) estimated the annual impact of antibiotic-resistant related infections in the USA to be 20–35 billion dollars in direct health costs, 8 million additional hospital days and 35 billion dollars resulting from lost productivity (PCAST 2014).

Vertebrate animals play host to an immensely diverse population of microorganisms that fulfil a spectrum of niches ranging from exclusively commensal to exclusively pathogenic (Woolhouse and Gowtage-Sequeria 2005a). A portion of these organisms can transfer from their original host species to colonise and infect a second animal species. Organisms that are capable of transferring to, and subsequently cause disease in humans, are known as zoonotic pathogens. It has been estimated that almost 75% of all emerging human pathogens originate from animals with no obvious indication of the taxonomic relatedness between the original and the new host species (Woolhouse et al. 2005a, 2005b).

The unpredictability of pathogen emergence means that the first line of defence has to be effective surveillance. In Australia, the continued operations of the Australian Group on Antimicrobial Resistance, Gram-positive Bacteria Typing Laboratory and Australian Collaborating Centre for Enterococcus and Staphylococcus Species Typing and Research centres provide and maintain valuable sources of baseline antibiotic resistance and *S. aureus* molecular

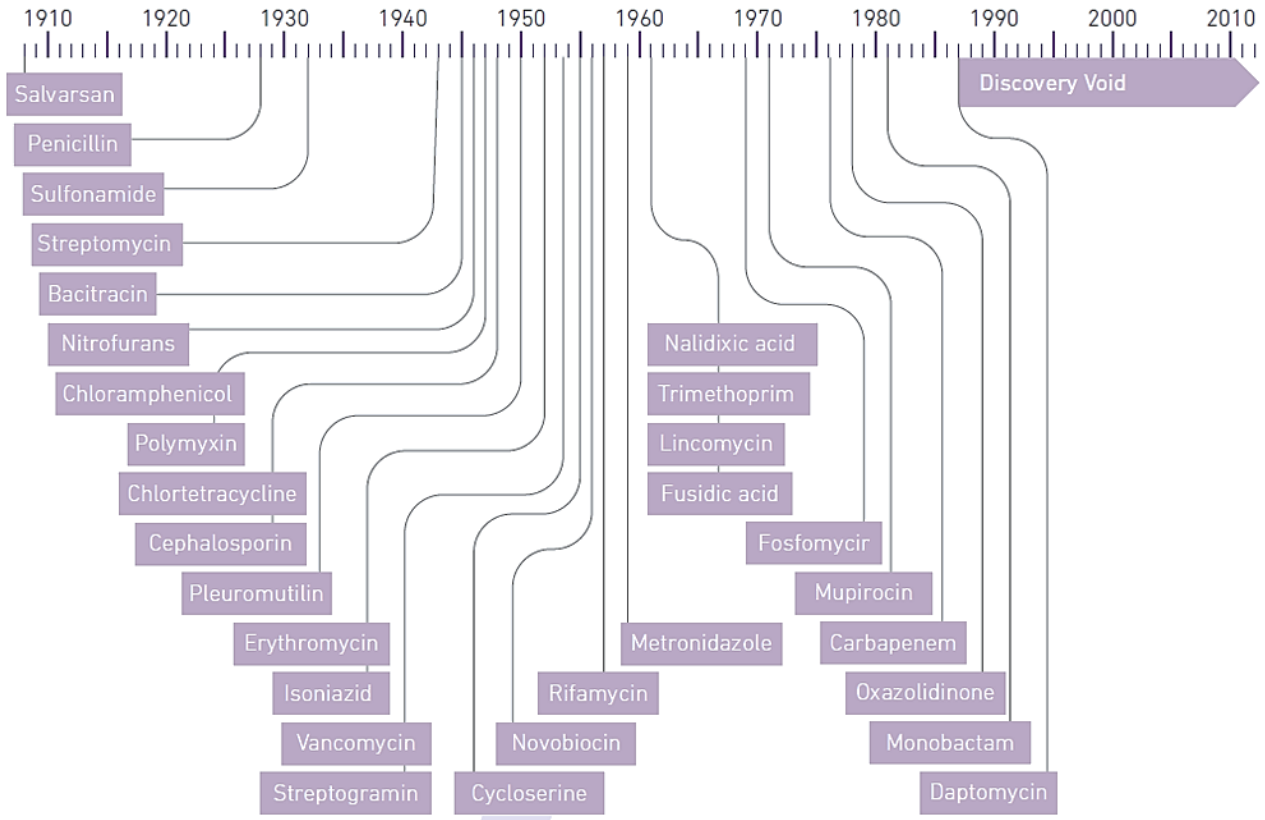


Figure 1.1: Dates of discovery for distinct classes of antimicrobial agents

Timeline depicting the dates of the initial patent or discovery of each antimicrobial class. A discovery void has been present for close to 3 decades.

Figure reproduced from WHO (2014).

typing data for methicillin-resistant *S. aureus* (MRSA) isolates from both human and animals. These laboratories primarily characterise MRSA isolates using multi-locus sequence typing and multiplex PCR (see Sections 1.4.2 and 1.8).

This study will focus on the detection of staphylococcal species recovered from captive and free-ranging wallabies and the characterisation of their antibiotic resistant profiles by biochemical and molecular methods. Specifically, this thesis will focus on the detection of MRSA and other methicillin-resistant staphylococci (MRS). Both MRSA and MRS are prominent examples of antimicrobial resistant bacteria disseminating in both human and animal populations. Currently three types of MRS(A) are recognised (and discussed further in Section 1.9): healthcare-acquired [HA-MRS(A)], community-acquired [CA-MRS(A)] and livestock-associated [LA-MRS(A)] (Purrello et al. 2014).

1.2 Staphylococcus as a pathogen

Staphylococci are Gram-positive organisms that belong to the Staphylococcaceae family in the phylum Firmicutes. The genus *Staphylococcus* is presently divided into 47 species and 24 sub-species (LPSN 2014). Division is primarily based on their ability to coagulate plasma by the enzyme coagulase. This enzyme mediates the conversion of fibrinogen to fibrin resulting in the clotting of blood and has long been associated with pathogenicity (Rayman et al. 1975). Staphylococci with this enzyme are termed coagulase-positive staphylococci (CPS) and include *S. aureus, S. delphini, S. intermedius, S. pseudintermedius, S. lutrae, S. schleiferi* subsp. *coagulans* and some strains of *S. hycius* (LPSN 2014). Of these, only *S. aureus* is a common coloniser of humans; other strains, such as *S. pseudintermedius*, are more readily identified as pathogenic species in dogs (Weese and van Duijkeren 2010). The remaining staphylococci that do not possess the coagulase enzyme are known as coagulase-negative staphylococci (CNS) and make up the majority of the genus. Whilst CPS are regarded as important pathogens, CNS have historically being described as benign commensal organisms (Pulverer and Pillich 1971). Today, CNS represent one of the major nosocomial pathogens in human and animal medicine (Huebner and Goldmann 1990, Becker et al. 2014b), particularly to dairy cattle (Piessens et al. 2011).

1.2.1 Carriage of staphylococci

It has been estimated that approximately 30% of the human population are persistent asymptomatic nasal carriers of *S. aureus*, transient carriers make up approximately 60% of the population and MRSA carriers comprise <1-15% of the population (Gould and Chamberlaine 1995, Kennedy and Deleo 2009, Cohn and Middleton 2010, Graves et al. 2010, Tang and Stratton 2010). However, certain populations in the community, such as those with insulin-dependent diabetes, long-term indwelling catheters and injecting drug users have been found to have *S. aureus* nasal carriage rates up to 50% (Tang et al. 2010).

The nasal passages are considered to be the most important colonisation site for *S. aureus* in humans, because the elimination of *S. aureus* from the nares results in the subsequent disappearance of the organism from other sites of the body (Tang et al. 2010). In addition, individuals with *S. aureus* nasal colonisation have a higher risk of infection with the same strain in other body sites (Safdar and Bradley 2008, Kennedy et al. 2009, Datta et al. 2013). Other body sites such as the hands, perineal region, skin wounds, throat, genitourinary tract and the digestive tract can all be colonised with *S. aureus* (Sanford et al. 1994).

1.2.2 Staphylococci can cause a wide range of infections

Staphylococci, in particular *S. aureus*, have been referred to as ‘persistent pathogens’ due to its high prevalence over many decades and its causation of many diseases. Clinical infections can be associated with skin and soft tissue, bone and joint, heart valves, blood stream and cerebrospinal fluid (Stevenson and Wang 2014).

This causation of disease in animals was first noted in 1914 by two research groups, where firstly (Nicolle and Césare 1914) from the Pasteur Institute reported that staphylococcal strains from infected farm animals closely resembled cultures from human lesions. The second paper attributed sporadic bouts of gastroenteritis to visitors that had visited a farm in the Philippines to the presence of staphylococci in the milk of a specific cow (Barber 1914). Since then, numerous reports have identified staphylococci as an aetiological agent of infections in cattle (Devriese et al. 1972, Sawant et al. 2009, Feβler et al. 2010), poultry (Bertolatti et al. 2003, Bystron et al. 2010), pigs (de Neeling et al. 2007, Neela et al. 2009, Larsen et al. 2012), domestic cats and dogs (Davis et al.

2014, Harrison et al. 2014, Loncaric et al. 2014b), and various wildlife (Gomez et al. 2014).

1.3 Staphylococcal identification

Bacterial identification can be broadly grouped into two categories, phenotypic and genotypic. Phenotypic identification involves biochemical assays, evaluation of bacterial growth in different media and/or determining what sugars it is able to utilise for growth. These methods are laborious, time- and resource-intensive and the results are subjective in nature. Genotypic identification usually involves screening isolates for specific genes, sequencing and comparing the resulting amplicons to global databases.

1.3.1 Phenotypic identification

The ability to accurately and efficiently identify organisms is imperative to the function of both clinical and research laboratories. However, many phenotypic identification kits demonstrate poor performance when applied to animal isolates. These kits employ a number of differential biochemical and metabolic tests and perform well in identifying human staphylococcal isolates, although limitations have been reported (Cunha et al. 2004). Due to their convenience and ease of use, these commercial systems have commonly been used for the speciation of staphylococci from animals. However, these kits are not designed to identify all organisms of interest to the veterinary or research microbiologist. Within the staphylococcal genus, the major pathogen *S. aureus* can be discriminated from CNS by its pigmented, coagulase-positive and DNase-positive phenotype (Rayman et al. 1975). Identification of closely related CNS species using phenotypic kits can be difficult, if not impossible, as species identification is based on a limited number of phenotypic traits which can be expressed at variable intensities among different strains within the same species (Heikens et al. 2005).

1.3.2 MALDI-TOF

More recently the use of mass spectrometry, in particular matrix-assisted laser desorption ionisation-time of flight mass spectrometry analysis (MALDI-TOF), has been used to accurately identify bacteria and yeast isolates. This method is highly discriminatory as it generates unique fingerprints based on

analysing the protein fractions of entire cells as opposed to conventional methods which analyse genomic DNA alone (Hsieh et al. 2008).

Two main approaches exist for using MALDI-TOF for bacterial identification, library-based and bioinformatics-enabled. The library-based methodology compares the spectra of an unknown bacteria to a collection of reference strains, similar to traditional methods utilising 16S ribosomal RNA (rRNA) gene sequencing and is the most commonly used approach (Sandrin et al. 2013). However, the accuracy of the bacteria identified is inherently underpinned by the quality and accuracy of the data stored in the library. The bioinformatics-enabled approach addresses concerns surrounding spectra reproducibility and coupled with the availability of an increasing number of fully-sequenced bacteria genomes, some investigators have opted to identify bacteria by identifying peaks in the MALDI-TOF profiles to particular proteins from data available in public databases (Sandrin et al. 2013)

In the clinical and diagnostic laboratory, commercially available systems complete with a built-in library are becoming popular. These laboratories report a long term cost and time saving when using MALDI-TOF compared to traditional methodologies. As an example, one Australian laboratory in New South Wales, recently compared the cost of traditional methodologies with MALDI-TOF. Costs involved were a function of the complexity of tests required and ranged from AUD$1.55 (staphylococcal latex and DNase only) to AUD$31.76 (organisms requiring conventional tube biochemistry and/or serological confirmation). Furthermore, the costs associated with identifying a coliform using VITEK2 was reported to be AUD$10.00. By comparison, the total consumable and labour cost for the identification of a single isolate by MALDI-TOF was AUD$0.45 with results available up to 20 hours earlier than conventional assays (Neville et al. 2011).

Of particular importance to staphylococcal identification, most commercial systems provide differentiation between *S. aureus* and CNS but are unable to accurately identify CNS. MALDI-TOF has been shown to not only be capable of differentiating between staphylococcal species (Dubois et al. 2010) but to be significantly better (93.2%) than currently available staphylococcal identification systems such as BD Phoenix (75.6%) and Vitek-2 (75.2%) for speciating CNS species (Croxatto et al. 2012). Very recently it has become possible to use

MALDI-TOF to accurately identify and discriminate between *S. aureus* clonal complexes and sequences types including CC5, CC8, CC22 and CC398 (Camoez et al. 2016, Sauget et al. 2016). Identification of CC398 isolates can be achieved in under 10 minutes compared to the standard 2 hours and 30 mins by PCR (Sauget et al. 2016)

1.3.3 Molecular identification

To overcome the constraints associated with phenotypic species identification and initial capital cost of MALDI-TOF, many molecular methodologies have been adopted for CNS differentiation. Further driving the shift towards molecular identification is the rapidly declining cost of DNA sequencing thus making sequence-based methods an attractive alternative to commercially available kits for organism identification with the whole genome sequences of the first two MRSA isolates, N315 and Mu50, resolved in 2001 (Kuroda et al. 2001).

The sequencing of housekeeping genes such as the 16S rRNA gene is the most popular amongst users and has the most extensive database (Clarridge 2004). This popularity stems from its ubiquitous and slow-evolving nature. Other genes used for staphylococcal species identification include the heat shock proteins (*hsp60, cpn60, groL*), beta subunit of RNA polymerase (*rpoB*), superoxide dismutase A (*sodA*), glyceraldehyde-3-phosphate dehydrogenase (*gapDH*), elongation factor Tu (*tuf*) and catalase (*katA*) (Drancourt and Raoult 2002, Kwok and Chow 2003, Ghebremedhin et al. 2008, Blaiotta et al. 2010, Bergeron et al. 2011).

Early studies on the taxonomy of the *Staphylococcus* genus using 16S rRNA gene sequence analysis indicated the existence of species-specific regions within the gene. This observation led to one of the first studies to investigate the subtle differences between staphylococcal species by comparing the patterns present in their 16S rRNA gene in both human and veterinary medicine (Takahashi et al. 1997, Takahashi et al. 1999). A total of nine species-specific regions termed ‘hypervariable’ regions have since been identified in bacterial 16S rRNA genes (Chakravorty et al. 2007). These hypervariable regions are flanked by conserved stretches of DNA in most bacteria, allowing PCR amplification of target sequences using universal primers. Chakravorty et al. (2007) reported that the combination of hypervariable regions 3, 4, 5 and 6

allowed for the amplification and speciation of 110 bacterial species including 11 species of staphylococci of pathogenic interest.

1.4 Molecular epidemiological typing techniques

The relationship between staphylococcal isolates of the same species can be investigated using techniques such as pulse-field gel electrophoresis (PFGE) and multi-locus sequence typing (MLST) whilst sequencing of protein A, *spa* typing, and DNA microarray are specific applications for *S. aureus* identification. These tools allow for the examination of both long-term and short-term epidemiology otherwise known as global and local epidemiology, respectively and a review by Stefani et al. (2012) highlights the benefits of each while pushing for a global harmonisation of typing methods for MRSA.

1.4.1 Pulsed-field gel electrophoresis

PFGE is very useful for detecting genetic variation which accumulates relatively rapidly making it the predominant technique of choice when studying local epidemiology such as in the case of a nosocomial outbreak (Peacock et al. 2002) where high resolving power is required. However, PFGE is marred by issues related to poor reproducibility and the ability to compare data between institutions (Peacock et al. 2002, Melles et al. 2007). However, it is not limited to a specific staphylococcal species and is thus more broadly applicable.

1.4.2 Multi-locus sequence typing

MLST is a nucleotide sequence-based approach that was designed to provide accurate and portable data which is appropriate for local and global epidemiological studies of bacterial pathogens and is regarded as the ‘gold standard’ for population analysis (Mellmann et al. 2008).

This method assesses variations in 450 – 500 bp fragments of seven housekeeping genes (*arc, aroE, glpF, gmk, pta, tpi* and *yqiL*) and compares them to an online database (http://www.mlst.net). This comparison to the online database allows different sequences for each housekeeping gene to be assigned as distinct alleles, and for each isolate, the alleles at each of the seven loci define the allelic profile of sequence or strain type (ST). These ST are unambiguous and place no weight for the number of nucleotide differences between alleles (Enright and Spratt 1999). Groups of similar ST are further grouped into clonal complexes (CC) using the eBURST software. CC are

generally comprised of a single dominant ST with a number of less common close relatives of this ST (Feil et al. 2004).

1.4.3 *Spa* typing

Typing of the 200 bp to 600 bp polymorphic X region of the staphylococcal protein A gene *spa* is a method which is less discriminatory than PFGE, but unlike PFGE, results are highly reproducible and comparable across laboratories. *Spa* typing is usually performed as part of a battery of molecular characterisation assays such as SCC*mec* typing, lineage-specific virulence or resistance gene amplification (Szabo 2014). Results from *spa* typing correlate well with DNA microarray analysis, primarily due to the Based Upon Repeat Pattern (BURP) algorithm. The BURP algorithm groups *spa* sequences with more than five repeats as new *spa* types (Strommenger et al. 2008). It is important to note that it is the composition and organisation of the repeat region rather than the number itself which allows *spa* typing data to be correlated with DNA microarray data (Koreen et al. 2004).

1.4.4 DNA microarray

DNA microarray is a hybridisation test which contains immobilised sequence-specific DNA probes covalently bonded to a substrate. It allows for the detection of a large number of genes in a single assay.

One of the most widely used arrays in the literature for human samples, companion animal, livestock and domestic animal *S. aureus* samples was developed by Monecke et al. (2008). This particular microarray covers 334 target sequences across 170 distinct genes. These genes include *S. aureus* species markers, MLST markers, SCC*mec*, various antibiotic resistance genes, virulence genes and genes encoding microbial surface components recognising adhesive matrix molecules of the host (MSCRAMM) (Monecke et al. 2008, Monecke et al. 2012). The Alere array was modified to detect the new *mecC* element in 2012 and was the first to discover *mecC* in European hedgehogs (Monecke et al. 2013).

Interpretation of the results followed the guidelines developed and implemented by Monecke et al. (2008) and Monecke et al. (2012). Briefly, results were regarded as negatives if the normalised intensity for a given probe was below 25% of the median value of the staphylococcal species markers

(*coa, eno, fnbA, gapA, latA, nuc, rrn, sarA, sbi, spa* and *vraS*) and a biotin staining control. If the normalised intensity of a given probe was higher than 50% of this breakpoint, it was regarded as positive. If it was between 25% and 50% the result was considered ambiguous. A different approach was taken for alleles that differed by a single nucleotide polymorphism (SNP) (*bbp, clfA, clfB* and *fnB*). The allele which elicited the highest intensity was regarded as positive, provided it exceeded the 50% biotin and staphylococcal species marker control sample breakpoints. All others were regarded as ambiguous, or if below the 35% breakpoint, as negative (Monecke et al. 2008).

In this thesis, the Alere StaphType DNA microarray was employed to determine the clonal complexes of all *S. aureus* isolates recovered from wallaby nasal samples using protocols and procedures previously described (see Chapter 5).

1.5 Mechanisms for bacterial gene transfer

Many bacterial lineages have an extensive and ongoing history of gene transfer and loss, as evidenced by the significant differences in genome content even amongst closely related isolates. This transfer of genetic material is known as horizontal gene transfer (HGT) and various mechanisms will be discussed below. Recent comparisons indicate that on average 20% of a bacterial genome is comprised of mobile genetic elements (MGE). In *S. aureus*, these elements include bacteriophages, plasmids, transposons, *S. aureus* pathogenicity islands and staphylococcal cassette chromosomes (SCC) (Lindsay 2010). Whole genome sequencing (WGS) has revealed that MGEs, estimated to account for 20% of the bacteria genome, can vary substantially in *S. aureus* and indicates frequent transfer and loss of entire elements (Polz et al. 2013).

The identification of genes carried on MGEs is important because it reveals the presence of toxin-encoding genes, important resistance and host-adaptation mechanisms. Genes include the Panton-Valentine Leucocidin (PVL) which is associated with primary skin and soft tissue infections (Bouchiat et al. 2015) and observed in the majority of CC1 isolates which includes the first known PVL-positive CA-MRSA, CC5 isolates such as CC5-MRSA-VI, first described as the Paediatric Clone with SCC*mec* IV from Portugal (see Section 1.9.1.2), CC8, a group of pandemic MRSA lineages including both CA-MRSA, HA-MRSA and LA-MRSA amongst others (Monecke et al. 2011).

Studies in *S. aureus* CC398 host adaptation have revealed surprisingly similar genomes between human and animal *S. aureus* CC398 isolates with no single (non-MGE) gene found to be characteristic for human or pig colonisation, however the MGE content of CC398 was highly variable (McCarthy et al. 2011). Some MGE content was varied between certain mammalian hosts indicating that these may be potential candidates for rapid gene loss or acquisition as a strain adapts to a new mammalian host (McCarthy et al. 2011). A recent study has demonstrated extremely high levels of HGT and MGE loss between CC398 isolates in gnotobiotic piglets *in vivo*. Elements such as bacteriophages and plasmids carrying resistance genes were transferred, even in the absence of antibiotics thus highlighting the limitation of relying solely on antibiotic resistance and MGE profiling as a means to differentiate isolates (Stanczak-Mrozek et al. 2015). Interestingly, *S. aureus* clones co-existed and diversified rather than allowing a single clone to dominate and limited transmission was seen between hosts with each animal harbouring unique clone populations (McCarthy et al. 2014). It is clear that MGEs play a significant role in the evolution of new clones adapted to new niches.

1.5.1 Bacteriophages

Lysogenic double-stranded DNA bacteriophages are common in *S. aureus* with some isolates carrying up to four different phage types. At least eight families have been described, each with a unique integrase gene and corresponding insertion sites in the bacterial chromosome (Lindsay 2014). One of the more notable phages is φ3 which is present in *S. aureus* CC398. This phage carries the immune evasion gene cluster encoding chemotaxis inhibitory proteins, staphylokinase, staphylococcus enterotoxins A involved in tempering the immune system to facilitate staphylococcal adaptation to humans (Utter et al. 2014). This cluster was found in all human-invasive isolates but absent from pig isolates, indicating it is not required for pig infection (McCarthy et al. 2011).

1.5.2 Genomic islands

Genomic islands are regions of the chromosome which are of external origin. These regions frequently carry genes related to virulence or toxin genes and are thus termed pathogenicity islands (Ito et al. 2003). Recently, Novick and Ram (2016) published a review which discusses *S. aureus* pathogenicity islands (SaPIs) in great detail. Briefly, these are small stretches of DNA

(generally 15-18 kb) which are preferentially packaged together containing virulence and pathogenicity genes such as *tst*, responsible for causing toxic shock syndrome and the PVL genes (Ruzin et al. 2001, Ito et al. 2003).

SaPIs are capable of autonomous replication only in the presence of a helper phage, one example is SaPI1 and its relationship with phage 80α (Ruzin et al. 2001, Chen et al. 2015a). All SaPIs are flanked by direct repeat sequences encoding integrases and are inserted in a single orientation at a specific site in the chromosome (Mir-Sanchis et al. 2012, Chen et al. 2015a). Currently, six different SaPI insertion sites, each characterised by a 15 - 22 nucleotide sequence, have been identified in *S. aureus* (Mir-Sanchis et al. 2012). SaPIs are present in many successful MRSA lineages such as USA300 and ST398, notable strains include COL, Mu50, N315 and MW2 (Larsen et al. 2009, Schijffelen et al. 2010, Alibayov et al. 2014).

In contrast to these pathogenicity islands, MRS(A) also carry a large genomic island known as cassette chromosome *mec* which preferentially carries genes related to antibiotic resistance as opposed to virulence and is described in detail by Ito et al. (2003) and discussed in Section 1.8.

1.5.3 Transposons and insertion sequences

Transposons (Tn) and insertion sequences (IS) are discrete segments of DNA which can integrate into any site within a genome by illegitimate recombination, i.e. in the absence of extensive DNA sequence homology, and do not need to be ‘carried’ by a plasmid or phage element (Baba et al. 2002).

Transposons carry accessory genes, not essential to normal bacterial cell function, for example antibiotic-resistance determinants (Mahillon and Chandler 1998). Some examples include transposons Tn*554* (erythromycin and spectinomycin resistance), Tn*552* (penicillin resistance) and Tn*5801* (tetracycline resistance) which are particularly relevant to staphylococci as they form components of the SCC*mec* element in MRS (see Section 1.8.2) (Lindsay and Holden 2004).

Insertion sequences are short stretches of DNA which encode functions essential to their own mobility (Mahillon et al. 1998). Such examples IS*431,* IS*1272*, IS*256* or IS*257* contained around the *mec* gene complex are

predominantly associated with antiseptic, aminoglycoside and mercury resistance (Kobayashi et al. 2001).

1.5.4 Plasmids

Plasmids are responsible for the carriage and transfer of a diverse range of antibiotic (aminoglycosides, β-lactams and macrolides); biocide in *S. aureus* (quaternary ammonium compounds) and heavy metal (cadmium) resistance genes (Nucifora et al. 1989, McCarthy and Lindsay 2012a, Marchi et al. 2015).

Staphylococcal plasmids can be classified into three classes based on their size and genes carried (Malachowa and DeLeo 2010). Class I plasmids are small 1 – 5 kb multi-copy (15 – 60 copies per cell) elements such as pUB110; class II plasmids are larger 15 – 30 kb low-copy elements that carry transposable elements containing resistance genes such as pMW2, pN315 and pMu50; class III plasmids are large, generally over 45 kb, conjugative plasmids which carry conjugative transfer *tra* genes and a variety of resistance markers including Tns and IS elements. Formerly, plasmids which were unable to be characterised in any of the aforementioned three classes were placed into class IV (Ito et al. 2003, Lindsay 2014).

Integrated plasmids are a key component of the staphylococcal genome, see Malachowa et al. (2010) for a summary of plasmids and their mechanism of action that can be integrated into the *S. aureus* genome, and SCC*mec* elements with integrated copies of pUB110 responsible for aminoglycoside resistance in SCC*mec* type II and pT181 responsible for tetracycline resistance in SCC*mec* type III. MRSA strains MW2 and N315 carry plasmids (pMW2 and pN315) that encode β-lactamases, the Mu50 plasmid (pMu50) however, carries Tn*4001* encoding aminoglycoside resistance and the efflux-mediated antiseptic resistance gene *qacA* (Ito et al. 2003).

1.5.5 Gene cassettes

Gene cassettes are promoter-less genes with a recombination site, known as the *attC* site. These cassettes can exist transiently in free circular form but cannot move independently and are thus found in association with integrons (gene capture and expression elements) (Partridge et al. 2009).

Of note, it has been found that gene cassettes conferring resistance to clinically redundant antimicrobial agents such as streptomycin and spectomycin

were commonly found to be the last cassette in an array. In comparison, cassettes encoding resistance towards gentamicin and β-lactams appeared in the first or second position of the array (Partridge et al. 2009). Cassettes have also been observed to 'move up' to the first position in an array following exposure to the relevant antibiotic (Rowe-Magnus and Mazel 2002).

1.6 Antibiotic resistance in staphylococci

1.6.1 Staphylococci possesses a broad arsenal of resistance mechanisms

Similar to other bacteria, staphylococci employ multiple mechanisms for nullifying foreign toxic compounds such as antibiotics, disinfectants and antiseptics (Costa et al. 2013). Mechanisms such as efflux mediated systems, antibiotic modifying enzymes and target alteration will be discussed.

1.6.1.1 Efflux-mediated systems

Efflux systems in bacteria can either expel a range of chemically distinct compounds or be target specific. The former are generally known as MDR efflux pumps. These MDR pumps have been classed into six families according to their structure and energy requirements namely, the major facilitator superfamily (MFS); the small multidrug resistance family (SMR); the multidrug and toxic compound extrusion family (MATE); the adenosine-triphosphate binding cassette superfamily (ABC); the resistance-nodulation-cell division superfamily (RND) and most recently, the proteobacterial chlorhexidine efflux (PACE) family (Costa et al. 2013, Hassan et al. 2013). These pumps collectively have the ability to export an immense variety of compounds and in staphylococci include quaternary ammonium compounds such as benzalkonium chloride and cetrimide; dyes such as ethidium bromide and fluoroquinolones such as ciprofloxacin (Paulsen et al. 1996, Costa et al. 2013).

1.6.1.2 Antibiotic modifying enzymes

At the fundamental level, antimicrobial agents are chemical entities that contain a core functional group. This functional group is both responsible for the effectiveness of the antimicrobial agent at inhibiting bacterial growth and is susceptible to hydrolysis. Unsurprisingly, there are enzymes which have evolved to specifically target and cleave these entities as a means of inactivating antimicrobials. Likely to be the first antibiotic resistance mechanism

to be reported, the β-lactamase enzyme from the amidase family remains widely studied in various pathogenic bacterial species (Wright 2005).

β-lactamase enzymes function by cleaving the β-lactam functional group of penicillins through one of two mechanisms: through the action of an active serine site or via the activation of water by a Zn^{2+} centre (Livermore 1998). The former converts the biologically active penicillin into harmless penicilloic acid (Purrello et al. 2014). The latter are known as metallo-β-lactamases and will not be discussed further as they are more prominent in Gram-negative organisms (Bebrone 2007). The genetic regulation of the β-lactamase mechanism will be discussed in Section 1.7.2. Additionally, β-lactamases have been classified based on one of two mechanisms: the functional characteristics of the enzyme or its primary structure (Section 1.7.3).

1.6.1.3 Target alteration

Antimicrobial compounds have been designed to target sites in the bacteria that are vital to their growth and survival. Prominent agents include the β-lactam antimicrobials that target the growing peptidoglycan side chains in Gram-positive bacteria. Other examples include antimicrobials such as aminoglycosides, tetracyclines and macrolides which interfere with protein synthesis. Owing to the critical nature of these systems to cellular function and survival, the organism cannot evade antimicrobial action by simply removing these systems from itself. Bacteria can however, acquire mutations in the intended target region which would lower its susceptibility to inhibition whilst retaining cellular function. Alternatively, organisms can acquire an entirely new structure to compensate for the presence of the antimicrobial agent (Lambert 2005). This is the case with staphylococcal isolates which have acquired an altered penicillin-binding protein (PBP)2a. This protein is up to 500 times less susceptible to the actions of penicillin G (Lu et al. 1999) and can thus continue to assemble the peptidoglycan component of the bacterial cell wall in otherwise lethal concentrations of β-lactam antibiotics. Strains of staphylococci which have acquired this altered penicillin-binding protein are known as MRS.

1.6.1.4 Multi-drug resistant staphylococci

The international bodies of the CDC and the European Centre for Disease Prevention and Control (ECDC) have proposed a standard terminology to be applied when describing resistance profiles in problematic bacteria. Bacteria

that were considered problematic generally were seen to be responsible for causing nosocomial infection and had a tendency to being multi-resistant. Epidemiological and clinically significant antibiotic groups or classes were defined for each bacterial genus or species. MDR was defined as resistance to three or more antibiotic classes. Extensive drug resistance (XDR) was defined as resistance to all except two or fewer classes of antibiotics. The final group was defined as resistance to all antibiotics and is known as pandrug-resistant (PDR). Therefore, a bacterium which is XDR is also MDR, however, an XDR strain is not the same as PDR strain (Magiorakos et al. 2012). In accordance with international bodies and existing literature, in this thesis, MDR is defined as resistance to three of more classes of antimicrobial agents.

1.7 β-lactam antimicrobial agents

β-lactam antimicrobial agents, especially penicillins and cephalosporins, have emerged as drugs of choice to treat many bacterial infections primarily because of their broad spectrum of bactericidal activity and low toxicity to the host (Hubschwerlen 2007). Penicillin was discovered by Alexander Fleming and subsequently introduced into clinical practice in 1940; the chemical structure is shown in Figure 1.2. Fleming discovered that the penicillin mould lysed colonies of *Staphylococcus* on solid media, was generally effective against Gram-positive organisms and showed no activity against Gram-negative organisms in broth culture. Penicillin and other β-lactam antimicrobial agents are structural analogues of the D-Ala^4-D-Ala^5 (Figure 1.3) extremity of the peptidoglycan precursor and act as a suicide substrate for the growing peptidoglycan chain.

1.7.1 Cell wall synthesis in Gram-positive bacteria

The bacterial cell wall is a rigid membrane which both shapes and protects microorganisms from the external environment (Strominger et al. 1959). In Gram-negative bacteria, peptidoglycan polymerisation occurs in the periplasmic space which is shielded from the environment by the outer cell membrane. This outer membrane also effectively excludes some β-lactam antimicrobial agents from entering the cell by porin alteration. These protective mechanisms are not available to Gram-positive organisms thus the peptidoglycan and its associated polymerisation complexes are vulnerable to attack by bactericidal agents such as β-lactam agents (Piessens et al. 2011).

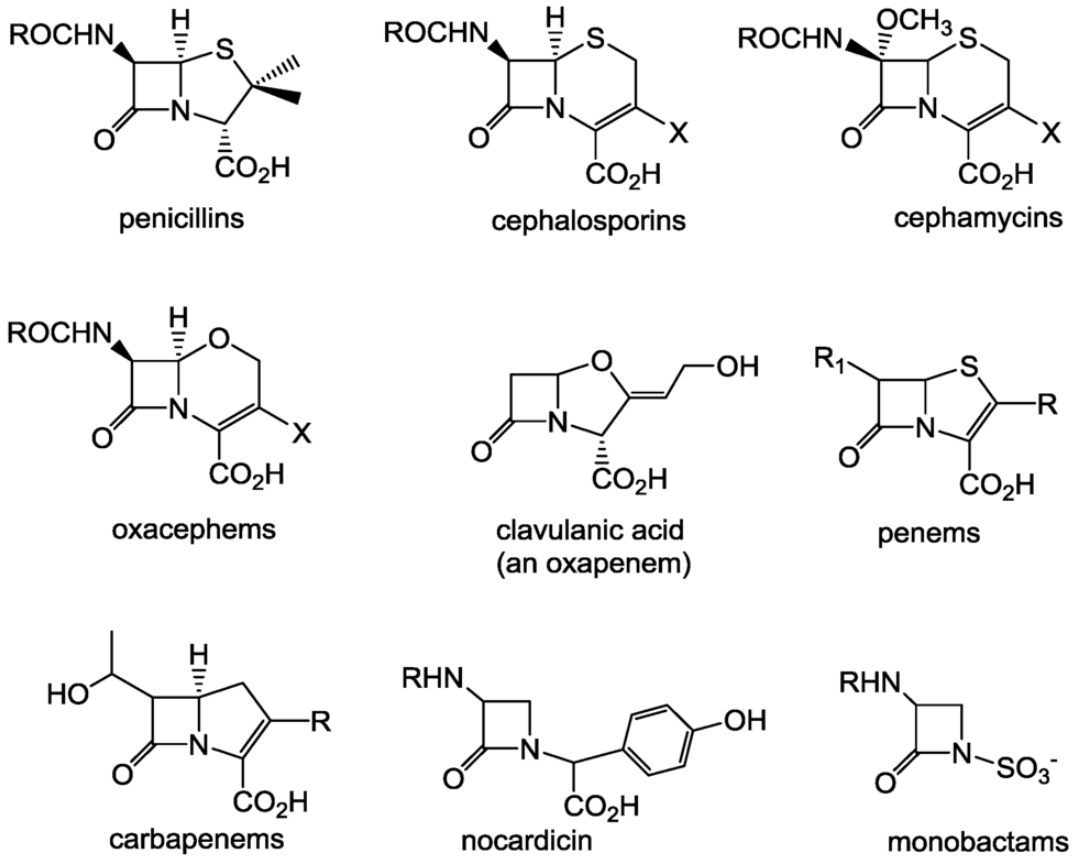


Figure 1.2: Chemical structure of β-lactam antimicrobial agents

Shown is the penicillin structure indicating the β-lactam ring structure that acts as the structural analogue to the terminal D-ala[4]-D-ala[5] of the growing peptidoglycan chain.

Figure reproduced from Konaklieva (2014)

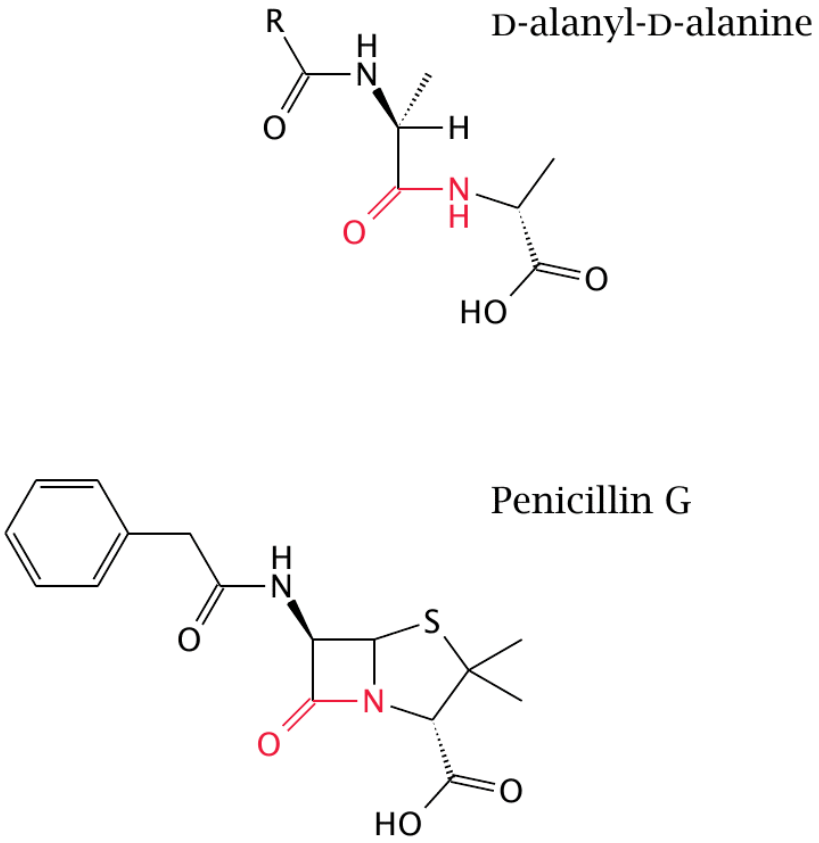


Figure 1.3: Structural similarity between β-lactam antimicrobials and D-Ala^4- D-Ala^5 terminus of the stem peptide.

Regions in red illustrate the structural similarities between β-lactam antimicrobial agents exemplified here by Penicillin G and the D-Ala^4- D-Ala^5 stem peptide.

Figure reproduced from Palmer et al. (2012).

S. aureus has five native PBPs of varying size; PBP1 (85 kDa), PBP2 (81 kDa), PBP3 (75 kDa), PBP3' (70 kDa) and PBP4 (45 kDa) and one non-native PBP found exclusively in MRSA, PBP2a (Georgopapadakou 1993, Fan et al. 2007). This non-native PBP was imported from a non-*S. aureus* source and will be discussed further in the following section. PBPs 1, 2 and 3 are essential for cell growth whereas PBP2 and PBP4 are speculated to participate in peptidoglycan cross-linking where PBP4 mediates the production of highly cross-linked peptidoglycan component (Fan et al. 2007). It should be noted that although these are called 'penicillin-binding' proteins, their function is associated with cell wall synthesis, as summarised in Figure 1.4, rather than penicillin resistance.

1.7.2 β-lactamase production and regulation

Resistance to β-lactam antimicrobial agents is mediated by one of two mechanisms in staphylococci. This section will discuss the enzymatic pathway encoded by the *bla* operon. The second mechanism is mediated by the production of an altered PBP which is encoded by *mecA* and will be discussed in Section 1.8. The *bla* operon consists of the structural gene *blaZ* which is regulated by a DNA binding protein BlaI and a membrane-spanning signal transducer BlaR1 (Olsen et al. 2006).

In the absence of β-lactams, BlaI homodimer binds to the operator region of *blaZ* and the *blaR1-blaI* complex to repress their transcription. In the presence of β-lactams the extracellular sensor region of BlaR1 is acylated and the signal can be transduced by the membrane-spanning domain to activate the intracellular zinc metalloprotease domain. The acylated BlaR1 then acts as a protease to disrupt the formation of the BlaI homodimer. This in turn triggers the transcription of both *blaZ* and *blaR1-blaI* for the formation of the β-lactamase protein (Hao et al. 2012).

1.7.3 β-lactamase classification schemes for staphylococci

As previously discussed in Section 1.6.1.2, β-lactamases are enzymes produced by staphylococci to cleave the β-lactam ring and render the antibiotic ineffective. Many typing schemes have been proposed but the Ambler and Bush systems for high level characterisation of β-lactamases from both Gram-positive and Gram-negative bacteria have gained prominence.

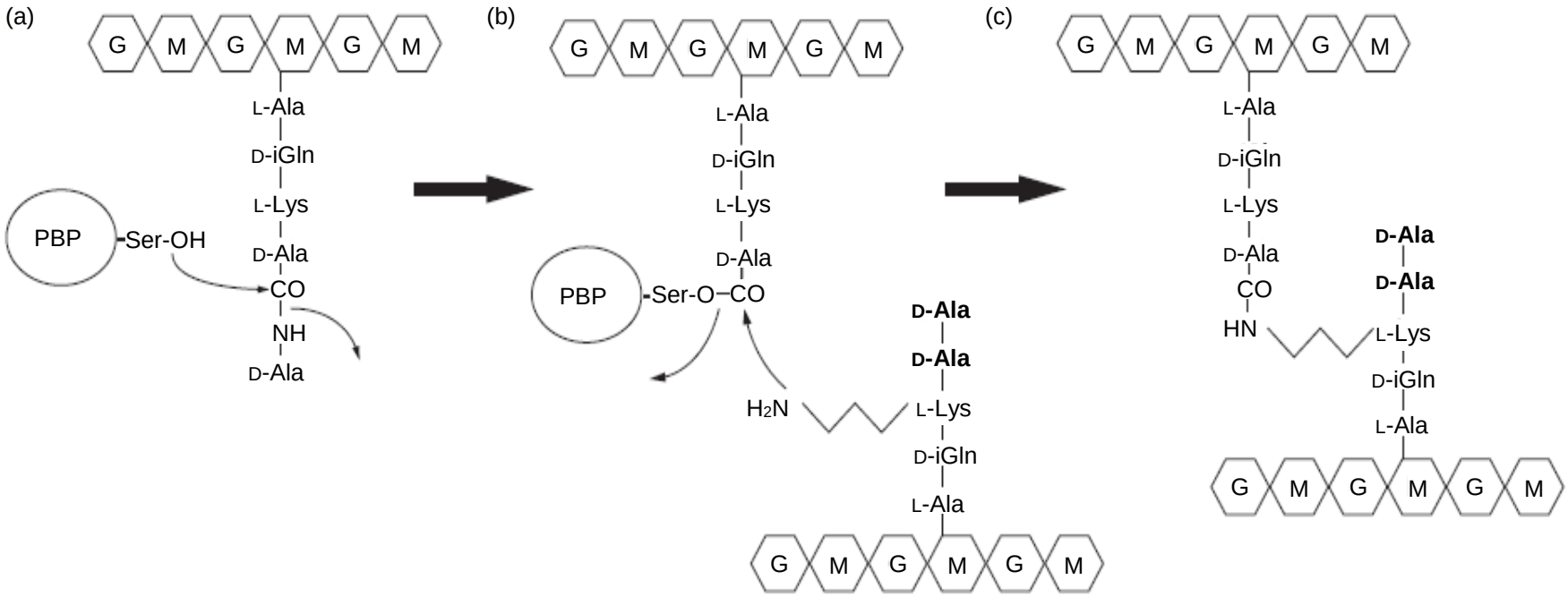

(a)
G M G M G M
L-Ala
D-iGln
L-Lys
D-Ala
PBP
Ser-OH
CO
NH
D-Ala
(b)
G M G M G M
L-Ala
D-iGln
L-Lys
D-Ala
PBP
Ser-O-CO
D-Ala
D-Ala
H2N
L-Lys
D-iGln
L-Ala
G M G M G M
(c)
G M G M G M
L-Ala
D-iGln
L-Lys
D-Ala
CO
HN
D-Ala
D-Ala
L-Lys
D-iGln
L-Ala
G M G M G M

Figure 1.4: Catalysis of transpeptidation during late stage peptidoglycan synthesis in Gram-positive bacteria

Glycan strands are represented by chains of hexagons representing the hexoses *N*-acetyl glucosamine (G) and *N*-acetyl muramic acid (M). The penicillin binding protein is indicated by ovals (PBP). The 'donor' pentapeptide is shown as the upper glycan strand and the 'acceptor' on the lower strand.

In normal cell wall synthesis, the serine (Ser) attacks the carbonyl group of the penultimate D-Ala amino acid from the stem peptide (a), which in turn releases the last D-Ala acid from the 'donor' peptide thereby forming covalent acyl-enzyme complex (b). In the transpeptidase case, the carbonyl from the D-Ala amino acid forms an ester linkage with the active serine site which is then attacked in various mechanisms by a primary amine linked to the third residue of a second 'acceptor' stem peptide (b). This creates a peptide bridge between two stem peptides forming a link between glycan strands (c).

β-lactam antimicrobial agents contain structural analogues of D-Ala^4- D-Ala^5 (shown in **bold**) with the addition of a β-lactam ring (see Figure 1.3). This additional β-lactam ring prevents the cross-linking of the growing side chain which leads to a severe loss in bacterial cell wall integrity and eventuates in cell lysis.

Figure reproduced from Zapun et al. (2008).

1.7.3.1 Ambler system

The Ambler system characterises the enzymes based on their primary structure. This system exploits conserved and distinguishing amino acid motifs to classify β-lactamases into four molecular classes, A, B, C and D. Molecular classes A, C and D include enzymes which hydrolyse their substrates through an active serine site, whereas class B enzymes are metalloenzymes which use at least one active-site zinc ion to facilitate β-lactam hydrolysis (Bush and Jacoby 2010). As previously mentioned, metallo-β-lactamases will not be discussed further as they are generally more applicable to Gram-negative organisms. Staphylococci possess molecular class A β-lactamases.

Molecular class A β-lactamases have undergone further characterisation and can be subdivided into four types, A, B, C and D based on their nucleotide sequence (Ambler et al. 1991). This sub-classification system has been adopted in Chapter 3 for the classification of staphylococcal β-lactamases.

1.7.3.2 Bush system

Bush et al. (2010) published a review and update of the Bush classification system. Briefly, the Bush system group β-lactamases into one of three groups based on their functional characteristics: group 1 cephalosporinases, group 2 serine β-lactamases and group 3 metallo-β-lactamases. A fourth group is proposed however insufficient data about these β-lactamases exists to classify them into one of the aforementioned three. Of these, group 2 is by far the largest and most diverse with over 600 unique enzymes from both Gram-positive and Gram-negative organisms. This is largely due to an increase in the identification of ESBLs from Gram-negative bacteria in the last 25 years (Bush et al. 2010).

Staphylococcal β-lactamases have a relatively limited spectrum of hydrolytic activity and are classified as sub-group 2a penicillinases. These enzymes are predominantly chromosomally encoded and only five unique enzymes have been reported between 1995 and 2009 (Bush et al. 2010). Subgroup 2a enzymes are inhibited by clavulanic acid, will preferentially hydrolyse benzylpenicillin derivatives and nitrocefin. They possess limited efficacy against cephalosporins, carbapenems or monobactams. Hydrolysis rates for these compounds occur at rates less than 10% of those for benzylpenicillin derivatives (Bush et al. 2010).

1.7.3.3 Alternate systems

In addition to the Ambler and Bush systems for the standardisation of β-lactamase classification, more recently, a third scheme has been proposed. This scheme groups similar β-lactamases into 'signature types' based on the deduced amino acid sequence of *blaZ* (Olsen et al. 2006). A difference of at least three amino acids is required to classify a novel signature type. Currently, 12 signature types of staphylococcal BlaZ exist; type strains encompass isolates from humans, cows and dogs from Europe and Australia (Olsen et al. 2006, Malik et al. 2007). Since the proposal of this typing strategy in 2006, little work has been done to elucidate the difference, if any, to the functional differences between the signature types. This sub-classification system has been adopted in Chapter 3 for the classification of staphylococcal β-lactamases.

1.7.4 Semi-synthetic penicillins and methicillin

Methicillin, 6(2:6-dimethoxybenzamido)-penicillanic acid, formerly known as celebnin, was the first of many derivatives synthesised after the isolation of 6-aminopenicillanic acid in 1957 (Figure 1.5). It was the first known penicillin that combined considerable antibiotic activity with almost complete resistance to the staphylococcal β-lactamase (Knox 1960). Methicillin was shown to be free from acute and chronic toxic effects to the patient and despite being poorly absorbed orally, however after intramuscular injection, its level in the serum and tissues were comparable to penicillin G (Acred et al. 1961).

Methicillin was introduced into clinical practice in 1959 and naturally occurring isolates of MRS were reported from London in 1961 and its presence was already being monitored in the mid 1960's (Sutherland and Rolinson 1964, Gravenkemper et al. 1965). Since these isolates were identified, a variety of MRSA clones have emerged and spread worldwide (Barber 1961). Many of these clones demonstrate resistance to several classes of antimicrobial agents (Chambers and Deleo 2009).

The current model of methicillin resistance states that in the presence of β-lactam antimicrobial agents, the low-affinity PBP2a takes over the cell wall biosynthetic functions of the five native PBPs which are rapidly inactivated by the antimicrobial agent (Pinho et al. 2001). MRSA strains grown in the presence of β-lactam antimicrobial agents are also shown to produce peptidoglycan strands which have greatly reduced peptide crosslinks. This indicates that

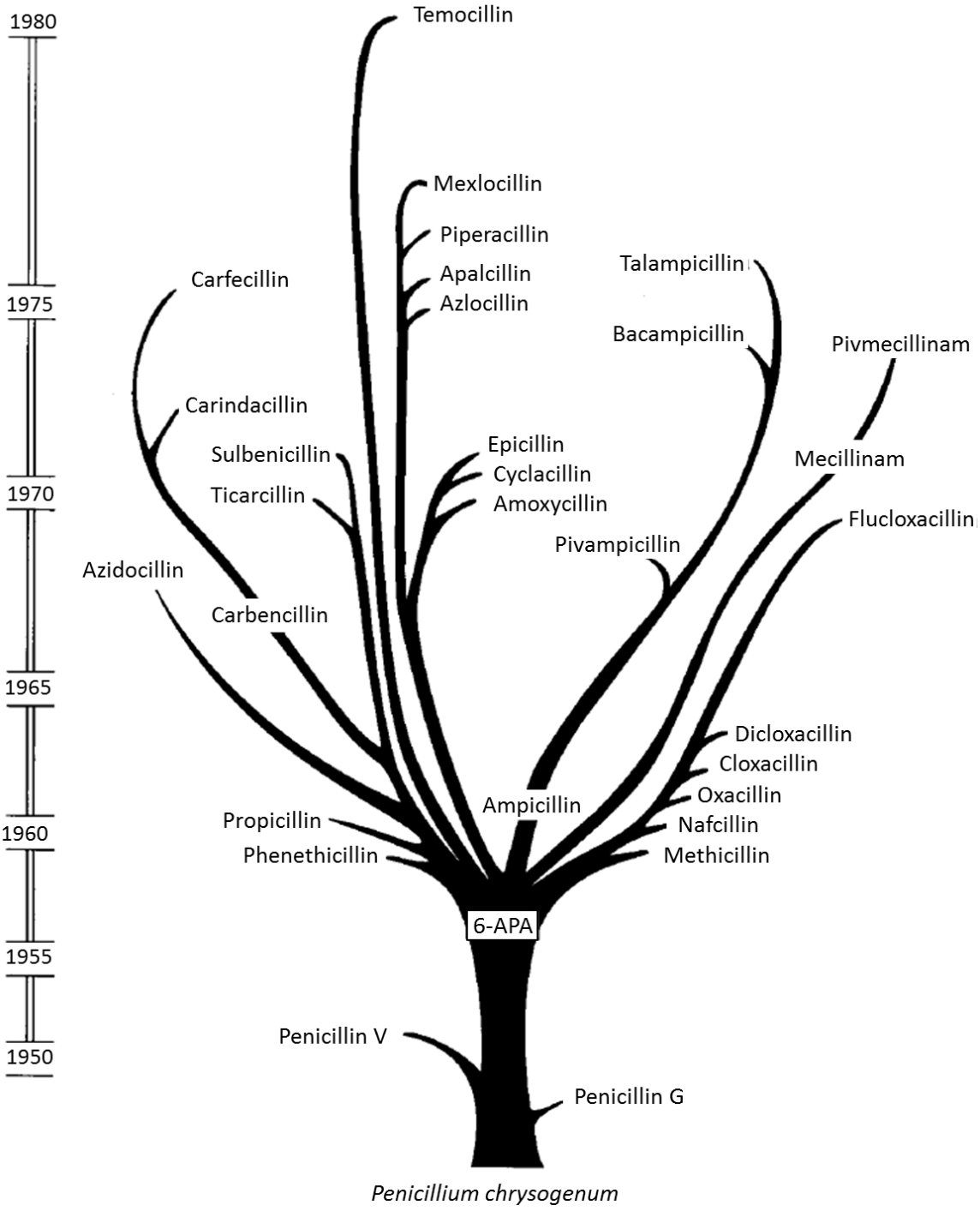


Figure 1.5: Semi synthetic derivatives of benzylpenicillin

Once the structure of the β-lactam ring of 6-aminopenicillanic acid (6-APA) of the penicillin nucleus was resolved in 1957, the production of semi-synthetic penicillins was possible via the addition of side-chains to the β-lactam ring.

Figure reproduced from Rolinson (1998)

MRSA produce longer glycan strands to compensate for the reduction in cell-wall linking (Pinho et al. 2001).

1.8 Methicillin-resistant staphylococci genotyping

Resistance to methicillin and all other semi-synthetic β-lactam compounds is modulated by an altered PBP2a. This 78 kDa PBP is encoded by the *mecA* gene and has an unusually low affinity for all β-lactam antimicrobial agents (Hiramatsu et al. 2001). The *mecA* gene is located on a mobile genetic element known as the Staphylococcal Cassette Chromosome *mec* (SCC*mec*) (Hiramatsu et al. 2001).

1.8.1 SCC*mec* element

These elements are a novel class of mobile genetic elements present predominantly in staphylococci and are characterised by the presence of site-specific integration and excision genes known as cassette chromosome recombinase (*ccr*) genes, regulatory elements and a variety of additional genes encoding resistance to other antimicrobial agents, heavy metals or virulence factors (Ito et al. 2009). An excellent comparison of the different multiplex PCR (MPCR)-based methodologies for typing SCC*mec* elements I – IX has been published (Turlej et al. 2011).

In this body of work, multiple PCR methodologies were trialled after which MPCR1, MPCR2 and MPCR3 from Kondo et al. (2007) was used in the final determination of SCC*mec* types (Chapter 4). Methodologies trialled included, but not limited to, those proposed by McClure et al. (2010), Lim et al. (2003) and Malik et al. (2006a).

1.8.1.1 mecA, mecB and mecC

Currently, aside from the prototype *mecA* from *S. aureus* N315, an additional six variants of the gene are recognised by the International Working Group on the Classification of Staphylococcal Cassette Chromosome *mec* (IWG-SCC*mec*) (Ito et al. 2012).

These variants are described in Table 1.1 and can be separated into three broad categories, *mecA, mecB* and *mecC*. Briefly, *mecA* which is present in all MRS species; the *mecA1* gene is described as always being present in *S. sciuri* isolates (Wu et al. 2001, Couto et al. 2003) and *mecA2* which was first isolated

Table 1.1: Standardised nomenclature system for reporting of novel *mec* genes from staphylococci and micrococci

Strain	Reported gene name	Proposed new name	Size (bp)	% identity with *mecA* in *S. aureus* N315
S. aureus N315[a]	*mecA*	*mecA*	2,007	100
Staphylococcal strains	*mecA*	*mecA*	2,007	98.3-100
S. sciuri K11[a]	*mecA*	*mecA1*	2,001	79.1
S. sciuri ATCC 70061	$mecA_S$	*mecA1*	2,001	80.2
S. vitulinus CSB08	*mecA*	*mecA2*	2,007	91
M. caseolyticus	$mecA_m$	*mecB*	2,025	61.6
S. aureus LGA251[a]	$mecA_{LGA251}$	*mecC*	1,998	68.7

[a] Prototype strains representing each *mec* gene

Table reproduced from Ito et al. (2012).

from three different staphylococcal species originating from five individual horses pre-hospitalisation (Schnellmann et al. 2006).

The *mecB* determinant, first identified by Tsubakishita et al. (2010a) in *Macrococcus caseolyticus* has been described as a discovery of 'academic' interest rather than one of veterinary and human medicine (Becker et al. 2014a). This *mecB* element has yet to be identified in staphylococci and to date, has only been isolated from a *M. caseolyticus* isolate in the nares of a dog with rhinitis (Gomez-Sanz et al. 2015).

The *mecC* gene, previously known as mec_{LGA251}, was simultaneously discovered by Garcia-Alvarez et al. (2011) and Shore et al. (2011) and shares 70% homology with the prototype *mecA* (Ito et al. 2012). This element has since been discovered in retrospective investigations of UK and Danish dairy cattle and humans dating back to 1975 and is the focus of current investigations involving human MRSA isolates in England (Paterson et al. 2014), European hedgehogs (Monecke et al. 2013) and more recently in Australia from a domestic cat (Worthing et al. 2016) to name a few.

1.8.1.2 mec gene complex

The *mec* complex is composed of *mecA* and its regulatory genes, *mecR1* and *mecI,* and associated insertion sequences. The *mecA* gene complex is regulated in a system that directly mirrors that of the *bla* operon (Section 1.7.2). However, the *mec* complexes of *mecB* or *mecC* are organised slightly differently to *mecA* with *mecB* being present on Tn*6045* with the following structure *blaZb-mecB-mecR1b-mecIb* and *mecC* in SCC*mec* type XI with the following structure *blaZc-mecC-mecR1c-mecIc* (Hiramatsu et al. 2013):

To date, a total of five *mec* complexes have been described in *S. aureus*. Each *mec* complex is composed of the following genes, *mecA*: encoding the altered PBP2a; *mecR1:* signal transducer gene encoding the sensor protein; and *mecI*: repressor gene encoding the repressor protein to prevent the translation of *mecA*.

Figure 1.6 is a summary diagram that depicts *mec* classes A, B and a generic *mec* class C (Malachowa et al. 2010). *Mec* class C is divided into C1 and C2, complex class C1 has both IS*431* sequences upstream and downstream of *mecA* in the same orientation whereas IS*431* sequences are in opposite orientations in complex class C2. The recently described class E *mec*

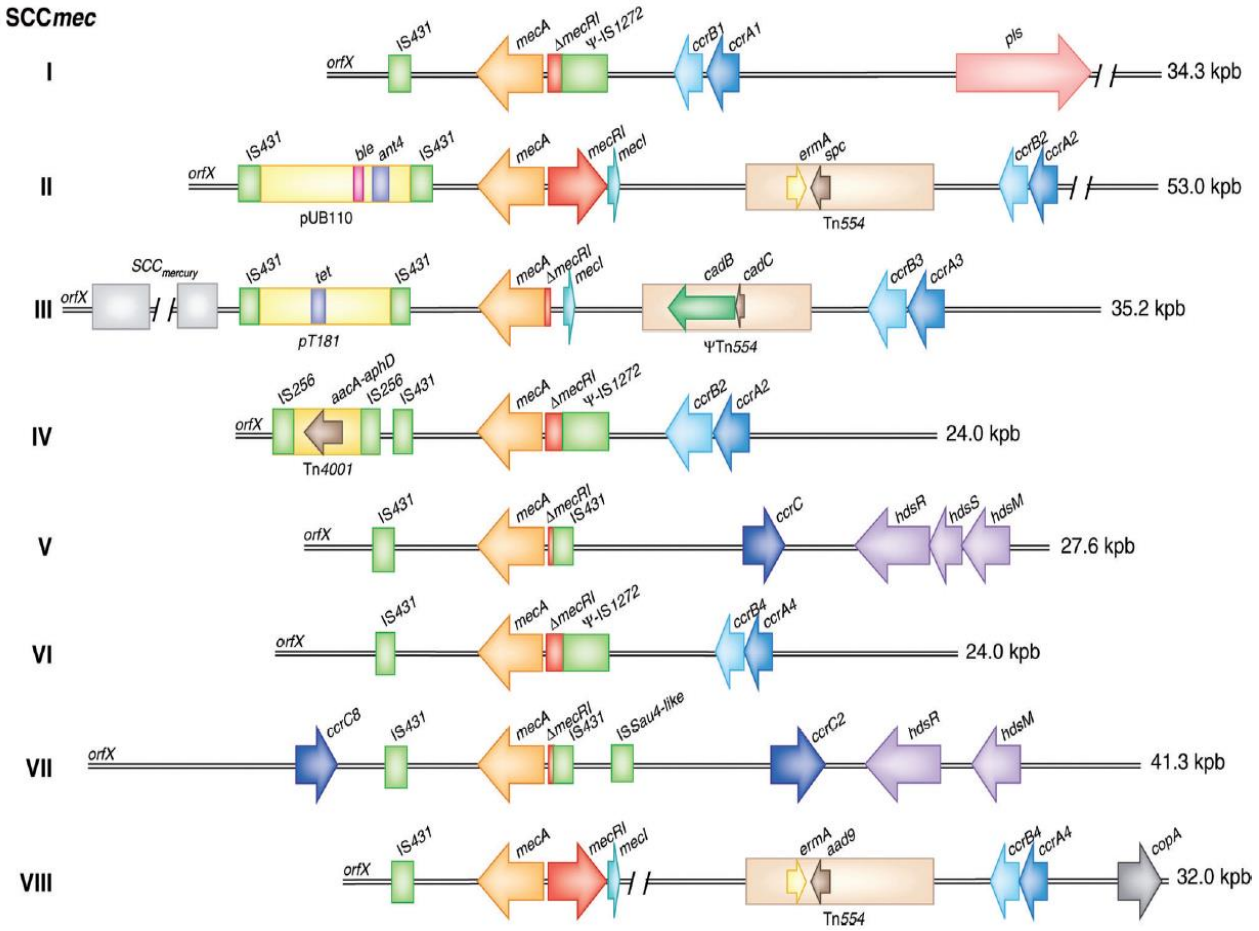
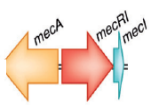
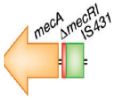


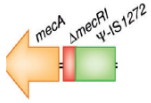
Figure key



Class A *mec* elements contains a complete *mecA* operon (*mec1-mecR1-mecA*)



Class B *mec* elements contains a disruption by insertion sequence 1272 (IS*1272-ΔmecR1-mecA)*



Class C *mec* elements contains a disruption by insertion sequence 431 (IS*431-ΔmecR1-mecA)*

Cassette chromosome recombinase genes (*ccr)* responsible for the movement of SCC*mec* into and out of genetic elements

Figure 1.6: Comparison of *S. aureus* SCC*mec* types (I-VIII)

Shown is a schematic comparison of *S. aureus* SCC*mec* types I – VIII highlighting the different *mec, ccr* regions and key genes present in the joining regions of each element. SCC*mec* types IX, X and XI have not been included here but are described in Table 1.2.

Abbreviations: Tn*554* – transposon 554 (erythromycin, *ermA*, and streptomycin/spectinomycin, *aad9* or *spc*, resistance); *copA* copper-transport ATPase; *hsdR, hsdM* and *hsdS* partial restriction-modification system type 1; Tn*4001* – transposon 4001 (aminoglycoside resistance operon, *aacA-aphD*; plasmid pT181 (tetracycline resistance, *tet*; ΨTn*554* – cadmium resistance, *cadB, cadC*; plasmid pUB110 (bleomycin, *ble*, and tobramycin, *ant4*, resistance).

Figure reproduced and adapted from (Malachowa et al. 2010).

Table 1.2: Currently identified SCC*mec* variants in *S. aureus*

SCC*mec* type	*ccr* gene complex	*mec* gene complex
I [1B][a]	1 (A1B1)	B (IS*431-mecA*-Δ*mecR1*-IS*1272*)
II [2A][a]	2 (A2B2)	A (IS*431-mecA-mecR1-mecI*)
III [3A][a]	3 (A3B3)	A (IS*431-mecA-mecR1-mecI*)
IV [2B][b]	2 (A2B2)	B (IS*431-mecA*-Δ*mecR1*-IS*1272*)
V [5C2][b]	5 (C1)	C2 (IS*431-mecA*-Δ*mecR1*-IS*1272*) two IS*431*s are arranged in opposite orientations
VI [4B][b]	4 (A4B4)	B (IS*431-mecA*-Δ*mecR1*-IS*1272*)
VII [5C1][b]	5 (C1)	C1 (IS*431-mecA*-Δ*mecR1*-IS*1272*) two IS*431*s are arranged in the same orientation
VIII [4A][b]	4 (A4B4)	A (IS*431-mecA-mecR1-mecI*)
IX [1C2][c]	1 (A1B1)	C2 (IS*431-mecA*-Δ*mecR1*-IS*1272*) two IS*431*s are arranged in opposite orientations
X [7C1][c]	7 (A1B6)	C1 (IS*431-mecA*-Δ*mecR1*-IS*1272*) two IS*431*s are arranged in the same orientation
XI [8E][c]	8 (A1B3)	E (*blaZ-mecA*$_{LGA251}$-*mecR1*$_{LGA251}$-*mecI*$_{LGA251}$)

[a] First three SCC*mec* elements were designated types I, II and III (Katayama et al. 2000, Ito et al. 2001).

[b] SCC*mec* types IV to VIII were described in quick succession (Ito et al. 2004, Boyle-Vavra et al. 2005, Oliveira et al. 2006a, Higuchi et al. 2008, Zhang et al. 2009). SCC*mec* I-VIII are also depicted in Figure 1.6.

[c] Three additional SCC*mec* elements have been identified in *S. aureus* since 2011; type IX and X (Li et al. 2011) and the simultaneous discovery of XI by two groups (Garcia-Alvarez et al. 2011, Shore et al. 2011) with the *mecC* element.

Table reproduced from Coombs (2012).

complex, described in Table 1.2, contains the highly divergent *mecC* (Garcia-Alvarez et al. 2011, Shore et al. 2011) (see Section 1.8.1.1).

1.8.1.3 ccr gene complex

The *ccr* genes belong to the large family of serine site-specific DNA recombinases and are located on the SCC*mec* element. These elements operate in a manner similar to bacteriophage integrases (Shore and Coleman 2013). Integration of SCC*mec* occurs through the recombination of the *attSCC* site on the circular SCC*mec* and the *attBscc* site, containing a core 15 bp sequence known as the integration site sequence, present at the 3' end of *orfX* by the *ccr* genes (Noto et al. 2008). This results in the insertion of SCC*mec* into the chromosome and has been demonstrated in a variety of *in vitro* and *in vivo* assays (Peacock and Paterson 2015).

Currently three phylogenetically distinct variations of *ccr* genes exist, *ccrA*, *ccrB* and *ccrC*. These genes were all identified in *S. aureus* and have DNA sequence similarities below 50%. Generally speaking, *ccr* genes that have nucleotide identities greater than 85% are assigned to the same allotype, whereas, those with nucleotide identities between 60% and 82% are assigned to a different allotype. *CcrA* and *ccrB*, present in SCC*mec* types I to IV, can excise SCC*mec* elements from each of these SCC*mec* types, demonstrating that the recombinase activity is not specific to their associated SCC*mec* types whereas, *ccrC* appears to be specific for SCC*mec* V (Peacock et al. 2015); a detailed description of the element is provided by Ito et al. (2004) and a proposed nomenclature and classification system has been proposed for these elements (Chlebowicz et al. 2011).

Currently six *ccrA/ccrB* gene allotypes have been characterised in *S. aureus*, Oliveira et al. (2008) have developed an online typing tool (http://www.ccrbtyping.net) to differentiate between *ccrA/ccrB* alleles or allotypes. To date, all *ccrC* variants identified have greater than 86% nucleotide identity and are thus all classified as *ccrC1* (Oliveira et al. 2006b, Oliveira et al. 2008).

1.8.1.4 J-regions

In addition to these core genes, SCC*mec* elements contain a variety of genes encoding resistance to other antimicrobial agents, such as aminoglycosides or macrolides; heavy metals; and virulence determinants such as *pls*, responsible

for promoting adhesion to epithelial cells (Roche et al. 2003). These additional genes are located before, between, and after, the *ccr* and *mec* genes and are designated the 'joining', 'junkyard' or 'J' regions, J1, J2 and J3, respectively (Garbacz et al. 2013, Shore et al. 2013). Genes in these J regions are non-essential but variations in the J regions are used for defining SCC*mec* subtypes (Szabo 2014).

1.8.2 SCC*mec* nomenclature

SCC*mec* types are defined by the combination of the *ccr* gene complex and the *mec* gene complex and as previously demonstrated in Figure 1.6, SCC*mec* elements are highly diverse in their structural organisation and genetic content To date, eleven SCC*mec* types have been identified in *S. aureus* and lodged with the IWG-SCC*mec* elements (Ito et al. 2009, Garcia-Alvarez et al. 2011, Li et al. 2011, Shore et al. 2011) (Table 1.2) and more than 15 subtypes recognised in the literature (Kondo et al. 2007). In addition to the elements described above, the literature also contains a large number of atypical SCC/SCC*mec* elements which appear to represent the various stages in the evolution of the element (Shore et al. 2013).

Due to the high diversity of the elements, it has become common practice to define MRSA clones by the combination of their SCC*mec* type (determined through MPCR methodologies previously published in the literature; (Kondo et al. 2007) and chromosomal background (determined by MLST or as part of the Alere DNA microarray for *S. aureus*) and usually expressed as a ST (Enright et al. 2000, Monecke et al. 2008). For example, ST22-SCC*mec* IV, abbreviated as ST22-IV.

A standardised reporting system for the classification and naming of SCC*mec* elements was proposed in 2009 by the IWG-SCC*mec* (Ito et al. 2009) and has been enhanced by the standardisation of naming and classification of *mec* elements in 2012 (Ito et al. 2012). The IWG-SCC*mec* have listed the representative strain for each type and subtype. Briefly, new SCC*mec* types and/or subtypes, those which contain a novel combination of *mec* and/or *ccr* element or variations of the J regions in existing *mec* and *ccr* gene combinations respectively, are numbered sequentially in ascending order. In its simplest form, the IWG-SCC*mec* has recommended that the structural type be indicated with a Roman numeral, with a lowercase letter indicating the subtype,

and the *ccr* complex and the *mec* complex indicated by an Arabic numeral and uppercase letter, respectively, in parenthesis (see Table 1.2). For example, SCC*mec* subtype type IVa (2B) indicates that the element harbours a type 2 *ccr* (A2B2) and class B *mec* complex with a subtype a J region (Ito et al. 2009).

1.9 Types of methicillin-resistant staphylococci

As previously discussed in Section 1.1, three divisions of MRS(A) are noted in the literature: hospital-associated MRSA (HA-MRSA), community-acquired MRSA (CA-MRSA) and livestock-associated MRSA (LA-MRSA). However, historically, MRSA has implicitly referred to HA-MRSA and, it is only in the last decade that terms such as CA-MRSA and subsequently LA-MRSA have become commonplace.

1.9.1 Hospital-acquired MRS(A)

HA-MRS(A) include SCC*mec* types I – III, VI and VIII. These are the older SCC*mec* types which had a global presence in the 1990's when they were referred to as 'epidemic MRSA' (EMRSA) and are relatively big and carry multiple antibiotic resistance determinants (Figure 1.6) (Hiramatsu et al. 2013).

An inverse relationship between the possession of SCC*mec* I and growth rate has been seen *in vitro*. It has been assumed that the presence of the MGE may confer a selective disadvantage in the absence of antibiotics (Ender et al. 2004). Thus, strains carrying these elements may be at a survival disadvantage when in an environment with faster growing wild type strains once antibiotic therapy is ceased which may help explain why they are not widely found amongst healthy individuals or in the broader non-hospital community. In fact, the HA-MRS(A) clone CC22 SCC*mec* IV is becoming the dominant clone in an increasing number of countries (Lindsay 2014).

1.9.1.1 SCCmec types I-III

SCC*mec* type I was identified from the first MRSA strain isolated in 1965 from the UK and named COL (Baba et al. 2002); SCC*mec* type II prototype strain N315 was recovered from a Nagasaki University Hospital in Japan (Baba et al. 2002); SCC*mec* type III prototype strain 85/2082 recovered in 1985 from New Zealand (Ito et al. 2001). Notable clones include ST5-I (EMRSA-3) in Europe and South America, ST5-II (USA100) in the USA, Canada, Japan,

South Korea, Australia and Europe and ST239-III across Asia, Australia, South Africa, South America and Europe (Stefani et al. 2012).

1.9.1.2 SCCmec type VI

SCC*mec* type VI is most recognised as the 'paediatric clone HDE288' from Portugal and was formerly classified as a subtype of SCC*mec* IV (Oliveira et al. 2006a). This clone has predominantly been found in Portugal, with sporadic hospital-associated isolates detected in Poland, Argentina, Colombia and the United States (McDougal et al. 2003, Oliveira et al. 2006a). More recently, SCC*mec* type VI has been recovered from the nasal cavity of two Wayampi Amerindian individuals residing in the remote communities of Trois-Sauts, French Guiana (Lebeaux et al. 2012).

1.9.1.3 SCCmec type VIII

SCC*mec* type VIII was first described in 2009 and is thought to have originally been characterised as SCC*mec* type II presumably as a result of incomplete sequencing of the SCC*mec* element (Zhang et al. 2009).

1.9.2 Community-acquired MRS(A)

As discussed above, the majority of MRSA infections up until 1980 were associated exclusively with hospitals or healthcare facilities. However, post 1980 MRSA infections emerged in community populations of intravenous drug users then more prevalently in apparently healthy community populations (Millar et al. 2007). These populations included prisoners, sports participants, children and the indigenous peoples of Australia, Alaska, America and the Pacific Islands (Millar et al. 2007). A variety of definitions for CA-MRSA exist in the literature, including but not limited to the following, community-onset; community-derived; community-associated and community-acquired, this thesis will refer to CA-MRSA isolates as community-acquired.

There is evidence to suggest that CA-MRSA isolates are more virulent and are replacing and behaving like traditional HA-MRSA isolates with clinical data indicating clear differentiation between CA-MSSA and CA-MRSA isolates could not be achieved (Popovich et al. 2008). CA-MRSA isolates predominantly include SCC*mec* types IV, V and VII.

*1.9.2.1 SCC*mec *IV*

SCC*mec* IV was simultaneously described by Ma et al. (2002) and Daum et al. (2002). These elements are amongst the shortest of all SCC*mec* identified (Figure 1.6) and typically only carry the *mec* gene complex with no other antibiotic resistance determinants (Hiramatsu et al. 2013). This small size is thought to contribute to its mobility and present a lower strain fitness cost to its host, further evidenced by its ability to grow significantly faster than HA-MRSA strains (Okuma et al. 2002, Smyth et al. 2011). Currently more than 8 subtypes of SCC*mec* type IV elements have been described (Kondo et al. 2007). Notable regional clones of SCC*mec* type IV MRSA which are now globally prevalent include ST1-IV (WA-1, USA400), ST8-IV (USA300), ST30-IV (South West Pacific clone) and ST80-IV (European clone) (Mediavilla et al. 2012).

Prevalence of these clones varies geographically with USA300 being the dominant clone present in North America and the South West Pacific clone being the primary CA-MRSA found in Australia, Asia, South America, Europe and the Middle East (Mediavilla et al. 2012). Other notable regional clones in Australia but not present on a global scale include, ST93-IV (Queensland clone) and ST75-IV which is restricted to remote Aboriginal communities (Chua et al. 2011, Holt et al. 2011).

Clone USA300 has received widespread attention due to its heightened transmissibility associated with the carriage of the arginine catabolic metabolic element (ACME) which used to be considered a marker for this lineage. Furthermore, there is evidence to suggest USA300 strains are acquiring high-level mupirocin resistance (Mediavilla et al. 2012). The genome of a USA300 strain was first resolved in 2006 and appears to be a close relative of the first MRSA strain, COL, differing by only 678 single-nucleotide polymorphisms (Diep et al. 2006, Enright 2006).

*1.9.2.2 SCC*mec *V*

SCC*mec* type V was first isolated by O'Brien et al. (1999) from the remote Aboriginal communities of Western Australia and subsequently genetically described by Ito et al. (2004). These isolates were the first description of the *ccrC* complex (Figure 1.6, Table 1.2). An unique feature of the SCC*mec* type V element is the presence of a second SCC*mec* element, type III, in the J3 region which is independent of the dominant SCC*mec* type V (Ito et al. 2004). Notable

regional clones of SCC*mec* type V include ST59-V(5C2&5) otherwise known as USA1000, ST772-V (Bengal Bay clone) and ST72-V (USA700) (Lim et al. 2012, Mediavilla et al. 2012). Of these, ST59-V(5C&2), previously named SCC*mec* type V_T and first described as SCC*mec* type VII by Higuchi et al. (2008), is the most notable clone and is found predominantly in Taiwan and to a lesser extent Australia, USA and the UK (Takano et al. 2008, Coombs et al. 2010, Coombs et al. 2011). It is also one of the largest SCC*mec* elements identified to date, smaller in size only in comparison to SCC*mec* type II (Figure 1.6) and is speculated to be the result of multiple recombination events (Higuchi et al. 2008).

*1.9.2.3 SCC*mec *VII*

SCC*mec* type VII was described by Berglund et al. (2008) and endorsed by the IWG-SCC (Ito et al. 2009). Despite first being identified in the community, one SCC*mec* type VII isolate was been identified in a retrospective analysis of 247 necrotising fasciitis cases in Taiwan from a total of 16 MRSA isolates (Changchien et al. 2011).

1.9.3 Livestock-associated MRS(A)

A third group of SCC*mec* types have emerged and been dubbed 'livestock-associated MRSA' and predominantly includes SCC*mec* types IX, X and XI, and to a lesser extent SCC*mec* types IV and V, due to their prevalence in livestock and animals (Hermans et al. 2008, Jansen et al. 2009, Garcia-Alvarez et al. 2011, Lim et al. 2012, Vestergaard et al. 2012, Loncaric et al. 2013). With the exception of reporting a novel combination or variant, these SCC*mec* types are collectively referred to as LA-MRSA. Notable mention is made to the diversity of SCC*mec* type XI isolates with variations in either the SCC or *mecC* portion of the SCC*mec* element from captive and free-ranging animals (Malyszko et al. 2014, Espinosa-Gongora et al. 2015).

1.9.3.1 Clonal complex 398 in animals

The first case of LA-MRSA was first identified in 2003 cultured from pigs and pig farmers from the Netherlands and France. This clonal lineage was designated as MLST ST398 and belongs to the larger group of CC398 strains, including ST752 and ST753 (Kock et al. 2013). Since 2003, reports of both MRSA and MSSA CC398, predominantly ST398, isolated from horses, pigs, pig farmers, veal calves and poultry from Belgium, Netherlands, France, North

America and Asia (Nemati et al. 2008, Verkade and Kluytmans 2014) have increased. Multiple cases of *S. aureus* CC398 transmission from pigs to pig farmers have been reported indicating direct contact with livestock is a significant risk factor for human colonisation and infection with *S. aureus* CC398 (Graveland et al. 2011, Abdelbary et al. 2014).

In addition to MRSA CC398, other LA-MRSA clonal lineages have been detected. In a 2009 European baseline report, Germany was amongst the countries with the most frequent occurrence (3.9%) of non-CC398 MRSA in pigs. Clonal lineages CC9/ST9, CC97/ST97 and CC30/ST39 were amongst the most common non-CC398 LA-MRSA identified (Kock et al. 2013).

1.9.3.2 Clonal complex 398 in humans

Aside from livestock, humans have been found to be colonised with *S. aureus* CC398 in New Zealand (Williamson et al. 2014a). Additionally, nosocomial transmission of LA-MRSA CC398 have been documented in the Netherlands (Bosch et al. 2016). One study suggesting that *S. aureus* CC398 originated in humans as MSSA before being transmitted to livestock where it acquired the *mecA* gene and became MRSA (Price et al. 2013). It is possible that this adaptation to animal hosts has impacted on the ability of LA-MRSA CC398 to infect and colonise humans with one study finding LA-MRSA CC398 transmissions at a Dutch hospital to be 72% less than other circulating MRSA genotypes (Wassenberg et al. 2011). Comparative analysis of the most frequently occurring *spa* types from LA-MRSA (CC398), CA-MRSA (CC8, CC30 and CC80) and HA-MRSA (CC5, CC22 and CC45) isolates in Europe recovered in 2010-2011, revealed that LA-MRSA CC398 was less efficient at adhering to human cells and human/bovine plasma fibronectin when compared to CA-MRSA and HA-MRSA isolates (Ballhausen et al. 2014). However, variations within LA-MRSA CC398 were present; *spa* type t108 had increased adhesive and invasion potential paired with an increased ability to evade phagocytosis compared to *spa* types t034 and t108 (Ballhausen et al. 2014).

1.10 Methicillin-resistant staphylococci in veterinary medicine

Advances in molecular techniques and global epidemiological typing of MRSA isolates, allowing for the differentiation and categorisation of strains into zoonotic, zooanthroponotic and/or host specific lineages (Grema et al. 2015). A total of 17 strains of human MRSA have been described in the UK. Of these,

EMRSA15 and EMRSA16 are the most dominant, with the latter representing the majority of human MRSA infections in Europe and America (Holden et al. 2004). Common lineages in humans include CC1, CC5, CC8, CC22, CC30 and CC45 whilst CC398 is dominant in pigs and other food-producing animals (Witte et al. 2007, Feβler et al. 2012).

Similar to humans, staphylococci can also be isolated from animals as part of their normal microbiota or as the causative agent of infection. However, the difference lies in the wealth of information regarding these isolates including antimicrobial susceptibility, lineages and molecular characteristics. This is particularly true for non-MRS or *S. aureus* species. In humans, a causal relationship between antibiotic usage and the occurrence of MRS(A) has been demonstrated for antimicrobial compounds such as quinolones, glycopeptides and β-lactams (Tacconelli et al. 2008). It is plausible that similar conditions apply to animals. It is important to note that whilst most animals with MRS are merely colonised and asymptomatic, a wide range of clinical infections can occur under specific host conditions. Conditions such as pyoderma, urinary tract infections and otitis can occur in addition to the infection of skin and soft tissue, wounds and post-operative sites (Weese et al. 2010).

The first isolation of MRSA from non-human sources was reported in 1972. These isolates were recovered from milk samples from cows suffering mastitis (Devriese et al. 1972). Since then, MRS(A) have been isolated from a variety of animals as both commensal and disease-causing agents. Animals including, but not limited to, dogs (O'Mahony et al. 2005, Vengust et al. 2006), cats (Malik et al. 2006b), horses (Yasuda et al. 2002), sheep (Alves et al. 2009), pigs (Huijsdens et al. 2006, de Neeling et al. 2007), chickens (Huber et al. 2011, Wang et al. 2013) and wildlife (Siqueira et al. 2010, Wardyn et al. 2012). This has led to a surge of interest and subsequent reports of MRS(A) colonisation and infection in animals. Furthermore, a distinction needs to be made between animals raised for food production and those which are kept for companionship. Food production animals are generally housed in an industrialised manner in high stocking densities as opposed to companionship animals which are kept at a few per household (Verkade et al. 2014).

1.10.1 Methicillin-resistant staphylococci in companion animals

Since the late 1990s, the role that livestock and companion animals play as reservoirs and vectors for the transmission of MRS has become clearer (Scott et al. 1988, Leonard and Markey 2008). Companion animals, such as cats, dogs, and horses, have frequently colonised by MRS which can lead to infection. Some evidence points towards the emergence of 'zooanthroponosis' whereby humans are the source of infection or colonisation for companion animals which then act as vectors to pass the infecting strain to other humans (Strommenger et al. 2006). Molecular studies lend support to zooanthoponosis where companion animal MRS isolates have been found to mirror the dominant lineages found in human populations in the same geographical area (van Duijkeren et al. 2004a, Harrison et al. 2014).

1.10.1.1 Methicillin-resistant staphylococci in household pets

Pets that are apparently healthy and not exhibiting signs of clinical disease can be asymptomatically colonised with staphylococci for variable periods of time. Staphylococci, such as *S. aureus* and *S. pseudintermedius*, have been frequently isolated from the nares, oral cavity, skin and anus of healthy cats and dogs (Malik et al. 2005, Garbacz et al. 2013, Davis et al. 2014).

Prevalence data for MRS colonisation in healthy cats and dogs are variable and direct comparison between studies is not advisable. This is predominantly related to different populations and methods, however, the majority of population-based studies in cats and dogs have reported colonisation rates of between 0 – 4% (Malik et al. 2006b, Abbott et al. 2010, Vanderhaeghen et al. 2012a, Loncaric et al. 2014b). Some exceptions have been noted such as a 7.3% and 13% prevalence of MRS in healthy dogs (Vengust et al. 2006, Garbacz et al. 2013). Isolation rates in animals attending veterinary clinics are generally higher with isolation rates of 15.4% of dogs in Turkey (Aslantas et al. 2013), 8.1% in Dublin (Abbott et al. 2010) and 7.8% and 16.7% of dogs at rescue shelter locations (Epstein et al. 2009, Loeffler et al. 2010b) reported.

1.10.1.2 Methicillin-resistant staphylococci in horses

Similar to prevalence data in cats and dogs, MRS prevalence data in horses are also variable and direct comparisons are both difficult to make and not advised. MRS colonisation rates in healthy horses vary widely from 1.7% (Loncaric et al. 2014b), 13% (Yasuda et al. 2002),15.8% (Corrente et al. 2009),

35.2% (De Martino et al. 2010) to 42% (Vengust et al. 2006). These differences are most likely attributed to differences in sampling and culturing methodologies in addition to geo-spatial variation and potential sampling bias.

Sampling bias and difference in culture technique may help us understand the differences in MRS colonisation rates from various tertiary care veterinary referral clinics. Colonisation rates vary from 2.7% in a Canadian clinic (Weese et al. 2006b) to 10.9% in a Belgian study (Van den Eede et al. 2009). Whilst horses exhibiting clinical signs of disease in Australia reported a colonisation rate of 3.7% (Axon et al. 2011) and 4.6% in Austria (Loncaric et al. 2014b).

Most equine and equine personnel related MRSA infections in Canada and North America have been associated with MRSA ST8 (van Duijkeren et al. 2010). Although recently there have been reports of livestock-associated MRSA ST398 colonising and infecting horses (Van den Eede et al. 2009, Couto et al. 2012).

1.10.2 Methicillin-resistant staphylococci in food-producing animals

LA-MRSA has largely being associated with *S. aureus* ST398, first and predominantly isolated from pigs since the mid-2000s in The Netherlands, Denmark and France (Armand-Lefevre et al. 2005, Voss et al. 2005, de Neeling et al. 2007). Since then, reports of MRSA CC398 from many parts of the world have been published (Kock et al. 2009, Lo et al. 2012, Groves et al. 2014). Another MRSA clone, ST9, has also been reported in predominantly pig-associated environments in some Asian countries but is less commonly encountered on the European continent (Lo et al. 2012). Pigs appeared to be natural hosts of the CC398 lineage. Prevalence rates of MRSA in pigs have ranged from 39% in The Netherlands, 42.5% in Taiwan, 16% in Hong Kong, 11.4% in China, 1.4% in Malaysia, 0.9% in Japan and its presence has been established in Thailand (de Neeling et al. 2007, Cui et al. 2009, Guardabassi et al. 2009, Neela et al. 2009, Baba et al. 2010, Larsen et al. 2012, Lo et al. 2012). Despite many reports detailing the prevalence of MRSA in pigs, pig farms and abattoirs; surveillance studies have also revealed other food-producing animals carry LA-MRSA. These animals include broiler chickens, veal calves, dairy cattle and turkeys (Nemati et al. 2008, Spohr et al. 2011, van Cleef et al. 2011, Ritcher et al. 2012, van Duijkeren et al. 2014). A recent review by Verkade et al.

(2014) summarises the epidemiology of MRSA and, to a lesser extent, MSSA CC398 in both animals and humans.

1.10.3 Other reservoirs of methicillin-resistant staphylococci

As evidenced above, a multitude of studies detailing the prevalence of MRS(A) in domestic and farm animals exist in the literature. However, data on the prevalence of MRS(A) in zoo animals, free-ranging wildlife and urban pests are scarce. This type of information is particularly relevant for veterinary staff in the administration of antibiotics to prevent dosing at sub-therapeutic levels (Vercammen et al. 2012). It is pertinent in the latter environments, home to wildlife and urban pests, to curb the dissemination of antibiotic-resistant staphylococcal strains to other host populations.

1.10.3.1 Methicillin-resistant staphylococci in zoos

Limited literature exists for the examination of MRS colonisation in captive zoo animals and existing literature is confined to screening studies for the presence of staphylococcal species in various animal species. One particular study sampled 93 different mammals over a 13 month time period and while staphylococci were isolated, none were identified as MRS (Vercammen et al. 2012). Sporadic cases of MRSA infections in captive zoo animals have been reported. Selected reports have included the isolation of a SCC*mec* IV MRSA in a parrot resulting in ulcerative bacterial dermatitis (Briscoe et al. 2008), MRSA in a Burmese python born in captivity and diagnosed with sub-spectacular eye abscess (Lee and Kim 2011) and MRSA in an African elephant calf which presented with a skin infection (Janssen et al. 2009).

1.10.3.2 Methicillin-resistant staphylococci in wildlife

Sporadic reports of MRS(A) colonising and infecting other hosts, including free-ranging wildlife, are present in the literature. To date, the majority of studies identify MRSA either by PCR screening for *mecA* or via the use of selective culture media. Wildlife sources of MRS(A) have included non-diseased red deers, Iberian ibex, wild boars and Eurasian griffon vultures (Porrero et al. 2013); faecal samples from voles, wood mice and brown rats (Gomez et al. 2014) and nasal swabs from injured rabbits and the wings of shore birds (Wardyn et al. 2012). Other sources have included, but are not limited to: dolphins, seals and walruses (Faires et al. 2009, Fravel et al. 2011); an eastern grey squirrel (Niemuth and Pilny 2012) and river water (Porrero et al. 2014).

1.10.3.3 Methicillin-resistant staphylococci in urban pests

Rats are recognised for their role in the transmission of zoonotic agents such as *Salmonella* spp., *Campylobacter* spp., *Toxoplasma gondii* and *Yersinia pestis* on livestock farms and in urban areas (Rollins et al. 2003, van de Giessen et al. 2009). Species such as the Norweigian rat are common urban pests and have been identified as carriers of MRS species such as methicillin-resistant *S. pseudintermedius* and *S. aureus* in an impoverished, inner-city Downtown Eastside neighbourhood of Vancouver, Canada (Himsworth et al. 2013, Himsworth et al. 2014). Rats carrying MRS(A) have also been isolated from pig farms in The Netherlands (van de Giessen et al. 2009). Furthermore, novel *S. aureus* ST carrying the *mecC* gene has been described in wild brown rats in Belgium (Paterson et al. 2012). Other urban pests such as the domestic housefly and foxes have also been implicated in the carriage of MRS (Rahuma et al. 2005, Carson et al. 2012).

1.10.4 Zoonosis, zooanthroponosis and interspecies transmission

There are significant public health concerns about the presence of MRS in animals. One of the main concerns involves the potential for animals to act as reservoirs or sentinels for MRS with subsequent transmission to humans. During the past 30 years, sporadic case reports of MRS(A) isolated from animals were published, predominantly in human medical journals with animals implicated as vectors for MRS(A) transmission. Several case reports and case series strongly indicate that the transmission of MRS(A) can be bidirectional (Guardabassi et al. 2004a, Loeffler and Lloyd 2010a, van Duijkeren et al. 2010). Whilst there have been studies that have found that MRSA isolates recovered from cats and dogs mirror those found in their local human hospitals, it does not prove that the MRSA transmission was from animals to humans (van Duijkeren et al. 2004b, van Duijkeren et al. 2005). Zoonotic infections have also been reported in people working with colonised or infected horses (Weese et al. 2006a). The spillage of isolates from the hospital to the community and eventually to pets could plausibly have occurred via patients, infected healthcare workers or urban pests such as rats and flies.

Numerous studies of MRSA colonisation in veterinary personnel have been conducted in the United States and Canada, Denmark and The Netherlands and Australia. These studies have found average prevalence rates ranging from

11.5%, 17% and 5.8% respectively (Weese et al. 2010, Jordan et al. 2011). It cannot be determined with certainty that these colonisation rates are reflective of MRSA acquisition from animals. However, the prevalence rates are higher than the general populace, particularly for equine and food animal veterinarians. This combined with typing data which shows strain similarity between human and animal strains provide further support of possible occupational origin. Typing data have revealed a higher incidence of equine-associated CA-MRSA CC8 in equine veterinarians (Weese et al. 2006a, van Duijkeren et al. 2010, Aslantas et al. 2012) and a predominance of MRSA ST398 in food production handlers. This was despite these clones being relatively uncommon in the general population (Wulf et al. 2012).

The literature reveals a wealth of specialised molecular techniques that can be relied upon to detect and characterise MSSA, MRSA and MRS isolates. The step-wise coupling of well-established MPCR, DNA sequencing techniques and array methodologies stands to produce reliable and internationally comparable typing data in a time and cost effective manner. These typing datasets allow for the unambiguous comparisons between Australian and international isolates in addition to inference with regard to a strains background (hospital, community or livestock).

1.11 Scope of this thesis

1.11.1 Rationale for thesis

Staphylococci are bacteria which can be opportunistic pathogens and are frequent colonisers of the nasal and epithelial layers of humans and animals. However, staphylococcal species diversity is grossly under-represented with a large portion of the literature focussed on the prevalence and characterisation of MRSA from humans and food-producing animals. The isolation of MRS(A) from different animal species has raised concerns over the role animals, particularly livestock and wildlife, play in the epidemiology and dissemination of MRSA in the wider community. For this reason, the research in this thesis focuses on: the detection of MSSA, MRSA and MRS isolates in wallabies; the determination of their antibiotic susceptibility with a focus on β-lactam antimicrobials; and the characterisation of the genetic background of *S. aureus* strains. Once complete,

this information can be pooled together to provide direct comparisons with staphylococcal strains both domestically and globally.

At the present time, it is unclear whether staphylococci, irrespective of methicillin resistance status, are present in captive or free-ranging populations of native Australian wallabies. This study represents the first of its kind to be conducted in Australia and as such, the data contained within this body of work acts as a baseline to which future studies can be compared to.

1.11.2 General aims

In order to test the overarching hypothesis of this thesis, four specific research aims have been formulated. In some instances, the research aims are addressed across multiple experimental chapters. The general aims of this thesis are:

1. To determine the presence and diversity of commensal staphylococcal species from apparently healthy captive and free-ranging wallabies and determine their antibiotic susceptibility.
2. To characterise the β-lactam resistant staphylococcal isolates associated with apparently healthy wallabies.
3. To evaluate the presence of and characterise the MRS isolates recovered from apparently healthy wallabies.
4. To investigate whether Australian wallaby *S. aureus* strains are discernibly different to strains currently circulating in Australia and globally.

1.11.3 Thesis style

The experimental chapters of this thesis (Chapters 2-5) follow a published paper format and have been submitted and subsequently published in peer-reviewed scientific journals.

CHAPTER 2

NASAL COLONISATION OF *STAPHYLOCOCCUS* SPP AMONG CAPTIVE AND FREE-RANGING WALLABIES IN SOUTH AUSTRALIA

Declaration for Thesis Chapter 2

Declaration by candidate

In Chapter 2 the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution
Experimental design, performance of experiments, writing, editing and revision of manuscript	80%

The following co-authors contributed to the work.

Name	Nature of contribution	Extent of contribution
Wayne Boardman	Editing and revision of manuscript	5%
Ian Smith	Wallaby nasal sample collection, provision of animal health data	5%
Amanda Goodman	Experimental design, editing and revision of manuscript	5%
Melissa Brown	Editing and revision of manuscript	5%

The undersigned hereby certify the above declaration correctly reflects the nature and extent of the candidate's and co-authors contribution to this work.

Candidate's signature		**01/07/2016**
Supervisor's signature		**01/07/2016**

2.1 Preface

This chapter is based closely on the paper by **Chen MMS**, Boardman WSJ, Smith I, Goodman AE and Brown MH. Nasal colonization of *Staphylococcus* spp among captive and free-ranging wallabies in South Australia. *Journal of Veterinary Sciences and Medical Diagnosis*, 3 (3): e1 – 9 (2014). The printed version of this article appears in Appendix F.

2.2 Abstract

Staphylococcal species diversity has been well studied with regard to antibiotic resistance in humans and animals of commercial or social value. However, studies of free-ranging wildlife and animals of conservation value are limited. In this study, multidrug resistant staphylococci were found exclusively in free-ranging wallabies indicating human activity and prior antibiotic exposure may not be significant contributing factors to the development of antibiotic resistance in staphylococci in animal reservoirs. Eight isolates of *S. aureus* were identified; one was resistant to ampicillin, penicillin and cefotaxime, one to ampicillin and penicillin and one to oxacillin, while the remaining five isolates were susceptible to all antimicrobial agents tested. Resistance to the β-lactam antibiotic family was the most prevalent with 37% of all isolates being resistant to one or more β-lactams. Fourteen species of *Staphylococcus* were identified from 89 strains isolated from 98 South Australian captive and free-ranging native wallabies. Among the identified isolates, *S. delphini* (18%) and *S. succinus* (17%) were the dominant species with single isolates of *S. cohnii*, *S. carnosus* and *S. hominis*. To our knowledge, this is the first to study to report the presence and diversity of commensal staphylococcal species in any member of the *Macropodidae* family and thus provides baseline data for future work on the prevalence and diversity of common microbial pathogens in macropods.

2.3 Introduction

The *Staphylococcus* genus comprises 45 species and 21 subspecies (Bergeron et al. 2011) and is widely distributed across a variety of environments and hosts including soil, water, skin and mucosal membranes of humans and other animals. A large portion of the literature regarding staphylococcal host colonisation and species diversity in animals is concerned predominantly with

cattle, pigs, horses, cats and dogs (Hajek et al. 1996, Bagcigil et al. 2007, De Martino et al. 2010). Moreover, staphylococcal species diversity is also grossly under represented because investigations have focused on the detection of common pathogenic species such as *S. aureus* (Siqueira et al. 2010). Studies involving free-ranging animals or animals of conservation value are scarce with the exception of a few which have focused on the prevalence of antibiotic resistance genes in various zoo animals and wildlife (Hauschild 2001, Hauschild et al. 2010, Vercammen et al. 2012).

Zoonotic infections resulting from wildlife reservoirs may become more prevalent as urbanisation causes changes in animal behaviour and movement (Daszak et al. 2001, Woolhouse et al. 2005b, Carson et al. 2012). In order to begin to detail our understanding of infectious disease in humans we need to gather knowledge about infections in other species with which we share our space and natural resources. It has been estimated that almost 75% of emerging human pathogens originate from animals (Woolhouse et al. 2005a). The One World, One Health concept encourages healthcare workers from human and veterinary medicine to share scientific data as there is growing evidence to show human and animal health can be negatively impacted by the same microorganisms (Health 2010).

In this study, Yellow-footed Rock-wallaby (*Petrogale xanthopus*) (YFRW), Black-flanked Rock-wallaby (*Petrogale lateralis*) (BFRW) and the mainland Tammar wallaby (*Macropus eugenii*) (TMW) were chosen to study the distribution of staphylococci in captive and free-ranging South Australian wallabies. Staphylococci diversity was examined using traditional biochemical methods in conjunction with partial 16S rRNA gene sequencing. We also determined the antibiograms for each staphylococcal isolate using Kirby-Bauer disc diffusion assay with 10 compounds across seven antimicrobial families and determined the presence of multidrug-resistant (MDR) staphylococci in these wallabies.

2.4 Materials and Methods

2.4.1 Sampling

A total of 30 free-ranging BFRW from the Anangu Pitjantjatjara Yankunytjatjara (APY) Lands (-26.08, 132.21) and 68 captive wallabies (16

BFRW, 28 YFRW, 24 TMW) from Monarto Zoological Park in South Australia, Australia were sampled between July 2009 and October 2010 during health examinations. The APY Lands, located in the far north-west corner of South Australia, covers 102,650 km^2 which is equivalent to approximately 10 percent of the state's area. It is home to 2,230 people across 33 communities and outstations (Ward et al. 2011). All wallabies were classified as apparently healthy at the time of sampling. Sterile single-use nasopharyngeal minitip swabs were used to swab both anterior nostrils and stored in 1 ml liquid Amies medium with 90% glycerol at -80°C until laboratory analysis. Ethical consent was obtained from the Flinders University Animal Welfare Committee.

2.4.2 Isolation and genus identification of *Staphylococcus* species

Swabs were grown in nutrient broth or tryptone soy broth and incubated at 37°C for 24 hr. A 100 µl inoculum was spread onto two selective media; staphylococcus number 110 (S110) and Oxacillin Resistance Screening Agar Base (ORSAB) and plates incubated for 24-48 hr. The S110 medium facilitates and favors the growth of staphylococci due to a high sodium chloride concentration. Sodium chloride concentrations up to 10% have been shown to have a protective effect for staphylococci and aid in the preservation of specific characteristic traits of different staphylococcal strains (Parfentjev and Catelli 1964). Chromogenic ORSAB medium facilitates the selection of methicillin resistant staphylococci (MRS) due to the presence of polymixin B and oxacillin in the medium. At the end of the incubation period, distinct colonies were purified and subjected to Gram-staining, catalase and coagulase tests for discrimination purposes. In order to concentrate on the *Staphylococcus* genus, isolates which were not Gram-positive and catalase positive were not analysed further.

2.4.3 Species-specific identification of *Staphylococcus* species

Samples received in 2009 were subjected to the Microbact 12S kit to determine the *Staphylococcus* species present; results were confirmed by partial sequencing of the 16S rRNA gene. Staphylococcal species were identified from samples obtained in 2010 only by partial sequencing of the 16S rRNA gene using universal primers V3V6F 5'-CCAGACTCCTACGGGAGGCAG-3' and V3V6R 5' ACATTTCACAACACGAGCTGACGA-3' to give a 752 bp product (Chakravorty

et al. 2007). Nucleotide sequences were determined by the Australian Genome Research Facility, University of Adelaide or First Base Laboratories, Malaysia. Sequences obtained were analysed with ClustalW, PhyML and TreeDyn 198.3 and then compared with published sequences in the National Center of Biotechnology Information database (accessed August 26 2013). Staphylococcal species were determined on the basis of 97% or higher similarity to a type strain (Aslantas et al. 2013). Genomic DNA was extracted using the HiYield™ Genomic Mini kit (Bio-Deal, New Zealand) according to the manufacturer's instructions for Gram-positive bacteria.

We tested the hypothesis that staphylococcal species diversity will not be impacted by the wallaby breed or captivity status. The significance of these data was determined by using chi-square analysis. A *p*-value ≤0.05 was regarded as being statistically significant. The *p*-values are shown only for results that were statistically significant (Dahiru 2008).

2.4.4 Antimicrobial susceptibility testing

The standard agar disc diffusion technique was performed according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI 2007), to determine the antibiotic susceptibility profile of all isolates. Briefly, overnight cultures were adjusted to 0.5 McFarland standard with 0.085% NaCl and 3 ml was spread onto a Mueller-Hinton agar plate in triplicate. The antibiotics used included β-lactams penicillin G (PEN) (10 units), ampicillin (AMP) (10 µg), oxacillin (OX) (1 µg); aminoglycosides gentamicin (CN) (30 µg), streptomycin (S) (10 µg); vancomycin (VA) (30 µg), cefotaxime (CTX) (30 µg), tetracycline (TE) (30 µg), erythromycin (E) (15 µg) and chloramphenicol (C) (30 µg). Multidrug resistance was defined as resistance to three or more antimicrobial families. A clinical methicillin-resistant *S. aureus* (MRSA) from the Flinders Medical Centre, *S. aureus* ATCC 6538 and *S. epidermidis* ATCC 12228 were used as reference strains.

We tested the hypothesis that antibiotic resistant staphylococci would be recovered more readily from captive wallabies compared to free-ranging wallabies. Raw disc diffusion diameter measurements from the Kirby-Bauer disc diffusion assay were averaged and assigned a qualitative value of R, iR and S according to the human specific CLSI guidelines M100-S17 (CLSI 2007). No difference was observed between the veterinary specific and human specific

CLSI guidelines for the antibiotics tested (CLSI 2008). The significance of these data was determined using chi-square. A *p*-value ≤0.05 was regarded as being statistically significant (Dahiru 2008).

2.5 Results

2.5.1 Isolation and identification of *Staphylococcus* species

A total of 28 YFRW were sampled, from these 19 were positive for bacterial growth resulting in the isolation of 48 Gram-positive cocci; six (12%) isolates were tentatively identified as MRS due to growth on ORSAB medium. From the 48 Gram-positive cocci, 28 were catalase positive and of these 2 were coagulase positive. A total of 10 different staphylococcal species were identified from these 28 catalase positive strains by partial 16S rRNA gene sequencing.

Species identified included *S. succinus, S. warneri, S. xylosus*, *S. delphini*, *S. fleurettii, S. vitulinus, S. saprophyticus*, *S. simulans* and *S. sciuri* (Table 2.1). In addition, two *S. aureus* strains were detected in 28 YFRW (carriage rate of 7%). No *S. epidermidis* strains were found.

From 24 TMW sampled, 24 Gram-positive isolates were isolated from the nasal cavities of five animals. Of these 24 isolates, 23 were recovered from S110 medium with one (4%) presumptive MRS isolated from ORSAB medium. Catalase production was detected in 10 isolates; no coagulase positive isolates were identified. These 10 catalase positive isolates were identified as belonging to four staphylococcal species, namely *S. delphini, S. epidermidis, S. hominis* and *S. succinus* (Table 2.1). TMW were the only captive wallabies to harbour *S. epidermidis* and the only wallaby species to carry *S. hominis*. No *S. aureus* were recovered from the TMW (Table 2.1).

All 16 captive BFRW were found to carry Gram-positive coccoid-shaped bacteria. A total of 31 isolates were recovered from S110 medium and 15 (32%) presumptive MRS were isolated from ORSAB plates. Biochemical tests identified 32 catalase positive strains, of which three were identified as *Enterobacter cloacae* and *Aerococcus viridans* (data not shown) and thus not further analysed. From 29 catalase positive strains, nine staphylococcal species were identified ranging from six isolates of *S. succinus* to a single isolate of *S. carnosus* which was the sole *S. carnosus* strain to be isolated in the study. Other species identified included *S. xylosus*, *S. fleurettii, S. sciuri, S. simulans*,

S. delphini and *S. saprophyticus* (Table 2.1). Coagulase production was detected in 10 isolates of which four were identified as *S. aureus* giving a carriage rate of 12% for *S. aureus* (Table 2.1). The remaining six coagulase positive species were identified as *S. xylosus*, *S. sciuri* and *S. saprophyticus*.

Of the 30 free-ranging BFRW sampled, 18 harboured cultivable bacteria resulting in the isolation of 24 Gram-positive cocci from S110 medium and 10 (41%) presumptive MRS from ORSAB medium. Catalase production was seen in 22 isolates, of which two were coagulase positive. From these 22 catalase positive isolates, six staphylococcal species were identified which were *S. aureus, S. delphini*, *S. warneri, S. epidermidis, S. cohnii* and *S. simulans* (Table 2.1). Two isolates of *S. aureus* were recovered giving a carriage rate of 9% (Table 2.1). Interestingly, despite the prevalence of *S. succinus* in the captive BFRW population, no *S. succinus* isolates were found in the free-ranging population ($p \leq 0.05$). Furthermore, 90% of all *S. warneri* recovered in this study originated from the free-ranging BFRW ($p \leq 0.05$).

2.5.2 Antimicrobial susceptibility testing

Of the 32 isolates recovered from ORSAB medium, nine were identified as staphylococci. These nine strains were identified as *S. aureus* (A74), *S. cohnii* (A31), *S. warneri* (A16 and A17) and *S. fleurettii* (M31, M47, A61, A69 and A72). Of these strains, seven were resistant to AMP, PEN, OX and demonstrated intermediate resistance to CTX, one was resistant only to OX. The *S. aureus* strain A74 was sensitive to all antimicrobial agents tested. See Table 2.2 and Table 2.3.

In the BFRW and YFRW populations, 30% of staphylococcal strains were resistant to at least one β-lactam antimicrobial compound compared to 60% of strains from TMW (Table 2.2 and Table 2.3). This translated to 33 (37%) out of 89 strains demonstrating β-lactam resistance. Another three TE resistant strains in addition to a single E resistant strain were detected (Table 2.2 and Table 2.3), bringing a total of 37 strains which were resistant to at least one antimicrobial compound. Resistance to CTX was seen in 12 strains with more than half (58%) comprised of *S. fleurettii* strains. Interestingly, from all *S. fleurettii* strains isolated only one was susceptible to CTX.

Table 2.1: Staphylococcal species recovered from wallaby nasal swabs

Staphylococcal species	**F-BFRW** (n=30)	**C-BFRW** (n=16)	**C-YFRW** (n=28)	**C-TMW** (n=24)	**Animals** (n=89)
S. aureus	2	4	2	0	**8**
S. carnosus	0	1	0	0	**1**
S. cohnii	1	0	0	0	**1**
S. delphini	8	2	3	3	**16**
S. epidermidis	3	0	0	4	**7**
S. fleurettii	0	4	3	0	**7**
S. hominis	0	0	0	1	**1**
S. saprophyticus	0	2	2	0	**4**
S. sciuri	0	3	1	0	**4**
S. simulans	1	2	2	0	**5**
S. succinus	0	6	7	2	**15**
S. vitulinus	0	0	3	0	**3**
S. warneri	7	0	1	0	**8**
S. xylosus	0	5	4	0	**9**
Total strains	**22**	**29**	**28**	**10**	**89**

Abbreviations: F-BFRW, Black-flanked Rock wallaby (*P. lateralis*) from a free-ranging environment; C-BFRW, Black-flanked Rock wallaby (*P. lateralis*) from captivity; C-YFRW, Yellow-footed Rock wallaby (*P. xanthopus*) from captivity; C-TMW, Mainland Tammar wallaby (*M. eugenii*) from captivity.

Table 2.2: Source of isolates, resistance profiles and staphylococcal species identification from Black-flanked rock wallabies

Strain	Species	Location and wallaby specie	Antibiogram									
			AMP	OX	PEN	CN	S	VA	CTX	TE	E	C
A1	*S. warneri*	F-BFRW	R	-	R	-	-	-	-	-	-	-
A2	*S. epidermidis*	F-BFRW	R	-	R	-	-	-	-	-	-	-
A4	*S. delphini*	F-BFRW	-	-	-	-	-	-	-	-	-	-
A5	*S. delphini*	F-BFRW	-	-	-	-	-	-	-	-	-	-
A7	*S. aureus*	F-BFRW	R	-	R	-	-	-	-	-	-	-
A8	*S. aureus*	F-BFRW	R	-	R	-	-	-	iR	-	-	-
A10	*S. delphini*	F-BFRW	-	-	-	-	-	-	-	-	-	-
A11	*S. delphini*	F-BFRW	-	-	-	-	-	-	-	-	-	-
A14	*S. warneri*	F-BFRW	-	-	-	-	-	-	-	-	-	-
A15	*S. warneri*	F-BFRW	-	-	-	-	-	-	-	-	-	-
A16*	*S. warneri*	F-BFRW	R	R	R	-	-	-	iR	R	R	-
A17*	*S. warneri*	F-BFRW	R	R	R	-	-	-	iR	R	R	-
A18	*S. epidermidis*	F-BFRW	-	-	-	-	-	-	-	-	-	-
A19	*S. delphini*	F-BFRW	-	-	-	-	-	-	-	-	-	-
A24	*S. warneri*	F-BFRW	R	-	R	-	-	-	-	-	-	-
A25	*S. simulans*	F-BFRW	-	-	-	-	-	-	-	-	-	-
A30	*S. delphini*	F-BFRW	-	-	-	-	-	-	-	-	-	-
A31*	*S. cohnii*	F-BFRW	R	R	R	-	-	-	R	-	R	-
A32	*S. epidermidis*	F-BFRW	R	-	R	-	-	-	-	-	-	-
A33	*S. delphini*	F-BFRW	-	-	-	-	-	-	-	-	-	-
A34	*S. warneri*	F-BFRW	-	-	-	-	-	-	-	-	-	-
A35	*S. delphini*	F-BFRW	-	-	-	-	-	-	-	-	-	-
A37	*S. succinus*	C-BFRW	-	-	R	-	-	-	-	-	-	-
A38	*S. succinus*	C-BFRW	-	-	-	-	-	-	-	-	-	-

A40	*S. sciuri*	C-BFRW	R	R	R	-	-	-	-	-	-	-
A41	*S. xylosus*	C-BFRW	-	-	-	-	-	-	-	-	-	-
A44	*S. xylosus*	C-BFRW	-	-	-	-	-	-	-	-	-	-
A45	*S. simulans*	C-BFRW	-	-	-	-	-	-	-	-	-	-
A46	*S. succinus*	C-BFRW	-	-	-	-	-	-	-	-	-	-
A48	*S. xylosus*	C-BFRW	-	-	-	-	-	-	-	-	-	-
A50	*S. saprophyticus*	C-BFRW	-	R	-	-	-	-	-	-	-	-
A51	*S. saprophyticus*	C-BFRW	-	-	-	-	-	-	-	-	-	-
A55	*S. xylosus*	C-BFRW	-	-	-	-	-	-	-	-	-	-
A58	*S. sciuri*	C-BFRW	-	R	-	-	-	-	-	-	-	-
A59	*S. fleurettii*	C-BFRW	R	R	R	-	-	-	iR	-	-	-
A61	*S. fleurettii*	C-BFRW	R	R	R	-	-	-	iR	-	-	-
A64	*S. carnosus*	C-BFRW	-	-	-	-	-	-	-	-	-	-
A66	*S. simulans*	C-BFRW	-	-	-	-	-	-	-	-	-	-
A67	*S. succinus*	C-BFRW	-	-	-	-	-	-	-	R	-	-
A68	*S. succinus*	C-BFRW	-	-	-	-	-	-	-	-	-	-
A69	*S. fleurettii*	C-BFRW	R	R	R	-	-	-	iR	-	-	-
A70	*S. aureus*	C-BFRW	-	-	-	-	-	-	-	-	-	-
A71	*S. delphini*	C-BFRW	-	-	-	-	-	-	-	-	-	-
A72	*S. fleurettii*	C-BFRW	R	R	R	-	-	-	iR	-	-	-
A73	*S. aureus*	C-BFRW	-	-	-	-	-	-	-	-	-	-
A74	*S. aureus*	C-BFRW	-	-	-	-	-	-	-	-	-	-
A75	*S. succinus*	C-BFRW	-	-	-	-	-	-	-	R	-	-
A76	*S. delphini*	C-BFRW	-	-	-	-	-	-	-	-	-	-
A78	*S. aureus*	C-BFRW	-	R	-	-	-	-	-	-	-	-
A79	*S. xylosus*	C-BFRW	-	-	-	-	-	-	-	-	-	-
A81	*S. sciuri*	C-BFRW	-	-	-	-	-	-	-	-	-	-

Abbreviations: F-BFRW, Black-flanked Rock wallaby (*P. lateralis*) from a free-ranging environment; C-BFRW, Black-flanked Rock wallaby (*P. lateralis*) from captivity; C-TMW, Mainland Tammar wallaby (*M. eugenii*) from captivity; C-YFRW, Yellow-footed Rock wallaby (*P. xanthopus*) from captivity; -, sensitive; R, resistant; iR, intermediate resistant; * multidrug resistant; AMP, ampicillin; OX, oxacillin; PEN, penicillin; CN, gentamicin; S, streptomycin; VA, vancomycin; CTX, cefotaxime; TE, tetracycline; E, erythromycin; C, chloramphenicol.

Table 2.3: Source of isolates, resistance profiles and staphylococcal species identification from wallabies at Monarto Zoo.

Strain	Species	Location and wallaby specie	Antibiogram									
			AMP	OX	PEN	CN	S	VA	CTX	TE	E	C
M2	*S. warneri*	C-YFRW	-	-	-	-	-	-	-	R	-	-
M3	*S. vitulinus*	C-YFRW	-	-	-	-	-	-	-	-	-	-
M6	*S. sciuri*	C-YFRW	-	-	R	-	-	-	R	-	-	-
M7	*S. aureus*	C-YFRW	-	-	-	-	-	-	-	-	-	-
M8	*S. succinus*	C-YFRW	R	R	R	-	-	-	iR	-	-	-
M9	*S. aureus*	C-YFRW	-	-	-	-	-	-	-	-	-	-
M10	*S. simulans*	C-YFRW	-	-	-	-	-	-	-	-	-	-
M11	*S. fleurettii*	C-YFRW	-	-	-	-	-	-	-	-	-	-
M14	*S. xylosus*	C-YFRW	-	-	-	-	-	-	-	-	-	-
M15	*S. succinus*	C-YFRW	-	-	-	-	-	-	-	-	-	-
M17	*S. saprophyticus*	C-YFRW	-	-	-	-	-	-	-	-	R	-
M21	*S. saprophyticus*	C-YFRW	R	R	R	-	-	-	-	-	R	-
M26	*S. simulans*	C-YFRW	-	-	-	-	-	-	-	-	-	-
M27	*S. succinus*	C-YFRW	R	R	R	-	-	-	-	-	-	-
M28	*S. xylosus*	C-YFRW	R	R	R	-	-	-	-	-	-	-
M29	*S. xylosus*	C-YFRW	R	R	R	-	-	-	-	-	-	-
M30	*S. succinus*	C-YFRW	R	R	R	-	-	-	-	-	-	-
M31	*S. fleurettii*	C-YFRW	R	R	R	-	-	-	iR	-	-	-
M32	*S. delphini*	C-YFRW	-	-	-	-	-	-	-	-	-	-
M33	*S. delphini*	C-YFRW	-	-	-	-	-	-	-	-	-	-
M36	*S. xylosus*	C-YFRW	-	-	-	-	-	-	-	-	-	-
M37	*S. succinus*	C-YFRW	-	-	-	-	-	-	-	-	-	-

M38	*S. vitulinus*	C-YFRW	-	-	-	-	-	-	-	-	-	-
M39	*S. vitulinus*	C-YFRW	-	-	-	-	-	-	-	-	-	-
M40	*S. succinus*	C-YFRW	-	-	-	-	-	-	-	-	-	-
M41	*S. succinus*	C-YFRW	-	-	-	-	-	-	-	-	-	-
M47	*S. fleurettii*	C-YFRW	-	R	-	-	-	-	-	-	-	-
M48	*S. delphini*	C-TMW	-	-	-	-	-	-	-	-	-	-
M53	*S. delphini*	C-TMW	-	-	-	-	-	-	-	-	-	-
M54	*S. succinus*	C-TMW	R	R	R	-	-	-	-	-	-	-
M56	*S. delphini*	C-TMW	-	-	-	-	-	-	-	-	-	-
M57	*S. succinus*	C-TMW	R	R	R	-	-	-	-	-	-	-
M65	*S. epidermidis*	C-TMW	R	-	R	-	-	-	-	-	R	-
M66	*S. epidermidis*	C-TMW	R	-	R	-	-	-	-	-	R	-
M68	*S. epidermidis*	C-TMW	R	-	R	-	-	-	-	-	-	-
M69	*S. hominis*	C-TMW	-	-	-	-	-	-	-	-	-	-
M72	*S. epidermidis*	C-TMW	R	R	R	-	-	-	iR	-	-	-

Abbreviations: F-BFRW, Black-flanked Rock wallaby (*P. lateralis*) from a free-ranging environment; C-BFRW, Black-flanked Rock wallaby (*P. lateralis*) from captivity; C-TMW, Mainland Tammar wallaby (*M. eugenii*) from captivity; C-YFRW, Yellow-footed Rock wallaby (*P. xanthopus*) from captivity; -, sensitive; R, resistant; iR, intermediate resistant; * multidrug resistant; AMP, ampicillin; OX, oxacillin; PEN, penicillin; CN, gentamicin; S, streptomycin; VA, vancomycin; CTX, cefotaxime; TE, tetracycline; E, erythromycin; C, chloramphenicol.

A total of eight *S. aureus* strains were recovered. One was resistant to AMP, PEN and CTX, one to AMP and PEN and one to OX. The remaining five *S. aureus* strains in addition to 47 other non-S. *aureus* staphylococci were susceptible to all compounds tested (Table 2.2 and Table 2.3). Multidrug resistance was identified in *S. cohnii* strain A31 and *S. warneri* strains A16 and A17 (Table 2.2). These three strains were the only MDR staphylococci isolated in this study and were from free-ranging wallabies. This finding was considered statistically significant ($p \leq 0.05$).

All isolates were sensitive to CN, S, VA and C; sporadic TE and E resistance was observed. Antibiotic resistant staphylococci were equally distributed amongst the wallaby populations studied (p-value: <0.05) which indicates that captivity status is not a major contributor to the development of antibiotic resistance in staphylococci.

2.6 Discussion

This is the first study to provide detailed information on commensal staphylococcal carriage in apparently healthy captive and free-ranging wallabies in South Australia. We showed that staphylococcal species prevalence and diversity was higher in captive wallabies compared to their free-ranging counterparts. However, MDR was found only in isolates from free-ranging wallabies.

Bacterial growth on ORSAB medium was unable to definitively predict the OX resistance phenotype; 22 staphylococcal strains demonstrated OX resistance by Kirby-Bauer disc diffusion however only eight grew on ORSAB plates. Furthermore, 23 out of 32 (72%) bacterial strains recovered from ORSAB plates were biochemically identified as non-staphylococcal isolates indicating the limitations of this chromogenic medium when challenged with complex samples as was seen with MRSAselect agar (Vinh et al. 2006).

Resistance towards β-lactam antimicrobial agents (AMP, PEN and OX) was found in approximately one third of all isolates irrespective of captivity status and in concordance with studies in sub-clinical mastitis in cattle (Asfour and Darwish 2011). This is in contrast to previous findings in healthy and diseased domestic cats and dogs where less than 7% of all staphylococcal isolates were found to be resistant to β-lactam compounds (Malik et al. 2007). With the

exception of two strains, all antibiotic-resistant strains of staphylococci demonstrated resistance to at least one β-lactam compound which confirms the presence of β-lactam resistant staphylococci in animals that have had limited exposure to β-lactam antimicrobial agents. This limited exposure to β-lactam compounds is supported by the recovery of *S. aureus* strains which were sensitive to all antimicrobial agents tested as almost 90% of all *S. aureus* isolated from humans since 1980 have been described as penicillin-resistant (Livermore 2000). All isolates were susceptible to CN, S, C and VA, with sporadic cases of TE and E resistance.

The discovery of significant numbers of antibiotic-resistant coagulase negative staphylococci in conjunction with antibiotic-susceptible *S. aureus* suggests that coagulase negative staphylococci could serve as reservoirs for antibiotic resistance genes, as hypothesized in the case for the emergence of MRSA (Wu et al. 2001). Therefore, the roles and pathogenic potential of commensal non-*S. aureus* requires further attention by investigators as the acquisition of genetic elements, such as antibiotic resistance genes, are often methods by which microbes enhance their survivability (Mazel 2006). Furthermore, these antibiotic-resistant staphylococci were recovered in the absence of intensive antimicrobial therapy which highlights the need for research directed at commensal bacteria to further our understanding of the nasal microbiome of animals. The nasal microbiome has been shown to be an important site of infection as the elimination of *S. aureus* from the nasal passages prior to surgery is associated with a reduced risk of a hospital-acquired *S. aureus* infection (Bode et al. 2010).

We collected and analysed animal health records for each wallaby from the time they were introduced into the collection (minimum 12 months) at Monarto Zoological Park (Appendix B). We observed approximately equal numbers of staphylococcal isolates which were resistant to the first-line antibiotic TE irrespective of captivity status. In light of these results regarding the prevalence of β-lactam resistant staphylococci and given the importance and dissemination of MRS in human and veterinary medicine, future studies will focus on the detection, isolation and sequence analysis of the β-lactamase and methicillin resistance genes *blaZ* and *mecA*, respectively in the appropriate strains.

The discovery of three MDR staphylococci exclusively of free-ranging wallaby origin was statistically significant ($p \leq 0.05$). These three isolates (A16, A17 and A31) originated from two wallabies residing in separate communities approximately 150 km apart. Strains A16 and A17 were identified as *S. warneri* with identical antibiograms which is indicative of a duplicate isolate. However in the absence of pulse-field gel electrophoresis data, this remains unclear. The presence of MDR bacteria in apparently healthy animals has not been well-studied, however MDR staphylococci isolation rates of 17% in *S. pseudintermedius* in canine pyoderma and otitis externa infections have been reported (Yoon et al. 2010). Additionally, a 9 year study of canine and feline feces, urine, skin, upper respiratory tract and otitis externa cases were analysed retrospectively and revealed, on average, a MDR staphylococci frequency of 20% (Normand et al. 2000). It was interesting that apparently healthy wallabies, with little to no human interaction, carried antibiotic-resistant or MDR staphylococci. This is of epidemiological significance as it demonstrates prior exposure to antibiotics by the host is not essential for antibiotic resistance. Given the remote location of these animals, zoonotic transmission of MDR staphylococci to the general populace is unlikely.

The majority of staphylococci recovered in this study were coagulase-negative species, in agreement with previous reports where animals of non-commercial origin were sampled (Hauschild 2001, Malik et al. 2007, Hauschild et al. 2010). To our knowledge, this is the first study to investigate the commensal staphylococcal species diversity in any member of the *Marsupialia* clade, *Australidelphia* order which includes the *Macropodidae* family (Campbell and Francois-Joseph 2010). Staphylococci were recovered from 67% of YFRW, 100% of captive BFRW and only 20% of TMW. In contrast, only 46% of free-ranging BFRW were found to carry staphylococci in their nasal passages which could be a reflection on the levels of interaction between animals in their respective environments. Wallabies held in captivity have a higher incidence of interacting with one another as opposed to their free-ranging counterparts. In support of this, data obtained from radio collars fitted to BFRW in the APY Land New Well population indicate intermittent interaction between wallabies in the 15 km granite inselberg environment (Ruykys et al. 2012).

The hypothesis that wallabies in captivity are more likely to share a similar nasal microbiome compared to their free-ranging counterparts is supported by this study. We observed that captive wallabies harbored 12 staphylococcal species compared to only five in the free-ranging population ($p \leq 0.05$). In captive wallabies, *S. succinus* was the dominant species recovered and second most prevalent in this study, accounting for 22% (15 isolates) of the total staphylococci from captive wallabies. In contrast, no *S. succinus* was recovered from the free-ranging wallabies ($p \leq 0.05$) indicating the possible presence of an environmental selection pressure. *S. delphini* (8 isolates) and *S. warneri* (7 isolates) accounted for 67% of all staphylococci recovered from free-ranging wallabies. Whilst equal numbers of *S. delphini* were recovered in the captive population; 90% of all *S. warneri* isolates in this study were isolated from the free-ranging population ($p \leq 0.05$).

Interestingly, although staphylococci did not appear abundant in the anterior nares of TMW (10 staphylococci isolated from 27 animals) it was the only wallaby species to harbor *S. hominis* and *S. epidermidis* in captivity. This was a significant finding as we expect strains traditionally associated with humans to be present in captive wallaby populations more readily compared to those from a free-range environment. We did not sample workers at the Animal Health Laboratories at Monarto Zoo or veterinary staff involved in the sampling of APY Land wallabies, so we cannot rule out the possibility of transmission from human carriers. However, veterinarians employed aseptic techniques, including wearing gloves, when obtaining nasal samples thereby reducing the risk of human transmission or contamination.

In our study, *S. delphini* and *S. succinus* were the dominant species recovered, accounting for approximately 35% of all isolates from both captive and free-ranging wallaby origin. Recently Guardabassi et al. (2012) suggested animals from the order *Carnivora*, family *Mustelidae* (mink, badger and ferrets) be considered the natural hosts of *S. delphini*. However, *S. delphini* has previously been isolated from horses, pigeons, camels and cattle from England, Japan, France and Norway (Bannoehr et al. 2007, Ben Zakour et al. 2012), respectively. *S. succinus,* a novobiocin-resistant staphylococci which could only be differentiated from other novobiocin-resistant staphylococci on the basis of genomic methods (Lambert et al. 1998) has predominantly been isolated from

cheese and sausage production (Corbiere Morot-Bizot et al. 2006, Coton et al. 2010) after its first isolation from Dominican amber (Lambert et al. 1998) and the occasional human clinical urinary tract infection (Novakova et al. 2006). Hauschild et al. (2010) reported that *S. succinus* accounts for 28% of all staphylococcal isolates recovered from free-living insectivores such as shrews and voles from the *Soricidae* and *Cricetidae* families, respectively. Novobiocin is an important antimicrobial agent and has been shown to have positive activity against MRSA when used in conjunction with rifampicin (Walsh et al. 1993). *S. succinus* may be ubiquitous in nature therefore, and given its similarities to other human pathogens, its role in the microbial community and pathogenic potential needs to be determined. This study has found both *S. delphini* and *S. succinus* as the dominant staphylococcal species in wallabies (order *Diprotodontia*, family *Macropodidae*). Furthermore, the last common ancestor to be shared by *Macropodidae* and *Mustelidae, Soricidae* and *Cricetidae* was approximately 147.7 ± 5.5 million years ago when marsupials separated from placental mammals (Luo et al. 2011). We speculate that *S. delphini* has a broad host range and may not have co-evolved with the *Mustelidae* family as previously suggested (Guardabassi et al. 2012).

2.7 Conclusion

Wallabies in South Australia were found to be a significant reservoir of antibiotic resistance in a variety of staphylococcal species. Resistance to the β-lactam family was widespread. Free-ranging wallabies, with little or no contact with humans, carried MDR staphylococci. Wallabies taken into captivity from the wild, or bred in captivity, had an increased diversity of staphylococcal species due to the influence of man. Captive animals are housed in man-made environments which may have been subjected to pre-treatment which could expose wallabies to agricultural, chemical or waste water run-off. Despite this, carriage rates of antibiotic resistance in the staphylococci did not appear to be higher than those of free-ranging animals. *S. aureus* appears to be a member of the wallaby microbial biome but seemed to be relatively susceptible to antibiotics, apart from the β-lactam family.

CHAPTER 3

CHARACTERISATION OF β-LACTAM MEDIATED RESISTANCE BY *blaZ* IN STAPHYLOCOCCI RECOVERED FROM CAPTIVE AND FREE-RANGING WALLABIES

Declaration for Thesis Chapter 3

Declaration by candidate

In Chapter 3 the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution
Experimental design, performance of experiments, writing, editing and revision of manuscript	80%

The following co-authors contributed to the work.

Name	Nature of contribution	Extent of contribution
Wayne Boardman	Editing and revision of manuscript	5%
Ian Smith	Wallaby nasal sample collection, provision of animal health data	5%
Amanda Goodman	Experimental design, editing and revision of manuscript	5%
Melissa Brown	Editing and revision of manuscript	5%

The undersigned hereby certify the above declaration correctly reflects the nature and extent of the candidate's and co-authors contribution to this work.

Candidate's signature		**01/07/2016**
Supervisor's signature		**01/07/2016**

3.1 Preface

This chapter is based closely on the paper by **Chen MMS**, Boardman WSJ, Smith I, Goodman AE and Brown MH. Characterisation of β-lactam mediated resistance by *blaZ* in staphylococci recovered from captive and free-ranging wallabies. *Journal of Global Antimicrobial Resistance*, 3: 184 - 189 (2015). The printed version of this article appears in Appendix G.

3.2 Abstract

Staphylococci are commensal organisms of animals, but some species are opportunistic pathogens that are resistant to almost all antimicrobial agents in clinical use. Bacterial resistance to β-lactam antimicrobial agents is widespread and has been investigated in species isolated from humans in addition to food production and companion animals. However, minimal progress has been made towards identifying reservoirs of β-lactam-resistant staphylococci in wildlife. This study was aimed at investigating and characterising β-lactamase resistance from staphylococci of wallaby origin. Staphylococci from free-ranging and captive wallabies were assessed for their phenotypic susceptibility to β-lactam antimicrobial agents prior to sequence analysis of their *blaZ* and *blaR1* genes. Deduced amino acid sequences were classified according to the Ambler molecular characterisation method, assigned a protein signature type and compared with sequences generated from previous studies involving isolates from humans, cattle and companion animals. All BlaZ sequences identified in this study were assignable to a pre-existing β-lactamase class and protein signature type, including the more recently discovered protein signature type 12. Three major phylogenetic groups were resolved upon phylogenetic analysis against published BlaZ sequences. This study has found antibiotic-resistant staphylococci both in free-ranging and captive wallaby populations and these bacteria harbour *blaZ* variants that are different to those recovered from humans, cattle and companion animals. Further studies of staphylococci from non-traditional sources are required in order to enhance our knowledge of the epidemiology of antibiotic resistance genes.

3.3 Introduction

Staphylococci are commensal organisms that reside in the nasal cavities and on the skin of animals. Under immunocompromised host conditions, certain species such as *S. aureus*, *S. intermedius* and *S. pseudintermedius* can become opportunistic pathogens in humans and other animals (Wendlandt et al. 2013). An ecological approach should be applied when furthering our understanding of infectious agents in humans. It has been estimated that almost 75% of emerging human pathogens originated in animals. Whilst there have been numerous studies investigating the incidence of staphylococci, in particular carriage of *S. aureus*, in humans, livestock and pets, the same information is lacking for native wildlife and animals in zoological park collections. Constraining epidemiological studies to animals that have the most direct contact with humans provides us with a narrow snapshot of the overall ecology of these pathogens. This was evident during the 1976 Nigerian brucellosis outbreak in the Ibarapa District, which resulted in >45% of cattle in nomadic herds testing positive for acute bovine brucellosis (Alausa 1979). In contrast, all government and privately owned farms were *Brucella*-free. However, the flow-on effects of this outbreak, which effectively halved the number of cattle in the area, resulted in acute meat shortages, malnutrition in the general populace and over 100 human cases of brucellosis (Alausa 1979, Ducrotoy et al. 2014).

Since its introduction into clinical medicine in 1940, penicillin has been the drug of choice for the treatment of staphylococcal infections (Fuda et al. 2005). However, penicillin-resistant staphylococci were reported as early as 1942 (Fuda et al. 2005) and ongoing studies have found the prevalence of penicillin-resistant *S. aureus* to be between 84% and 88% in Australia (Coombs et al. 2014). Penicillin resistance in staphylococci can be mediated by the production of an altered form of penicillin binding protein 2A (PBP2A), encoded by the *mecA* gene, or as a result of enzymatic inactivation of the drug by the β-lactamase enzyme. This enzyme is encoded by *blaZ*, which is part of the three-membered *bla* operon also containing a repressor (*blaI*) and signal transducer/sensor protein (*blaR1*) (Fuda et al. 2005). Under the simplest classification system, four classes (A, B, C and D) of the staphylococcal β-lactamase gene product, BlaZ, have been reported based on conserved and

distinguishing amino acid motifs in the protein sequence (Bush et al. 2010). Classes A, C and D hydrolyse their substrates through a serine active site, whereas class B β-lactamases function through an active-site zinc ion (Olsen et al. 2006, Bush et al. 2010). A detailed typing scheme that groups BlaZ on the basis of their amino acid sequence into one of 12 signature types has been proposed (Olsen et al. 2006). Novel protein signature types are formed when three or more deviations from an existing type are detected (Olsen et al. 2006).

As *blaZ* can be carried on the chromosome and on mobile genetic elements (Livermore 1998), to investigate its spread within a bacterial genus and between strains of the same species it is necessary to combine traditional microbiological techniques with bioinformatic tools. Numerous methods for the phenotypic detection of β-lactam resistance in staphylococci have been established; all had a sensitivity of <72%. This has led to the detection of *blaZ* by PCR to be the recommended gold standard (Pereira et al. 2014). The presence of *blaZ* has been well documented in staphylococci of human and cattle origin (Olsen et al. 2006, Asfour et al. 2011) as well as in cats and dogs (Malik et al. 2007). However, to our knowledge, there are no reports describing *blaZ* in staphylococci of wallaby origin. This study aimed to investigate the natural diversity of the *blaZ* gene from staphylococci of captive Black-flanked Rock wallaby (*P. lateralis*) (BFRW), captive Yellow-footed Rock wallaby (*P. xanthopus*) (YFRW) and captive Mainland Tammar wallaby (*M. eugenii*) (TMW) in addition to free-ranging BFRW both by traditional and molecular techniques.

3.4 Materials and Methods

3.4.1 Bacterial isolates

A total of 89 staphylococcal isolates (56 penicillin-susceptible and 33 penicillin-resistant) were used in the first phase of this study to detect the presence of the *blaZ* gene. These strains originated from a previously described collection of staphylococcal strains isolated from anterior nasal swabs obtained from 68 captive and 30 free-ranging wallabies in South Australia (Chen et al. 2014). Staphylococci were preliminarily identified by Gram-staining, catalase and coagulase production. DNA was extracted using the HiYield™ Genomic Mini kit (Bio-Deal, New Zealand) and identified to species level by 16S rRNA gene sequencing. All Gram-positive, catalase-positive isolates which were

identified as staphylococci by 16S rRNA gene sequencing were added to the strain collection.

3.4.2 PCR detection of the *bla* operon

All 89 isolates were tested using primer pairs 486-488, 487-373 and 487-531 to amplify a 1.16 kb cumulative fragment of the *blaR1-blaZ* genes as previously described (Olsen et al. 2006). This fragment contained the first 209 bp of *blaR1,* a 106 bp non-coding intergenic region and the complete 846 bp *blaZ* gene. As a supplement to primer pair 487-531, primers to amplify a 861 bp fragment (B861) of the *bla* region encompassing *blaZ* (Nannini et al. 2003, Malik et al. 2007) were used on a limited number of isolates. The *blaI* and *blaR1* genes were amplified from strains demonstrating the presence of the *blaZ* gene in addition to selected controls with oligonucleotides *blaI*F 5'-CTAATTTAATAAGAGTCAA GC-3', *blaI*R 5'-TGTTTGGACTTGACCGACAT-3', *blaR1*F 5'-TCCATGACATAC GTGAATTTT-3' and *blaR1*R 5'-ATAATCAAGCGCCCACAGTT-3' to give products of 979 bp and 1033 bp, respectively. These oligonucleotides were designed based on the *bla*-operon from *S. epidermidis* (GenBank accession number X52734).

Each PCR reaction contained 12.5 µl GoTaq® Green Master Mix (Promega, Madison, Wis), 25 pmol each of forward and reverse primer, 5 µg of DNA and 9.5 µl sterile nuclease free water (Promega, Madison, Wis); all reactions were performed with 1.5 mM $MgCl_2$. The PCR cycle conditions for these sets of primers included an initial denaturation of 95°C for 5 minutes, followed by 30 cycles of 94°C for 1 minute, 54°C for 1 minute, 72°C for 1 minute and a final extension at 72°C for 5 minutes. PCR products were separated on 1% agarose gels (Promega, Madison, Wis) in 0.5 x Tris-acetate-EDTA buffer. Resulting gels were stained with ethidium bromide, visualised under UV light and the image analysed on an EZ Imager Gel-Doc (Bio-Rad). PCR products resulting from primer pairs 486-488 and 487-531 as well as representative amplicons from *blaI* and *blaR1* were purified from reaction components using Wizard® SV Gel and the PCR Clean-up System by centrifugation (Promega, Madison, WI) according to manufacturer's instructions and were sequenced by First Base Laboratories, Selangor, Malaysia. The forward primers were also used for determining the DNA sequence.

3.4.3 Nucleotide analysis of *blaZ* and protein signature typing

DNA sequences were assembled manually using BioEdit (version 7.1.11), subjected to homology analysis on NCBI and multiple alignments performed with Clustal X2 with bootstrap 1000. The *bla* operon from *S. epidermidis* (Accession number X52734) was used as a reference in addition to other published *blaZ* sequences available in GenBank. From the 103 *blaZ* sequences available in the public database as of September 2013, 43 sequences with lengths greater than 780 bp were selected for further analysis. Approximate likelihood ratio test analysis was performed by PhyML 3.0 including bootstrap 100 analysis (http://www.phylogeny.fr/version2_cgi/one_task.cgi?task_type=phyml) using default settings. Dendograms were constructed in Newick format using TreeDyn 198.3 (http://www.phylogeny.fr/version2_cgi/one_task.cgi?task_type=treedyn). Nucleotide sequences of *blaZ* genes determined in this study were submitted to GenBank under accession numbers KM362524 - KM362542 and KM368805 - KM368812.

3.4.4 Antibiotic susceptibility testing

A total of 41 staphylococcal strains (n = 25 wallabies), comprising 33 β-lactam (AMP, PEN and OX) resistant isolates from a previous study and eight *blaZ*-positive, β-lactam sensitive isolates identified in Section 2.2 were challenged with the β-lactam antimicrobial agents amoxicillin-clavulanic acid (AMC) (30 µg) to ascertain β-lactamase activity and cefoxitin (FOX) (30 µg) to confirm OX results by Kirby-Bauer disc diffusion according to Clinical Laboratory Standards Institute guidelines (CLSI 2007). *S. aureus* ATCC 6538, *S. epidermidis* ATCC 12228 and *Escherichia coli* DH5α were used as reference strains.

3.4.5 Iodometric detection of β-lactamase activity

β-lactamase activity was detected using a method based on previous publications (Catlin 1975, Kilic and Yalinay Cirak 2006). Briefly, an overnight bacterial culture was pelleted, washed twice with 0.5 M phosphate buffered saline (PBS) and resuspended in 100 µl 0.5 M PBS in 96-well microtitre plates. The cell suspension was standardised to OD_{595} of 2 in a 100 µl volume containing 25 µl 400 µg/ml penicillin (Sigma-Aldrich, Australia) dissolved in distilled water. Cell suspensions were incubated at room temperature for 60 minutes prior to the addition of 20 µl 1 % starch and 10 µl iodine (5 g I_2 and 15 g

KI dissolved in 100 ml distilled water, stored in a dark bottle). Microtitre plates were read with a MultiSkan EX at 10 minute intervals for 70 minutes at 26°C. Assays were performed in triplicate. A positive reaction was recorded upon the colour change from purple to colourless indicating the starch-iodine complex was cleaved by the β-lactamase enzyme (Catlin 1975).

3.5 Results

3.5.1 Phenotypic assessment of β-lactam resistance

Of the 33 penicillin-resistant strains from the previously described strain collection (Chen et al. 2014), nine were found to be resistant to FOX and all were susceptible to AMC. Furthermore, nine strains demonstrated β-lactamase activity in the presence of starch and iodine upon exposure to penicillin (Table 3.1).

3.5.2 Presence of the *bla*-operon in staphylococci of wallaby origin

From the 89 purified strains, 27 demonstrated the presence of the structural *blaZ* gene and signal-transducer gene *blaR1* using PCR; the β-lactamase repressor gene was detected in 11 strains (Table 3.1).

3.5.3 Protein signature types from the BlaZ region

A total of 70 780 bp *blaZ* sequences (24 – 803 of X52734), 43 from the public database and 27 obtained from this study, were analysed. A dendogram was constructed using the deduced amino acid sequences of the 70 *blaZ* sequences and revealed three major phylogenetic groups (I, II and III; Figure 3.1) consisting of four protein signature types (3, 5, 6 and 12) (Table 3.2) encompassing three out of four β-lactamase molecular classes.

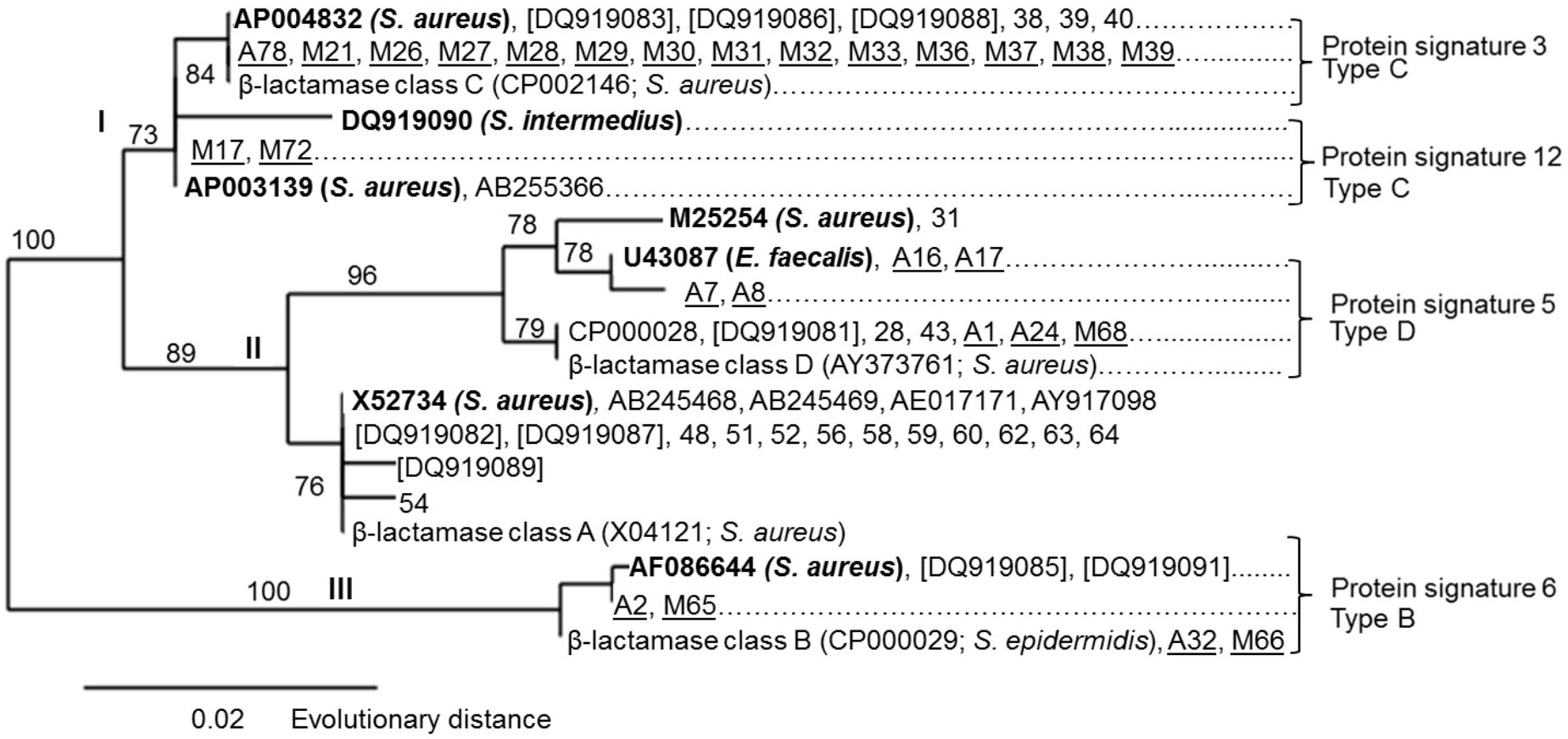
Group I strains were composed of protein signature types 3 and 12 from the β-lactamase class C family. A total of 14 BlaZ sequences, all recovered from captive wallabies, were identified as protein signature type 3 making it the most prevalent signature type in this study. These 14 strains were identical to type strain *S. aureus* AP004832, however only strain *S. aureus* A78, demonstrated the ability to produce β-lactamase (Table 3.1). The more recently identified

Table 3.1: Source of isolates, resistance profile and presence of *bla* genes in staphylococci of wallaby origin

Strain	Species	Location and wallaby specie	Resistance profile					β-lactamase activity	Presence of			Ambler class	Signature Type	*blaZ* accession number
			AMP	PEN	OX	FOX	AMC		*blaZ*	*blaR1*	*blaI*			
ATCC 12228	*S. epidermidis*	-	R	R	S	S	S	Yes	+	+	+	-	-	-
ATCC 6538	*S. aureus*	-	S	S	S	S	S	No	-	-	-	-	-	-
DH5α	*E. coli*	-	S	S	S	S	S	No	-	-	-	-	-	-
A1	*S. warneri*	F-BFRW	R	R	S	S	S	No	+	+	+	D	5	KM362524
A7	*S. aureus*	F-BFRW	R	R	S	S	S	Yes	+	+	+	D	5	KM362525
A8	*S. aureus*	F-BFRW	R	R	S	S	S	Yes	+	+	+	D	5	KM362526
A16	*S. warneri*	F-BFRW	R	R	R	R	S	Yes	+	+	+	D	5	KM362527
A17	*S. warneri*	F-BFRW	R	R	R	R	S	Yes	+	+	+	D	5	KM362528
A24	*S. warneri*	F-BFRW	R	R	S	S	S	No	+	+	+	D	5	KM362529
A78	*S. aureus*	C-BFRW	R	R	S	S	S	Yes	+	+	+	C	3	KM362530
M68	*S. epidermidis*	C-TMW	R	R	S	S	S	No	+	+	+	D	5	KM362531
M72	*S. epidermidis*	C-TMW	R	R	R	R	S	No	+	+	+	C	12	KM362532
A2	*S. epidermidis*	F-BFRW	R	R	S	S	S	Yes	+	+	-	B	6	KM362533
A32	*S. epidermidis*	F-BFRW	R	R	S	S	S	Yes	+	+	-	B	6	KM362534
M21	*S. saprophyticus*	C-YFRW	R	R	R	R	S	No	+	+	-	C	3	KM362535
M27	*S. succinus*	C-YFRW	R	R	S	S	S	No	+	+	-	C	3	KM362536
M28	*S. xylosus*	C-YFRW	R	R	S	S	S	No	+	+	-	C	3	KM362537
M29	*S. xylosus*	C-YFRW	R	R	S	S	S	No	+	+	-	C	3	KM362538
M30	*S. succinus*	C-YFRW	R	R	S	S	S	No	+	+	-	C	3	KM362539
M31	*S. fleurettii*	C-YFRW	R	R	R	S	S	No	+	+	-	C	3	KM362540

M65	*S. epidermidis*	C-TMW	R	R	S	S	S	Yes	+	+	-	B	6	KM362541
M66	*S. epidermidis*	C-TMW	R	R	S	S	S	Yes	+	+	-	B	6	KM362542
M17	*S. saprophyticus*	C-YFRW	S	S	S	S	S	No	+	+	-	C	12	KM368805
M26	*S. simulans*	C-YFRW	S	S	S	S	S	No	+	+	-	C	3	KM368806
M32	*S. delphini*	C-YFRW	S	S	S	S	S	No	+	+	-	C	3	KM368807
M33	*S. delphini*	C-YFRW	S	S	S	S	S	No	+	+	-	C	3	KM368808
M36	*S. xylosus*	C-YFRW	S	S	S	S	S	No	+	+	-	C	3	KM368809
M37	*S. succinus*	C-YFRW	S	S	S	S	S	No	+	+	-	C	3	KM368810
M38	*S. vitulinus*	C-YFRW	S	S	S	S	S	No	+	+	-	C	3	KM368811
M39	*S. vitulinus*	C-YFRW	S	S	S	S	S	No	+	+	-	C	3	KM368812

Abbreviations: F-BFRW, Black-flanked Rock wallaby (*P. lateralis*) from a free-ranging environment; C-BFRW, Black-flanked Rock wallaby (*P. lateralis*) from captivity; C-TMW, Mainland Tammar wallaby (*M. eugenii*) from captivity; C-YFRW, Yellow-footed Rock wallaby (*P. xanthopus*) from captivity; AMP, ampicillin; PEN, penicillin; OX, oxacillin; FOX, cefoxitin; AMC, amoxicillin-clavulanic acid; R, resistant;



S, sensitive; +, amplified; -, not amplified. **Figure 3.1: Phylogenetic analysis of *blaZ* sequences**

Strains in bold represent the type strain. DNA sequence types in numerals refer to Olsen et al. (2006); square brackets refer to Malik et al. (2007); underlined font refers to this study and accession numbers of sequences from GenBank.

Table 3.2: Protein signature types of BlaZ.

Signature type[b]	Strains	BlaZ variable position[a]																								
		1 9	1 0	2 0	2 2	2 4	2 5	5 5	5 7	7 6	8 2	1 1 9	1 1 1	1 3 1	1 3 9	1 4 1	1 4 2	1 4 5	1 9 3	1 9 6	2 0 3	2 0 7	2 1 7	2 2 0	2 4 1	2 4 5	2 5 1	2 6 8
1	**X52734 (*S. aureus*)**[c]	V	I	N	S	A	K	S	V	V	A	T	K	V	Q	R	E	E	K	L	S	P	Y	V	G	V	K
3[d]	**AP004832 (*S. aureus*)** M21[e], A78[g]	V	M	N	S	A	K	S	V	V	E	T	K	V	Q	R	E	K	N	F	N	P	Y	V	G	V	K
5	**U43087 (*E. faecalis*)** A16[g], A17[g]	A	I	N	P	A	K	S	V	I	E	T	K	V	Q	R	G	E	K	L	S	S	C	V	G	V	K
	A7[g], A8[g]																						Y				
	A1[g], A24[g], M68[f]											A															
6	**AF86644 (*S. aureus)*** A32[g], M66[f]	A	I	T	S	A	K	A	V	V	E	K	N	I	K	R	K	K	N	L	N	P	Y	V	N	I	K
	A2[g], M65[f]																							I			
12	**DQ919090 (*S. intermedius)*** M17[e], M72[f]	V	I	N	S	T	Q	S	V	V	E	T	K	V	Q	H	E	K	N	F	N	P	Y	V	G	V	K
											I																

[a] Variable positions are shown and refer to the deduced protein sequence of X52734

[b] Signature types are defined based on Olsen et al. (2006), Malik et al. (2007)

[c] Strains in bold represent the current type strain

[d] This is shared by 14 strains from captive wallabies

[e] Isolate from Yellow-footed Rock wallaby (*P. xanthopus*)

[f] Isolate from Mainland Tammar wallaby (*M. eugenii*)

[g] Isolate from Black-flanked Rock wallaby (*P. lateralis*)

protein signature type 12 DQ919090 from companion from dogs was found in two staphylococcal strains from a captive YFRW and a captive TMW (Table 3.2). These two strains differed from the type strain by a point mutation resulting in the change of valine 86 to isoleucine (Table 3.2).

Group II contained strains with β-lactamase classes A and D and multiple protein signature types. Here, however, only protein signature type 5 from β-lactamase class D was identified in this group (Table 3.2), and these strains were recovered primarily from BFRW. Of these seven strains, two *S. warneri* isolates were identical to type strain *Enterococcus faecalis* U43087 and produced β-lactamase (Table 3.1). Point mutations identified in five isolates of *S. aureus* and *S. warneri* resulted in two different amino acid substitutions (Table 3.2) which seem to have differing effects on β-lactamase activity (Table 3.1).

Group III consisted only of strains with β-lactamase protein signature type 6, belonging to β-lactamase variant class B family (Figure 3.1). Signature type 6 was identified in four *S. epidermidis* strains from two captive TMW and a single free-ranging BFRW (Figure 3.1; Table 3.1). Of the four strains, two were identical to type strain *S. aureus* AF086644 and the remaining two strains contained a single point mutation at residue 241 from a valine to isoleucine (Table 3.2). Irrespective of the mutation, all four strains demonstrated β-lactamase activity.

3.6 Discussion

The presence and diversity of the *bla* operon has been studied in staphylococci from humans, cattle, dogs and cats however this is the first study from commensal staphylococcal in apparently healthy captive and free-ranging wallabies.

This study emphasises the natural diversity of BlaZ amongst wallaby strains of staphylococci and takes the step in understanding the carriage of staphylococci in Australian macropods. The first study to investigate BlaZ diversity in staphylococci reported 69 unique sequences from 105 cattle strains (Olsen et al. 2006). Of these 69, 60 sequences were represented by a single bacterial isolate, with signature type 6 being the most prevalence. Other studies, such as those by Malik et al. (2007) who identified six unique

sequences from 13 cat and dog strains and Bagcigil et al. (2012) who identified 25 unique sequences from 78 cattle also found BlaZ signature type 3 to be most prevalence amongst both coagulase-positive and -negative staphylococci. Here, four unique BlaZ sequences were identified from 27 out of 89 wallaby strains studied with all sequences represented by at least two isolates. From these four sequences, one was unique to free-ranging BFRW and found in strains *S. aureus* A7 and A8. These two strains were isolated from two wallabies living independently of one another in two populations approximately 350 km apart. The second sequence, also a variant of signature type 5, was identified in two free-ranging BFRW sampled in 2009 and a captive TMW sampled in 2010. A third sequence that was a variant of signature type 12 was identified from staphylococci isolated from captive TMW and YFRW. Finally the last sequence, variant of signature type 6, was identified in staphylococci from one free-ranging BFRW and one TMW. Given the spatial and temporal distribution of these wallabies, the discovery of bacteria harbouring these identical signature types is unlikely to be the result of direct transmission and is indicative of the natural diversity of BlaZ. Our results support previous studies where very little exchange of *bla* genes between coagulase-positive and coagulase-negative staphylococci was observed (Olsen et al. 2006).

Furthermore, Milheirico et al. (2011) found no evidence of correlation between *bla* allotypes, the strain background, β-lactam resistance phenotypes or strain origin of *S. aureus* isolates, indicating that the *bla* genes have evolved independently of *S. aureus*. Whilst our study is not as detailed or broad as that described above, we also see some indication of signature type clustering with respect to wallaby species. For example, the majority of BlaZ signature type 5 staphylococci were recovered from free-ranging BFRW and staphylococci from YFRW were associated with either the common BlaZ signature type 3 or the more recently identified BlaZ signature type 12 from pet dogs. However, these observations are preliminary and based on a small sample size; large scale typing and epidemiological studies would need to be performed to substantiate these observations.

Two methods were used to detect β-lactamase activity. The first used a disc diffusion assay with AMC. However, when all strains returned a negative result, indicating that they were susceptible to the actions of clavulanic acid and thus

did not possess a functional β-lactamase enzyme an iodometric method was employed to detect β-lactamase activity. Several observations were made regarding the impact of these naturally-occurring BlaZ mutants. Firstly, β-lactamase activity was not observed in *S. warneri* (A1, A24) and *S. epidermidis* M68) strains carrying the genes encoding products with protein signature type 5 containing an alanine in place of a threonine at residue 119 whereas modification at residue 220 had no effect on β-lactamase activity. Secondly, all mutants of protein signature type 6 strains had demonstrable levels of β-lactamase activity. Thirdly, residue 86 in protein signature type 12 appears to play a minimal role in conferring β-lactam resistance; the two strains containing this mutation had opposing antibiograms, type strain *S. intermedius* DQ919090 was resistant only to penicillin (Malik et al. 2007).

Diversity amongst BlaZ sequences was demonstrated by phylogenetic analysis with the emergence of three main groups (Figure 3.1). All BlaZ sequences examined in this study belonged to one of the four molecular classes of β-lactamase and to a previously described signature type. Surprisingly no class A β-lactamase genes were identified in this study which was unusual given this group of enzymes are the predominant β-lactamases in Gram-positive cocci (Bush et al. 2010).

The expression of the β-lactam resistant phenotype could be a result of the expression of more than one gene. Our results indicating that the detection of *blaZ* could not be used to assume the presence of *blaI* and *blaR1*, is comparable to Milheirico et al. (2011). Similar to previous authors (Shuford et al. 2006, Malik et al. 2007), we detected staphylococcal strains which were β-lactam resistant but negative for the *blaZ* gene by PCR (data not shown). Conversely, detection of the *blaZ* gene does not guarantee the demonstration of a β-lactam resistant phenotype as was seen in eight isolates. Analysis of *blaZ* sequences from these eight β-lactam susceptible isolates revealed no variations compared to their β-lactam resistant counterparts. Likewise, other studies by Pereira et al. (2014) and Papanicolas et al. (2014) have also found strains that exhibited sensitivity to penicillin, did not produce the β-lactamase enzyme and yet were positive for the *blaZ gene* using conventional PCR, sequencing and real-time PCR. Genetic data found no differences in the *blaZ* region between the β-lactam resistant and sensitive isolates (Pereira et al. 2014). This finding

could be the result of DNA mutations in the regions encoding the repressor (*blaI*) or promoter (*blaR1*) with data indicating that the promoter region is the primary target for the accumulation of mutations, presumably in response to the different types of β-lactam compounds (Milheirico et al. 2011).

3.7 Conclusion

In summary, this study indicates that *blaZ* plays a role in β-lactam resistance in staphylococci isolated from wallabies and that it is comparable to the *blaZ* genes of human, cattle, dog and cat origin. Our finding of phenotypically penicillin-sensitive staphylococcal isolates presenting with *blaZ* gene sequences which were 100% identical to their penicillin-resistant counterparts is corroborated by other authors in the field. In light of the diversity of *blaZ* sequences uncovered from free-ranging BFRW, further longitudinal studies encompassing more native wildlife species are required in order to enhance our studies of the epidemiology of antibiotic resistance genes and how they may relate to their evolution or relationship with certain animal hosts.

CHAPTER 4

METHICILLIN RESISTANCE GENE DIVERSITY IN STAPHYLOCOCCI ISOLATED FROM WALLABIES

Declaration for Thesis Chapter 4

Declaration by candidate

In Chapter 4 the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution
Experimental design, performance of experiments, writing, editing and revision of manuscript	85%

The following co-authors contributed to the work.

Name	Nature of contribution	Extent of contribution
Wayne Boardman	Editing and revision of manuscript	5%
Melissa Brown	Editing and revision of manuscript	10%

The undersigned hereby certify the above declaration correctly reflects the nature and extent of the candidate's and co-authors contribution to this work.

Candidate's signature		**01/07/2016**
Supervisor's signature		**01/07/2016**

4.1 Preface

This chapter is based closely on the paper by **Chen MMS**, Boardman WSJ and Brown MH. Methicillin resistance gene diversity in staphylococci isolated from captive and free-ranging wallabies. *Infection, Ecology and Epidemiology*, doi: 10.3402/iee.v5.31507 (2016). The printed version of this article appears in Appendix I.

4.2 Abstract

Infection with methicillin-resistant staphylococci (MRS) can be life-threatening in humans and its presence in animals is a cause for public health concern. The aim of this study was to measure the prevalence of MRS in captive and free-ranging wallabies' over a 16-month period in South Australia, Australia. Eighty-nine purified staphylococcal isolates recovered from 98 captive and free-ranging wallabies anterior nasal swabs were used in this study. All isolates were tested for the presence of the *mecA, mecA1* and *mecC* genes. Multiplex PCR-directed SCC*mec* typing, *ccrB*-typing and determination of the minimal inhibitory concentration of oxacillin was performed on *mec*-positive isolates. A total of 11 non *S. aureus* MRS were isolated from seven out of 98 animals, corresponding to a 7.1% carriage rate. The SCC*mec* types I, III and V were identified by multiplex PCR and sequencing of the *ccrB* gene. This is the first report of MRS carriage in both captive and free-ranging wallabies in Australia. These data demonstrate a low prevalence of MRS and no association between wallaby captivity status and MRS carriage could be assigned. These animals may act as a reservoir for the exchange of genetic elements between staphylococci. Furthermore, the *mecA* genes of animal isolates were identical to that found in human MRS strains and thus the possibility of anthroponotic transfer must be considered

4.3 Introduction

MRSA is a frequent pathogen of humans and many animal species. Additionally, methicillin-resistant coagulase-negative staphylococci (MRCNS) has long been recognised as important human and animal pathogens. Both MRSA and MRCNS are of interest to human and animal medicine and are collectively known as MRS. The *mec* genes encoding resistance to methicillin and almost all β-lactam antibiotics, are carried by the large mobile genetic

element, SCC*mec*, which is able to integrate into the staphylococcal chromosome at a specific site within the 3' end of the ribosomal methyltransferase (Ito et al. 2001, Shore et al. 2013). The SCC*mec* element is highly variable in various staphylococcal species. To date, 11 SCC*mec* types (I – XI) and numerous subtypes have been identified in MRSA (Ito et al. 2009, Monecke et al. 2012, Shore et al. 2013). In 2011, a new divergent *mecA* homologue, designated *mecC*, located in a new SCC*mec* cassette designated SCC*mec* type XI, was described in *S. aureus* (Garcia-Alvarez et al. 2011, Shore et al. 2011). This homologue is not detectable using routine *mecA*-specific PCR approaches and various studies have searched for this new element in different animal hosts (Basset et al. 2013, Loncaric et al. 2013, Deplano et al. 2014, Porrero et al. 2014).

The origins of the SCC*mec* elements remain unknown, but it is believed that the *mecA* gene began with a single common ancestor. Homologues of the *mecA* gene have been found in *S. sciuri*, *S. vitulinus* and *S. fleurettii.* Furthermore, the *mecA* gene of *S. fleurettii* has 99 to 100% sequence homology to MRSA strain N315 thus indicating that a direct precursor to the methicillin resistance determinant for MRSA is present in *S. fleurettii* (Wu et al. 2001, Schnellmann et al. 2006, Tsubakishita et al. 2010b, Tulinski et al. 2012).

While studies on MRSA in humans, companion animals and livestock have been widely documented, there is still a scarcity of information on infections, carriage and the role of this particular pathogen in wildlife. Here, we report the assignment of mec types to staphylococcal isolates and from captive and free-ranging wallabies from South Australia, Australia.

4.4 Materials and methods

4.4.1 Bacterial isolates

A collection of 89 staphylococcal isolates obtained from captive and free-ranging wallabies in a surveillance study undertaken in 2009-2010 was used in this study (Chen et al. 2014). Free-ranging wallaby samples were obtained from three colonies living in the Anangu Pitjantjatjara Yankunytjatjara (APY) Lands (102,650 km^2). The APY Lands, classified as a 'managed conservation and natural environment resource' by the Australian Department of Agriculture, are located in the far north-west corner of South Australia, Australia and are home to approximately 2,230 people across 33 communities and outstations

(ABARES 2016). Anterior nasal swabs were collected from 68 captive and 30 free-ranging wallabies during routine health examinations. All animals were assessed as apparently healthy at the time of sampling. Staphylococcal species identification, antibiotic susceptibility profiles and the presence of the β-lactamase resistance operon from the 89 isolates have been reported previously (Chen et al. 2014, 2015b).

4.4.2 Detection of the *mecA1, mecA* and *mecC* elements

All 89 staphylococcal strains were screened for the presence of *mecA* as previously described (Malik et al. 2006b). Additional PCRs were performed for the detection of the *mecC* gene identified in all cefoxitin-resistant strains (Garcia-Alvarez et al. 2011, Shore et al. 2011). Furthermore, all *S. sciuri* isolates were screened for the presence of the ubiquitous *mecA1* homologue (Couto et al. 2003).

We tested the hypothesis that the carriage of *mecA* and *mecC* in staphylococci would be impacted by the captivity status of the wallaby host. The significance of these data was determined by using chi-square analysis. A *p*-value ≤0.05 was regarded as being statistically significant. The *p*-values are shown only for results that were statistically significant (Dahiru 2008).

4.4.3 Phenotypic screening of cefoxitin resistance

A total of 22 oxacillin-resistant isolates from the 2009-2010 surveillance study and 11 *mecA*-positive isolates identified and described above, were assessed for their ability to grow in the presence of cefoxitin by Kirby-Bauer disc diffusion test with a 30 µg cefoxitin disc (Oxoid, Basingstoke, UK). Inoculum preparation, inoculation and incubation were performed as recommended by the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI 2007). Instead of clinical breakpoints, for the present study, the results were evaluated according to the epidemiological cut-off values of the European Committee for Antimicrobial Susceptibility Testing (EUCAST) susceptibility breakpoints for *S. aureus* and coagulase-negative staphylococci. Data from the EUCAST disc diffusion distribution website was last accessed January 2016 (http://www.eucast.org). *S. aureus* strain ATCC 6538 and a clinical MRSA SCC*mec* II isolate were used as internal quality controls.

4.4.4 Determination of oxacillin minimum inhibitory concentration

Oxacillin MIC analysis was carried out on 11 MRS strains and two isolates that exhibited discordant results for *mecA* PCR and cefoxitin resistance. Bacterial suspensions were adjusted to 0.5 McFarland standard in 0.85% saline. A total of 14 two-fold dilutions of oxacillin were made to cover the concentrations 0.03125 to 256 µg/ml in Mueller-Hinton broth supplemented with 2% NaCl. The test was incubated aerobically at 37°C for 24 hours and results read with a MultiSkan® EX Type 355 (Thermo Fisher Scientific, SA, Australia) spectrophotometer at 595 nm. Interpretation of MIC breakpoints for all strains followed the guidelines provided by EUCAST; data from the EUCAST MIC distribution website was last accessed January 2016 (http://www.eucast.org). A clinical MRSA SCC*mec* II, *S. aureus* ATCC 6538 and *S. epidermidis* ATCC 12228 were used as quality control strains in addition to a sterility control.

4.4.5 Typing of SCC*mec* and *ccr* elements

All 11 *mecA*-positive isolates were typed using multiplex-PCR methodologies 1 and 2 with 1.5mM $MgCl_2$ to classify isolates into the main SCC*mec* types I, II, III, IV and V. SCC*mec* IV subtyping was performed with multiplex PCR 3 (Kondo et al. 2007). Supplementary SCC*mec* and *ccr* typing methodologies (Lim et al. 2003, Zhang et al. 2009) and (Oliveira et al. 2008) were used in selected *mecA*-positive strains that could not be assigned a SCC*mec* type with the aforementioned multiplex PCR. Appropriate control strains that have been previously assigned to *mec* classes from Malik et al (2007) were included for the SCC*mec* typing protocols (Malik et al. 2007).

4.4.6 Nucleotide analysis of *mec* and phylogenetic analysis

DNA sequences were assembled manually using BioEdit v.7.1.11 (Hall 1999) (available at http://www.mbio.ncsu.edu/bioedit/bioedit.html), subjected to homology analysis on National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/genbank/) and multiple alignments performed with Clustal X2 with bootstrap 1000 (Larkin et al. 2007) (available at http://www.clustal.org/clustal2/). The *mec* sequences from Ito et al. (2012) were used as references. Approximate likelihood ratio test analysis was performed using PhyML 3.0 with bootstrap 100 analysis (http://www.phylogeny.fr/one_ task.cgi?task_ type=phyml) using default settings. Dendrograms were constructed in Newick format using TreeDyn 198.3

(http://www.phylogeny.fr/one_task.cgi?task_type=treedyn). Nucleotide sequences of *mecA* and *ccrB* genes determined in this study were submitted to GenBank under accession numbers KT021005 – KT021015 and KT003668 and KT003669.

4.5 Results

4.5.1 Detection and diversity of staphylococcal *mec* genes from wallabies

Initially, the presence of *mecA* or *mecC* was set as the gold standard to consider an isolate as a MRS irrespective of its phenotype. The *mecA* gene was detected and subsequently sequenced from 11 staphylococcal isolates. These 11 isolates originated from seven out of the 98 sampled animals, corresponding to a MRS carriage rate of 7.1%. The *mecA1* gene purportedly present in every *S. sciuri* isolate was detected in only two out of the four *S. sciuri* isolates recovered in this study (data not shown) and the *mecC* gene was not identified in any isolates.

Sequencing and subsequent bioinformatic analysis of the 11 *mecA* and two *mecA1* genes revealed that irrespective of resistance phenotype all seven methicillin-resistant *S. fleurettii* isolates contained 19 nucleotide variations in the *mecA* gene and that these variations were comparable to those found in the NCBI database. The remaining four *mecA* sequences from *S. cohnii, S. epidermidis* and *S. warneri* were comparable to human-derived MRSA strains found in NCBI. Finally, the *S. sciuri mecA1* genes recovered in this study demonstrated 97 to 99% sequence homology to type strains K11 and ATCC 700061 across 1016 bp (data not shown).

4.5.2 Antimicrobial susceptibility testing

All MRS isolates were found to be susceptible to gentamicin, streptomycin, vancomycin and chloramphenicol (Table 4.1) as assessed by Kirby-Bauer disc diffusion. Interestingly, two isolates of *S. fleurettii* (M11, M47) were identified to be β-lactam sensitive by disc diffusion and three cefoxitin-sensitive *S. fleurettii* isolates were identified. Despite their phenotypic resistance profiles, all five isolates demonstrated oxacillin MIC values greater than 16 µg/ml (Table 4.1). Conversely, *mecA*- and *mecC*- negative isolates M21 (*S. saprophyticus*) and M54 (*S. succinus*) which demonstrated resistance towards AMP, PEN, OX and FOX and had MIC values of 32 µg/ml (Table 4.1). These two isolates were

Table 4.1: Phenotypic and genotypic characterisation of methicillin-resistant staphylococcal isolates from wallabies

Strain	Specie	Location and wallaby specie	OX MIC (µg/ml)	Resistance profile												SCC*mec* typing				
				AMP	OX	PEN	AMC	FOX	CN	S	VA	CTX	TE	E	C	*ccr* complex		*mec*	SCC*mec*	*mecA* accession number
																Multiplex PCR [a]	*ccrB* typing [b,c]			
MRSA	*S. aureus*		8	R	R	R	S	R	R	S	S	S	S	S	S	A2B2	ND	A	II	NA
ATCC 6538	*S. aureus*		0.047	S	S	S	S	S	S	S	S	S	S	S	S	-	-	-	NA	NA
A31	*S. cohnii*	F-BFRW	0.625	R	R	R	S	R	S	S	S	R	S	R	S	-	A1B1 (117)	A	I	KT021007
M72	*S. epidermidis*	C-TMR	1.5	R	R	R	S	R	S	S	S	iR	S	S	S	A3B3 + C	A4B4 (602)	C C	III	KT021015
A59	*S. fleurettii*	C-BFRW	16	R	R	R	S	R	S	S	S	iR	S	S	S	-	-	A	NT	KT021008
A61	*S. fleurettii*	C-BFRW	32	R	R	R	S	R	S	S	S	iR	S	S	S	-	-	A	NT	KT021009
A69	*S. fleurettii*	C-BFRW	32	R	R	R	S	S	S	S	S	iR	S	S	S	-	-	A	NT	KT021010
A72	*S. fleurettii*	C-BFRW	32	R	R	R	S	S	S	S	S	iR	S	S	S	-	-	A	NT	KT021011
M11	*S. fleurettii*	C-YFRW	16	S	S	S	S	S	S	S	S	iR	S	S	S	-	-	A	NT	KT021012
M31	*S. fleurettii*	C-YFRW	16	R	R	R	S	S	S	S	S	iR	S	S	S	-	-	A	NT	KT021013
M47	*S. fleurettii*	C-YFRW	16	S	R	S	S	S	S	S	S	S	S	S	S	-	-	A	NT	KT021014
A16	*S. warneri*	F-BFRW	4.5	R	R	R	S	R	S	S	S	iR	R	R	S	5	-	C	V	KT021005
A17	*S. warneri*	F-BFRW	4	R	R	R	S	R	S	S	S	iR	R	R	S	5	-	C	V	KT021006
M21	*S. saprophyticus*	C-YFRW	32	R	R	R	S	R	S	S	S	S	S	R	S	-	-	-	-	NT
M54	*S. succinus*	C-TMR	32	R	R	R	S	R	S	S	S	S	S	S	S	-	-	-	-	NT

[a] Multiplex PCR performed as recommended by Kondo et al (2007)

[b] *ccrB* typing performed as recommended by Oliveira et al (2008)

[c] *ccrB* sequence submitted to Genbank under accession numbers: KT003668 and KT003669

Abbreviations: F-BFRW, Black-flanked Rock wallaby (*P. lateralis*) from a free-ranging environment; C-BFRW, Black-flanked Rock wallaby (*P. lateralis*) from captivity; C-TMW, Mainland Tammar wallaby (*M. eugenii*) from captivity; C-YFRW, Yellow-footed Rock wallaby (*P. xanthopus*) from captivity; R, resistant; iR, intermediate resistant; S, susceptible; -, no amplification; NA, not available; NT, not typeable; AMP, ampicillin; OX, oxacillin; PEN, penicillin; AMC, amoxicillin-clavulanic acid; FOX, cefoxitin; CN, gentamicin; S, streptomycin; VA, vancomycin; TE, tetracycline; E. erythromycin; C. chloramphenicol

included in subsequent SCC*mec* typing protocols to aid in the determination of the genetic mechanism behind this irregular phenotypic profile.

4.5.3 Typing of SCC*mec* elements from MRS

A total of four different staphylococcal species were identified from 11 *mecA* positive isolates (Table 4.1) with the majority dominated by *S. fleurettii* from the *S. sciuri* species group. Overall, five different *ccr-mec*-complex combinations were detected in these 11 MRS isolates. These combinations could be broadly separated into three categories based on the method of classification. Firstly, SCC*mec* types III (M72) and V (A16 and A17) were identified by multiplex PCR. Secondly, 2 novel variants were identified. Novel variant one (A31) was identified by the unique combination of a novel *ccrAB1* allele 117 and a class A *mec* element. Novel variant two was characterized by the detection of a type 4 *ccrB* element from the aforementioned SCC*mec* III strain M72. Finally, a single non-typeable variant was identified. This non-typeable element accounted for over half (63.6%) of all SCC*mec* elements identified and all were found in *S. fleurettii* strains (Table 4.1). No SCC*mec* element genes could be detected for the two *mecA* and *mecC*-negative cefoxitin-resistant strains (M21 and M54).

4.6 Discussion

A systematic review of the literature investigating antimicrobial-resistant bacteria in wildlife populations yielded 210 studies up until mid-2015 (Vittecoq et al. 2016). Studies investigating the presences of *E. coli* (115 studies), *Salmonella spp* (54 studies) and *Enterococcus spp* (43 studies) comprised the bulk of the literature. However, analysis of staphylococcal carriage involving free-ranging animals are scarce as the majority of studies conducted have focused on captive animals which have had regular contact with humans (Aslantas et al. 2012, Vercammen et al. 2012, Wardyn et al. 2012). However, in recent years, a trend of sampling free-ranging animals for the purpose of determining the prevalence of MRS has emerged (Loncaric et al. 2013, Gomez et al. 2014, Loncaric et al. 2014a). Most recently, analysis of faecal pellets from captive and free-ranging brush-tailed rock wallabies revealed the presence of class 1 integrons via PCR amplification (Power et al. 2013). However, the present study is the first to investigate the carriage of MRS using nasal swabs collected from apparently healthy captive and free-ranging wallabies.

Worldwide, a large range of MRS incidence rates have been reported ranging from 15.4% in Turkish dogs (Aslantas et al. 2013), 28.6% in Polish riding horses (Karakulska et al. 2012), 29.5% in Belgian pigs (Vanderhaeghen et al. 2012b) and up to 43.0% in Danish goat and sheep (Eriksson et al. 2013). Previous studies in Australia has found MRS carriage rates to be 4.0% in South Australian cats and dogs (Malik et al. 2006b), 3.7% in horses admitted to a veterinary intensive care unit in New South Wales (Axon et al. 2011) and 0.9% in a nationwide study of pigs (Groves et al. 2014). In the present study, surveillance of healthy wallabies revealed MRS carriage rates of 7.4% for captive wallabies (five out of 68 animals) and 10.0% for free-ranging animals (three out of 30 animals). These carriage rates however, were not considered statistically significant and thus indicate that the carriage of MRS was not affected by the captivity status of the wallabies. This finding is significant as we had expected wallabies with close association with humans to carry MRS more readily compared to those in a low-human environment.

The CLSI now recommends the use of cefoxitin discs as oppose to oxacillin discs for the detection of methicillin resistance in staphylococci by Kirby-Bauer disc diffusion and the amplification of the *mecA* gene by PCR as the gold standard (CLSI 2010). However, similar to other authors Cuirolo et al. (2010) we detected staphylococcal isolates which contained the *mecA* gene but were susceptible to both oxacillin and cefoxitin by disc diffusion. Our strains had a non-induced oxacillin MIC value of 32 µg/ml compared to 1 µg/ml observed by Cuirolo et al. (2010). This demonstrates the limitations of using cefoxitin as a predictor of *mecA*-mediated methicillin resistance.

Many SCC*mec* typing strategies have been developed since the publication of its discovery in 2000 (Katayama et al. 2000). However, the majority of studies have been conducted with human samples and thus most methodologies were designed specifically for the detection of MRSA. As all MRS identified in this study were CNS, a range of multiplex PCR strategies were selected from the literature and trialed in this study. No PCR amplicons could be obtained from the 11 MRS isolates using PCR methodologies from Lim et al (2003) or Zhang et al (2009). SCC*mec* typing by the Kondo multiplex PCRs showed the carriage of known types (III and V). However, the majority of *mec* elements, identified as containing class A *mec* elements, were unable to be classified by this method.

MRCNS are considered to be a source of SCC*mec* elements by horizontal gene transfer to *S. aureus* and the diversity of SCC*mec* element types amongst CNS is larger than that among *S. aureus* (Tulinski et al. 2012). Whilst *S. aureus* was not found in co-existence with MRCNS in our nasal swabs, native wildlife have already been shown to potentially act as a reservoir for multidrug-resistant staphylococci (Chen et al. 2014) and could function as a reservoir for the evolution of novel SCC*mec* types. This is extremely worrisome given that *S. epidermidis* and the *S. sciuri* species group are also opportunistic pathogens and their zoonotic potential cannot be discounted (Argudin et al. 2015). In this study two novel variants of existing SCC*mec* types were identified by sequencing the *ccrB* gene. Variant one contained a class A *mec* element with a previously undescribed variant of *ccrAB1* thus leading to the formation of a novel SCC*mec* I variant (*S. cohnii* strain A31). Variant two was identified by the discovery of a type 4 *ccrAB* element in *S. epidermidis* SCC*mec* III strain M72. Furthermore, two MRS isolates (*S. warneri* strains A16 and A17) from a single free-ranging wallaby were identified as harbouring community-acquired SCC*mec* type V. This SCC*mec* type was first identified in the Australian indigenous population in 2005 (O'Brien et al. 2005) and the discovery of this element in free-ranging wildlife living in indigenous land is further evidence of its community origin.

4.7 Conclusion

To the best of our knowledge, this is the first report of MRS in captive and free-ranging wallabies in Australia. Our data demonstrates the absence of MRSA and a low prevalence of MRCNS in both captive and free-ranging wallabies, indicating that MRS occurs naturally even in the absence of human intervention. However, the presence of multi-drug resistant staphylococci carrying the *mecA* gene isolated from indigenous land is worrisome may have implications for wildlife rehabilitation and subsequent antimicrobial treatment in cases of wound infections, localised inflammation and systemic bacterial infections. This study highlights the need for further longitudinal and environmental studies involving a larger range of native wildlife species. These studies are required in order to increase our understanding regarding the epidemiology of resistance genes in non-human populations.

CHAPTER 5

CLONAL DIVERSITY OF METHICILLIN-SENSITIVE *STAPHYLOCOCCUS AUREUS* FROM SOUTH AUSTRALIAN WALLABIES

Declaration for Thesis Chapter 5

Declaration by candidate

In Chapter 5 the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution
Performance of experiments, result interpretation, writing, editing and revision of manuscript	60%

The following co-authors contributed to the work.

Name	Nature of contribution	Extent of contribution
Stefan Monecke	Experimental design, performance of experiments, results interpretation, editing and revision of manuscript	30%
Melissa Brown	Editing and revision of manuscript	10%

The undersigned hereby certify the above declaration correctly reflects the nature and extent of the candidate's and co-authors contribution to this work.

Candidate's signature		**01/07/2016**
Supervisor's signature		**01/07/2016**

5.1 Preface

This chapter is based closely on the paper by **Chen MMS**, Monecke, S and Brown MH. Clonal diversity of methicillin-sensitive *Staphylococcus aureus* from South Australian wallabies. *One Health*, doi: 10.1016/j.onehlt.2015.12.001 (2015). The printed version of this article appears in Appendix H.

5.2 Abstract

Seven methicillin-susceptible *S. aureus* nasal isolates from apparently healthy captive and wild wallabies were characterised by DNA microarray and antibiotic susceptibility assays. Isolates were found to belong to uncommon clonal complexes including those previously associated with birds, pigs and humans.

5.3 Introduction

S. aureus is a versatile bacterium which can infect or colonise a variety of mammals, including humans. With the use of molecular techniques, *S. aureus* strains can be differentiated assigning them to clonal complexes (CC) (Monecke et al. 2008). Clonal complexes group bacterial isolates from the same species based on the genetic variation present in seven housekeeping genes. This variation can be used to infer the relationship of all isolates within a particular CC to a common ancestor (Spratt et al. 2004). Some CCs have been identified predominantly in specific hosts, such as CC692 in birds whereas others, such as CC15, are less specific (Monecke et al. 2011, Monecke et al. 2014).

5.4 Materials and methods

5.4.1 Bacterial isolates

Anterior nasal swabs were collected from 68 captive and 30 free-ranging wallabies (*P. lateralis*, *P. xanthopus* and *M. eugenii*) at two locations in South Australia, the Monarto Zoo (a 1,000 hectare open range zoo) and the APY Lands (a lightly populated remote indigenous land), during routine health examinations between July 2009 and October 2010. From these 98 nasal swabs, a total of seven *S. aureus* isolates were identified from five captive animals and two free-ranging animals (Table 5.1). Isolates were identified with a

combination of biochemical assays and 16S rRNA gene sequence analysis (Chen et al. 2014).

5.4.2 Antibiotic susceptibility testing

Antimicrobial susceptibility tests revealed that four strains were susceptible to every antimicrobial agent tested (Chen et al. 2014, 2015b). Antibiotics tested included β-lactams, aminoglycosides, macrolide, glycopeptide, cephalosporin, tetracycline and chloramphenicol. Three strains, A7, A8 and A78, exhibited ampicillin and penicillin resistance with A8 also demonstrating intermediate resistance towards cefotaxime (Table 5.1).

5.4.3 Molecular characterisation of *S. aureus* clonal complexes

Molecular characterisation of the *S. aureus* isolates was performed by DNA microarray analysis (StaphyType, Alere Technologies, Jena, Germany) using previously described protocols and modified to include probes for *mecC* and the SCC*mec*-XI-associated *blaZ* allele (Monecke et al. 2012). The assignment of isolates to a CC was determined by an automated comparison of hybridisation profiles to reference profiles (Monecke et al. 2008).

5.5 Results

5.5.1 Molecular characterisation of *S. aureus* clonal complexes

Analyses identified a single CC692 strain, three CC49 and three CC15 strains. All strains demonstrated the absence of methicillin-resistance genes and were thus classified as methicillin-sensitive *Staphylococcus aureus* (MSSA). Microarray analysis also revealed that these strains possessed limited antimicrobial resistance determinants confirming the above mentioned phenotypic analyses, with the *bla*-operon being the only one identified (Table 5.1) (see Appendix E for detailed results).

5.6 Discussion

Virulence genes common in staphylococci including those encoding enterotoxins and exfoliative toxins (*eta, etb* and *etd*), epidermal cell differentiation inhibitors (*edinA, edinB* and *edinC*), toxic shock syndrome toxin (*tst*) and the Panton-Valentine leucocidin toxin (*lukF*-PV and *lukS-PV*), were not found. These data combined with veterinary records which indicated these animals were in a non-diseased state support *S. aureus* status as commensal

Table 5.1: Microbiological characteristics of commensal MSSA in the nasal passages of South Australian wallabies

Isolate	Specie	Location and wallaby specie	Clonal complex	Antibiotic susceptibility profile [b]												Microarray-based analysis
				AMP	OX	PEN	AMC	FOX	CN	S	VA	CTX	TE	E	C	Resistance genes
A7[a]	*S. aureus*	F-BFRW	CC15	R	-	R	-	-	-	-	-	-	-	-	-	*blaZ, blaI, blaR1*
A8[a]	*S. aureus*	F-BFRW	CC15	R	-	R	-	-	-	-	-	iR	-	-	-	*blaZ, blaI, blaR1*
A78[a]	*S. aureus*	C-BFRW	CC15	R	-	R	-	-	-	-	-	-	-	-	-	*blaZ, blaI, blaR1*
A70[a]	*S. aureus*	C-BFRW	CC49	-	-	-	-	-	-	-	-	-	-	-	-	None detected
A73[a]	*S. aureus*	C-BFRW	CC49	-	-	-	-	-	-	-	-	-	-	-	-	None detected
M9[a]	*S. aureus*	C-YFRW	CC49	-	-	-	-	-	-	-	-	-	-	-	-	None detected
M7[a]	*S. aureus*	C-YFRW	CC692	-	-	-	-	-	-	-	-	-	-	-	-	*fosB*

[a] Data pertaining to antibiotic susceptibility profiles and isolate captivity status have been previously published (Chen et al. 2014) but have been included here to aid the reader in analysis.

[b] Disk diffusion breakpoints were determined as previously described (Chen et al. 2014).

Abbreviations: F-BFRW, Black-flanked Rock wallaby (*P. lateralis*) from a free-ranging environment; C-BFRW, Black-flanked Rock wallaby (*P. lateralis*) from captivity; C-YFRW, Yellow-footed Rock wallaby (*P. xanthopus*) from captivity; -, sensitive; R, resistant; iR, intermediate resistant; AMP, ampicillin; OX, oxacillin; PEN, penicillin; AMC, amoxicillin-clavulanic acid; FOX, cefoxitin; CN, gentamicin; S, streptomycin; VA, vancomycin; CTX, cefotaxime; TE, tetracycline; E, erythromycin; C, chloramphenicol

organism in this population. All isolates carried a variety of genes encoding proteins associated with adherence to host structures. The presence of these genes as well as their assignment to allelic variants depended on CC affiliation and not on host species as there was no difference to previously described isolates of the same CCs from other hosts (Monecke et al. 2008, Monecke et al. 2009, Overesch et al. 2011).

To date, CC692 has been isolated exclusively from various birds and as such, to the best of our knowledge, this represents the first report of CC692 from any mammalian species (Monecke et al. 2014). For CC49-MSSA, only a few human and veterinary cases have been reported from Western Europe. CC49-MRSA were observed in pigs and rats (the latter being *mecC*-positive) (Overesch et al. 2011, Paterson et al. 2012). CC15-MRSA is very common in humans; studies from Europe showed it to be one of the most prevalent lineages (Monecke et al. 2009). Isolates of CC15 were an unexpected finding in two free-ranging wallabies and a single captive wallaby. These three CC15 strains had very similar hybridisation profiles to the 115 *S. aureus* CC15 strains examined from asymptomatic human carriers in Germany, including the presence of immune evasion genes *chp* and *scn* and the absence of staphylokinase gene *sak,* both considered typical qualities of this lineage (Monecke et al. 2011). This provides further support to the notion that these strains are commensal organisms and are not involved in disease. Since immune evasion cluster genes are apparently host-specific and their carriage might rapidly change in adaption to a new host, this observation might suggest a recent transmission of CC15 from humans to wallabies.

5.7 Conclusion

This study describes the first genotyping data on commensal *S. aureus* from South Australian native wallabies. Results indicate that wallabies did not harbor unique host-specific strains as the three identified CCs have been described in other species. Reassuringly, resistance genes were rare with no MRSA recovered amongst the seven isolates from apparently healthy wallabies. The absence of both common virulence genes in conjunction with resistance genes provides further confirmation to support these strains status as commensal organisms. Additionally, given the *S. aureus* genotypes are typically associated with humans and birds, interspecies transmission from non-macropod hosts

cannot be ruled out. These findings also raise questions about which genotypes can be considered the indigenous flora of wallabies and there was no evidence for a zoonotic background of particular “Australian” clones of *S. aureus* such as ST93 and ST1850.

CHAPTER 6
GENERAL DISCUSSION AND CONCLUSION

Staphylococci are one of the most diverse and versatile bacterial species which can colonise its host in either a benign commensal or as a pathogen. This species has been documented to colonise hosts as diverse as humans, reptiles in addition to terrestrial and aquatic mammals. Staphylococcal species are generally known as opportunistic pathogens which by definition are: microbes capable of causing host damage as a result of direct microbial action or the host immune response (Casadevall and Pirofski 1999). Infections caused by *S. aureus* have been categorised as instigating a class 3 host response and are on-par with pathological conditions caused by *Mycobacterium tuberculosis*, *E. coli* 0157:H7, measles and influenza (Casadevall et al. 1999). Staphylococci, in particular *S. aureus,* has the ability to survive on almost any surface for many months (Wertheim et al. 2005). Furthermore, there is evidence to suggest that households comprising of five or more members are positively associated with *S. aureus* carriage and that the same strains were carried by mother and child (Wertheim et al. 2005).

Over the past decade, the number of reported zoonotic disease transmission events has increased, highlighting the limitations in our existing global disease surveillance capacity. Successful surveillance of zoonoses must not be limited to humans, companion animals and livestock. Zoonotic pathogens do not distinguish between humans, companion animals, livestock or other sentinels. Furthermore, zoonoses with wildlife origins have been identified as the most significant emerging disease threat to global public health (Jones et al. 2008). Surveillance needs to include all animals that have the potential to be sentinels for the carriage of antibiotic-resistant organisms. Animals that could act as sentinels include those in zoological collections and in repopulation programs.

This thesis has endeavoured to understand the prevalence and diversity of antibiotic–resistant staphylococci in Australia's native wildlife, with particular focus on β-lactam resistance. This study presents a novel method for the detection of the β-lactamase enzyme via biochemical means without the need for expensive reagents (Chen et al. 2015b). This represents the first body of work of its kind to be conducted in Australia with respect to macropods.

6.1 Staphylococcal species diversity in wallaby nares

6.1.1 Identification and confirmation of staphylococcal species

The misidentification of bacterial pathogens can have dire consequences for patients and is not conducive to epidemiological investigations. As previously discussed in Section 1.3.2, highly automated and accurate identification platforms such as MALDI-TOF are widely used in both clinical and research laboratories for the identification of bacterial species (Argemi et al. 2015, Deak et al. 2015, Randall et al. 2015). Molecular methodologies with high specificity and low 'per sample cost' used in this study reduced the time and labour costs associated with obtaining large data sets, see Section 1.4.4 and Chapter 5. Staphylococcal strains originating from free-ranging BFRW were preliminary identified with the Microbact 12S system. This biochemical staphylococcal identification system is comprised of a panel of 12 colourmetric tests based on sugar utilisation. It has the ability to differentiate between 22 clinically relevant species with the aid of proprietary software (Oxoid Microbact® 2000) (Oxoid 2015). Upon evaluation against 16S rRNA gene sequencing, the Microbact 12S species identification system performed poorly for isolates of veterinary origin, with many staphylococcal species, such as *S. fleurettii* and *S. succinus*, not included in the test panel (Oxoid 2015). All staphylococcal strains used in this study were identified with Gram stain, catalase and coagulase tests and 16S rRNA gene sequencing. Strains of *S. aureus* were further investigated with the Alere StaphyType DNA microarray platform (see Section 1.4.4). Out of the 89 staphylococcal isolates from 68 captive and 30 free-ranging wallabies, seven *S. aureus* strains and 82 CNS were identified.

6.1.2 Staphylococcal species diversity in captive animals

The majority of studies carried out in zoological parks and wildlife rescue centres focus on the detection of *S. aureus* or MRS(A) rather than staphylococcal species diversity. They include a Danish study which identified seven different *S. aureus* sequence types from 59 isolates from a zoological park collection (Espinosa-Gongora et al. 2012). Similar studies have also been conducted in central Iowa at a wildlife rescue centre where seven *S. aureus* and three MRSA were identified from 158 samples (Wardyn et al. 2012) and in Belgium where 40 species, 93 different mammals, were screened for the presence of MRS(A) (Vercammen et al. 2012). As such there is limited insight

into staphylococcal species diversity from non-companion, non-equine and non-livestock animals.

6.1.2.1 Staphylococcal species diversity in macropods

As previously mentioned, limited studies on bacterial diversity in macropods have been reported. Of the few available in the literature, one investigated the diversity of periodontal pathogens and reported the presence of *Fusobacterium necrophorum* subsp. *Necrophorum*, *F. necrophorum* subsp. *Funduliforme, Porphyromonas gingivalis* and *P. gulae*-like species in the oral cavity, suggesting an association between the debilitating condition of lumpy jaw and *F. necrophorum* subsp. *necrophorum* (Antiabong et al. 2013). In a separate study using non-culture-based methods to investigate the microbiome of the maternal pouch and saliva of TMW, a *Fusobacterium* clone was detected in the saliva of one adult female. No staphylococcal species were found in the saliva or the pouch (Chhour et al. 2009). A previous study involving 9 Bridled nailtail wallabies from a wildlife sanctuary was conducted in 2009. This study utilised the Microbact 12S staphylococcal species identification system discussed previously in Section 6.1.1. A total of 5 staphylococcal species (*S. capitis* subsp. *capitis, S. chromogenes, S. epidermidis, S. haemolyticus* and *S. lentus*) were isolated (Harris 2009).

This study identified a total of 14 species from 67 staphylococci from captive wallabies (Chapter 2; Figure 6.1). *S. succinus* was identified to be the most prevalent species, closely followed by *S. xylosus* (Chen et al. 2014). Both species are predominantly encountered in fermented foodstuffs such as sausages (Leroy et al. 2010) and cheese (Coton et al. 2010) but have also been the dominant species recovered from a large collection of staphylococcal isolates isolated from free-ranging shrews and rodents (Hauschild et al. 2010). With the exception of *S. succinus,* the distribution of staphylococcal species across the wallabies ranged from 2% through to 13%, indicating the absence of a greatly dominant specie or clone. This wide diversity of staphylococcal species could be the result of higher exposure to man-related feed, housing and environment.

These animals are also at higher risk of acquiring different staphylococcal species through human contact, in a phenomenon known as ‘reverse zoonoses’ or ‘zooanthroponosis’ (Messenger et al. 2014) (Chapter 1). Similar to

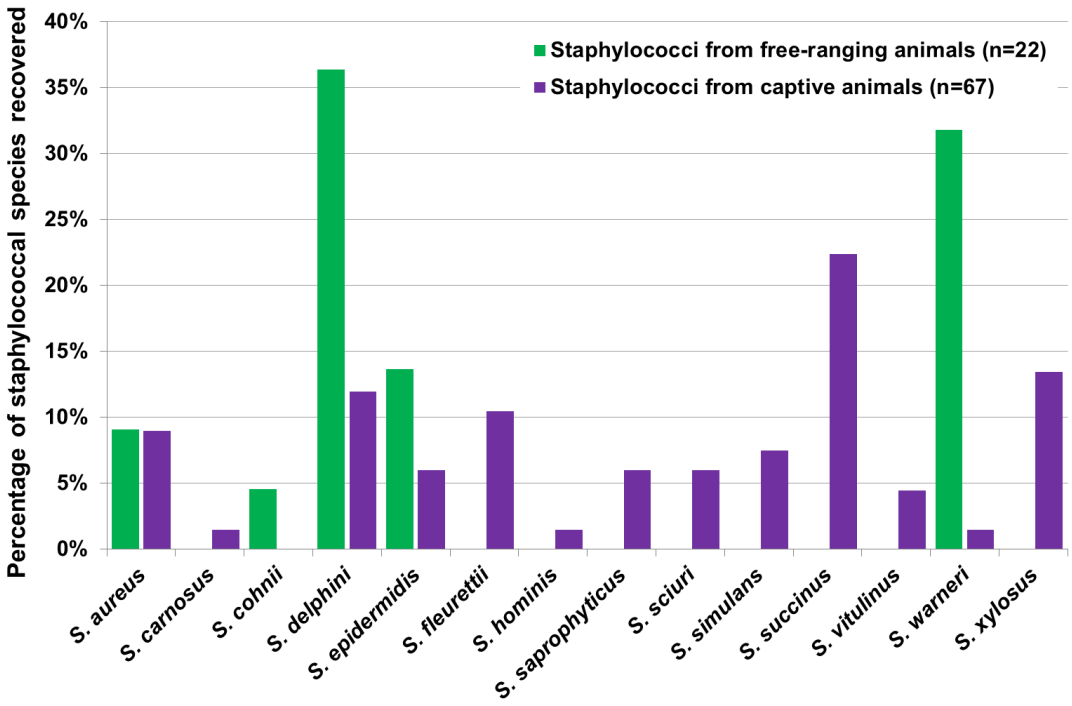


Figure 6.1: Prevalence and diversity of staphylococcal species from wallaby nasal passages

The frequency of different staphylococcal species, as identified by 16S rRNA gene sequencing, in the anterior nasal cavity of captive and free-ranging wallabies from South Australia was determined. Frequency has been expressed as a percentage with green indicating the isolate was recovered from a free-ranging wallaby from the APY Lands and purple indicating the isolate was recovered from a wallaby held at Monarto Zoo.

Wertheim et al. (2005), we were unable to conclude whether wallabies are persistent, intermittent or non-carriers of staphylococci because only a single nasal swab was taken from each wallaby. In addition, wallaby enclosures, hessian transfer bags, animal examination table and veterinary staff were not sampled in this present study but given the lack of close human contact with these wallabies this may not have been a limitation of the study.

6.1.3 Comparison of staphylococcal diversity between captive and free-ranging wallabies

Five staphylococcal species (*S. aureus, S. cohnii, S. delphini, S. epidermidis* and *S. warneri*) were recovered from the free-ranging wallabies sampled in the APY Lands of South Australia; sampling locations are highlighted in Appendix A. This was consistent with results from Harris (2009) where five staphylococcal species (*S. chromogenes, S. epidermidis, S. hominis, S. lentus* and *S. lugdenensis*) were identified from 20 free-ranging BFRW. Differences in the staphylococcal species isolated could be related to the identification method used, as discussed in Section 6.1.1, the Microbact 12S kit is designed to identify staphylococci of clinical relevance to human medicine. As such, only 22 out of 71 species and subspecies are identifiable with this kit (LPSN 2014, Oxoid 2015). Similar to Harris (2009) no difference in the recovery incidence of *S. aureus* relative to wallaby captivity status was observed.

Differences in both the number of staphylococcal species identified and their distribution across the wallaby population was also varied (Figure 6.1). The majority of staphylococcal isolates recovered from free-ranging BFRW were either *S. delphini* or *S. warneri* as opposed to *S. succinus* in the captive wallabies. *S. delphini* has previously been isolated from a broad range of hosts, including dolphins, domestic pigeons, camels, horses, minks, foxes and badgers (Bannoehr et al. 2007, Sasaki et al. 2007, Guardabassi et al. 2012). *S. delphini* has been implicated as an important pathogen in mink cultivation as it is frequently isolated from lesions (Guardabassi et al. 2012). More recently, S*. delphini* was recovered from the small intestines of deceased ferret kits with diarrhoea (Gary et al. 2014). Guardabassi et al. (2012) retrospectively analysed a collection of *S. intermedius* group organisms and suggested that carnivorous animals from the Mustelidae (including minks, ferrets and badgers) are the natural hosts of *S. delphini* organisms. However, as 36% of all staphylococcal

species recovered from free-ranging BFRW were identified to be *S. delphini* (Chapter 2) and the presence of members from the Mustelidae family in the Australia outback is yet undetermined, it is possible that wallabies, or macropods, are also natural hosts of *S. delphini.* However, their status as commensal or pathogenic organisms has not been determined for animals, aside from minks, and thus further studies encompassing multiple species of macropods are required to ascertain this information.

6.2 Antibiotic resistance in wildlife

The use of antimicrobial drugs is known to be an important selective force in bacterial ecology, and there is increasing evidence that staphylococci from animals constitute a reservoir of antibiotic resistance genes (Guardabassi et al. 2004b, Marshall et al. 2009). Staphylococci of animal origin have been found to share antimicrobial resistance genes with human staphylococci, genes including MDR genes such as *cfr* that confers resistance to phenicols, lincosamides, oxazolidinones, pleuromutilins and streptogramin A (Wendlandt et al. 2015).

As discussed previously (Section 4.6), a systematic review of the literature up until mid-2015 revealed a total of 210 independent studies were conducted to investigate antimicrobial-resistant bacteria in wildlife populations. This review revealed the majority of studies focused on *E. coli* (115 studies)*, Salmonella* spp (54 studies) and *Enterococcus* spp (43 studies) (Vittecoq et al. 2016). In contrast, only 34 articles investigated antimicrobial resistance in *Staphylococcus* spp. in relation to wildlife and antimicrobial resistance have been published since 2000 (Greig et al. 2015). Of these, only four reported wildlife as a risk factor with one article reporting rodents on a hog farm to be a potential sentinel of antimicrobial resistant *Staphylococcus* spp transmission between swine herds (van de Giessen et al. 2009). This is of particular importance as a Dutch study suggested that MRSA can be transmitted vertically through the pork production chain, from breeder to finishing farms as a result of animal acquisition (van Duijkeren et al. 2008). Rodents are well known for their role in the transmission and persistence of zoonotic bacteria species, such as *Y. pestitis* and MRSA (Himsworth et al. 2014) as a result of their foraging behaviour (Section 1.10.3.3). Pathogens carried by rodents are commonly indirectly transmitted to humans and other animals via the contamination of food-stuffs and the environment (Himsworth et al. 2013). Rock wallabies are

opportunistic feeders and will consume a wide range of grasses, monocots, forbs, chenopods, trees and woody shrubs (Miller 2001). Thus they are exposed to a wide variety of environmental factors which could contribute to the acquisition of antimicrobial resistant bacteria, including *Staphylococcus* spp. Furthermore, wallabies, like most macropods engage in frequent acts of nose-to-nose contact between individuals (Miller 2001) which gives rise to the potential of bacterial exchange between individuals.

6.2.1 Antibiotic resistance in *S. aureus* strains from captive wallabies

It has been estimated that up to 90% of all *S. aureus* strains isolated from humans since 1980 are penicillin resistant (Livermore 2000). A recent study of almost 7000 *S. aureus* isolates from patients in Europe reported 73% of isolates to be PEN resistant (den Heijer et al. 2013).

Generally, there is a higher prevalence of antibiotic resistance towards compounds commonly used in human and veterinary practice in the respective country (Gordon et al. 2014). Similar to the veterinary teaching hospital at the University of Zambia, veterinary staff at Adelaide and Monarto Zoo primarily use (oxy)-TE and PEN in the management of animal diseases. Despite these similarities, 63% of all *S. aureus* from pet dogs investigated were resistant to PEN (Youn et al. 2014). A Spanish study reported the incidence of β-lactam resistance to be 83% in nursery, breeder and finishing pigs. More worrisome was the finding that 99% of all *S. aureus* strains were resistant to at least one of the eight antimicrobial agents tested (Gordon et al. 2014). Therefore, the lack of any detectable level of antibiotic resistance to common antibiotic families (Figure 6.2), including the β-lactams, in 80% of *S. aureus* strains from captive wallabies is a strong indication that these isolates were present as a commensal organism and not of human origin or a product of indiscriminate antibiotic use by the zoo. The *S. aureus* antibiograms reported in this study are similar to those of opportunistically sampled injured wildlife (Wardyn et al. 2012) and are further evidence of their status as commensal organisms in wallabies.

6.2.2 Antibiotic resistance in CNS from captive wallabies

Prevalence and diversity data relating to CNS as an animal pathogen is predominantly linked to bovine mastitis and intimately linked to clinical isolates and thus are not representative of the commensal staphylococci population

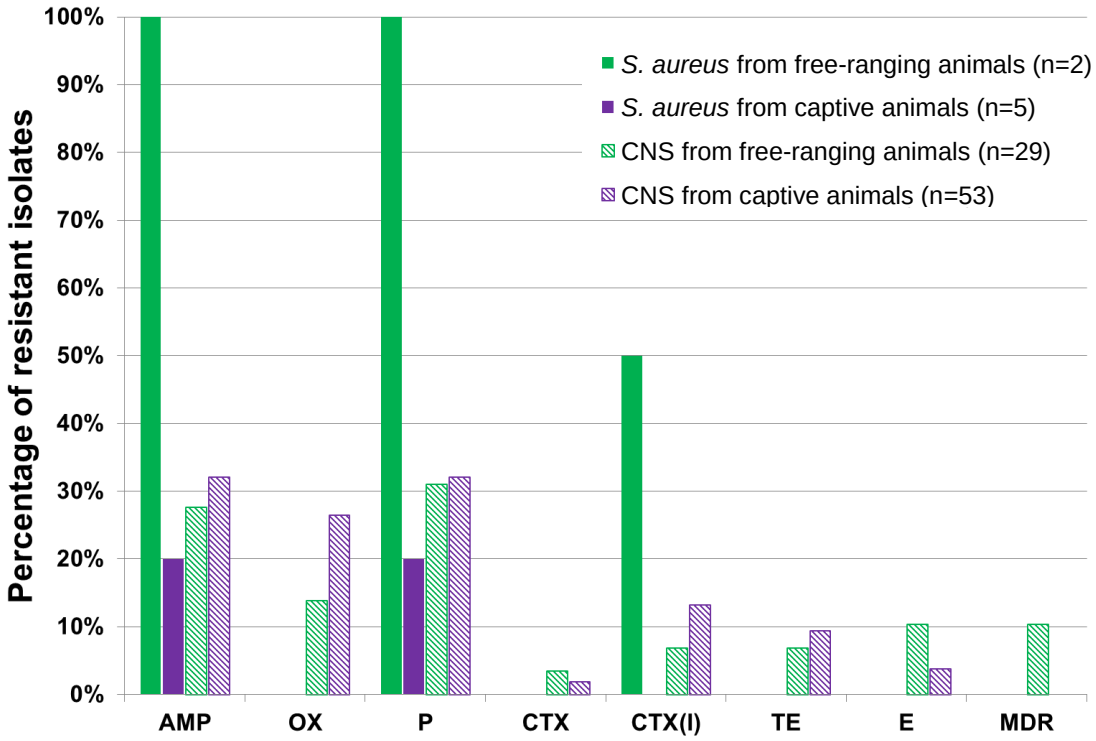


Figure 6.2: Prevalence of antibiotic resistant staphylococci from wallaby nasal passages.

The percentage of antibiotic resistant staphylococci was determined by Kirby-Bauer disc diffusion on Mueller-Hinton agar supplemented with 2% sodium chloride. Staphylococci have been differentiated based on their coagulase activity. All staphylococcal isolates were sensitive towards gentamycin, streptomycin, vancomycin and chloramphenicol (data not shown in this figure). Both wallaby populations carried staphylococci which had similar antibiotic resistance profiles, however, MDR staphylococci were recovered exclusively from free-ranging wallabies.

Abbreviations: AMP – ampicillin 10 µg; OX – oxacillin 1 µg; PEN – penicillin 10 units; CTX – cefotaxime 30 µg; TE – tetracycline 30 µg; E – erythromycin 15 µg; CTX(I) – intermediate resistance to cefotaxime; MDR – multidrug resistant.

(Taponen and Pyorala 2009, Zadoks and Watts 2009, Feβler et al. 2010, Waller et al. 2011).

This study identified CNS with demonstrated resistance towards common β-lactam agents, cephalosporins, macrolides and tetracyclines. However, no MDR staphylococci were recovered from captive wallabies (Figure 6.2).

6.2.3 Antibiotic resistance in *S. aureus* strains from free-ranging wallabies

Unlike the *S. aureus* isolates recovered from captive wallabies where 80% of all isolates to be sensitive to all antimicrobial agents tested, both *S. aureus* isolates recovered from free-ranging wallabies demonstrated resistance towards the β-lactam agents AMP and PEN with one isolate also exhibiting intermediate resistance towards third-generation cephalosporin CTX (Figure 6.2). This was an unexpected finding as these animals were not in receipt of antimicrobial treatment (Burow et al. 2014, Simoneit et al. 2015), nor were they in close human contact associated with providing care to animals, especially at the infant or juvenile stages of life (Graveland et al. 2011, Schaumburg et al. 2012).

6.2.4 Antibiotic resistance in CNS from free-ranging wallabies

CNS recovered from free-ranging wallabies demonstrated antibiograms not distinct from their captive counterparts. Resistance towards β-lactams, cephalosporins, macrolides and tetracyclines was observed (Figure 6.2). Most disturbing was the presence of MDR staphylococci from wallabies residing in two separate colonies. These colonies are approximately 150km apart and are well outside of the normal BFRW core home ranges of approximately 10 square kilometres (Ruykys et al. 2011). Furthermore, most studies suggest that rock-wallabies rarely move between colonies (Ruykys et al. 2011) lending further evidence towards the independent development or procurement of MDR staphylococci in these wallabies.

The presence of antimicrobial resistance in CNS is of particular importance to the dairy industry where CNS, as well as *S. aureus*, are responsible for causing clinical and subclinical mastitis (Feβler et al. 2010, Asfour et al. 2011). Both clinical and subclinical mastitis have been associated with a reduction in milk production (Gröhn et al. 2004, Halasa et al. 2009). Whilst a reduction in milk

production is economically costly, it is particularly harmful in the reproductive cycle of animals that raise their young on milk (Siqueira et al. 2010).

Samples were not collected from the mammary glands of lactating wallabies but veterinary staff assessed all animals to be apparently healthy at the time of sampling. However, the presence of CNS which were resistant to first-line antimicrobial agents may represent a risk towards the successful raising of future pouch young in this endangered wallaby specie.

6.2.5 Comparison of antibiotic susceptibility profiles for staphylococci isolated from captive and free-ranging wallaby populations

Staphylococci isolated from both wallaby populations demonstrated resistance towards the same antimicrobial compounds (Figure 6.3). Overall, all staphylococci recovered were susceptible towards CN, S, VA and C. Approximately one-third of all staphylococci recovered were resistant to at least one β-lactam antimicrobial agent with three MDR staphylococci isolated from two free-ranging BFRW.

Differences observed in antibiotic susceptibility patterns could also be the result of variations in wallaby behaviour as a result of captivity. In the wild, wallabies gain higher exposure to various herbaceous plants and make daily trips to the nearest water source (Miller 2001). This exposure to various environmental stimuli allows the opportunity for interaction with various antibiotic-producing soil fungi and bacteria, such as *Streptomyces* spp. A recent study investigating the composition of pathogenic bacteria in a zoological park sampled the air, water and soil of animal enclosures and found a high prevalence of *Staphylococcus* spp (61%) and *Enterobacter* spp (33%) present (Wu et al. 2011).

It has been strongly suggested that oral administration of antimicrobial agents to chicken and pigs result in the carriage of antimicrobial-resistant *E. coli* (Burow et al. 2014, Simoneit et al. 2015). However, our study identified MDR staphylococci exclusively from free-ranging BFRW rather than from captive wallabies. This was a highly unexpected finding as these free-ranging animals had no prior exposure to antimicrobial treatment, livestock, and domestic pets and had minimal human contact. We also found no correlation between prior exposure to antimicrobial agents and the carriage of antimicrobial-resistant staphylococci, even against the first line antimicrobial agent TE.

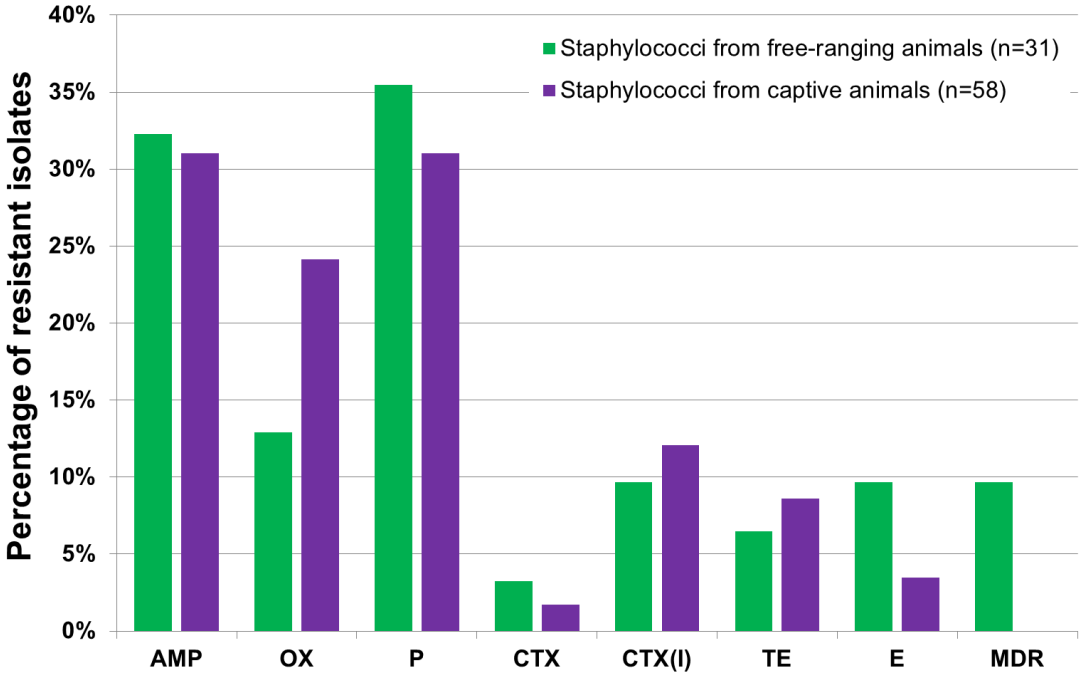


Figure 6.3: Percentage of staphylococci resistant to various antimicrobial agents

The percentage of antibiotic resistant staphylococci was determined by Kirby-Bauer disc diffusion on Mueller-Hinton agar supplemented with 2% sodium chloride. Staphylococci have been differentiated based on their captivity status. All staphylococcal isolates were sensitive towards gentamycin, streptomycin, vancomycin and chloramphenicol (data not shown in this figure). Both wallaby populations carried staphylococci which had similar antibiotic resistance profiles, however, multidrug resistant staphylococci were recovered exclusively from free-ranging wallabies.

Abbreviations: AMP – ampicillin 10 µg; OX – oxacillin 1 µg; PEN – penicillin 10 units; CTX – cefotaxime 30 µg; TE – tetracycline 30 µg; E – erythromycin 15 µg; CTX(I) – intermediate resistance to cefotaxime; MDR – multidrug resistant.

This is indicative of the development or acquirement of antimicrobial-resistant staphylococci is independent of captivity status.

All wallabies sampled in both the APY Lands and at Monarto Zoo were classified as healthy by veterinary personnel and had minimal exposure to antimicrobial agents in the 12 months preceding sample collection (see Appendices B and C). However, we are currently in a situation whereby staphylococci in the commensal flora of free-ranging wallabies have the capacity to respond to first-line antimicrobial agents used in veterinary practice.

6.3 Characterisation of β-lactam resistance

The molecular basis of resistance towards β-lactam antimicrobial agents was investigated in this study using both phenotypic and genetic methodologies. In contrast to human, companion and livestock animal isolates, the molecular basis of β-lactam resistance in staphylococci is poorly understood in macropods. Approximately 75% of diseases that have emerged in the past three decades originated from a wildlife source (Woolhouse 2002). The rational pre-emptive action to be taken in the face of this scenario is to increase disease surveillance and monitoring activities across a broad animal host range. This is in addition to implementing risk management strategies aimed at mitigating the risk of human infection (Bengis et al. 2004).

6.3.1 β-lactamase activity

The activity of the β-lactamase enzyme encoded for by *blaZ* was investigated using an iodometric assay, as outlined in Chapter 3. Due to a lack of cost-effective means to accurately detect β-lactamase production in both CPS and CNS (Kilic et al. 2006), an in-house assay was developed. This assay builds upon previous work which exploited the reaction between starch and iodine to form a colourless solution (Catlin 1975).

Using this method, a total of nine out of 33 PEN-resistant strains demonstrated β-lactamase activity. Of the 33 PEN-resistant strains, 27 contained the *blaZ* gene (Section 6.3.2). All nine strains demonstrating β-lactamase activity contained the structural *blaZ* gene (Chapter 3). The discrepancy between isolates possessing *blaZ*, demonstrating phenotypic resistance towards β-lactam compounds and β-lactamase activity could be the result of multiple factors. Factors including the inherent sensitivity of the

iodometric assay, presence of an intact *bla*-operon and mutations in the *bla* promoter region (Milheirico et al. 2011).

6.3.2 Comparison between the prevalence and diversity of the β-lactamase gene from captive and free-ranging wallabies

Of the 58 staphylococci identified from captive wallabies, 19 (33%) contained the *blaZ* gene. From these 19 isolates, 11 (58%) demonstrated both the ability to grow in the presence of β-lactam antimicrobial agents and possessed *blaR1* and *blaZ*. The remaining eight isolates were found to contain combinations of *blaR1, blaI* and *blaZ* but had no detectable ability to grow in the presence of β-lactam antimicrobial agents. This is similar to findings reported by others (Shuford et al. 2006, Malik et al. 2007, Papanicolas et al. 2014, Pereira et al. 2014). Comparatively, all eight (26%) out of 31 staphylococci recovered from free-ranging wallabies demonstrated the presence of *blaR1, blaZ* and had the ability to grow in the presence of β-lactam antimicrobial agents (Table 3.1).

6.3.2.1 Signature types of BlaZ

This study is one of a few to categorise *blaZ* into the corresponding protein signature types. A total of four signature types were identified from 27 staphylococcal isolates. Similar to our findings, previous studies also found signature type 3 to be the most commonly encountered amongst staphylococci of animal origin (Olsen et al. 2006, Malik et al. 2007). Our study found naturally occurring mutations were present for three out of four signature types; these mutations did not appear to impact upon β-lactamase production or phenotypic resistance to β-lactam antimicrobial agents.

6.3.3 Detection of methicillin-resistant staphylococci in animals

As many MRS demonstrate a hetero-resistance phenotype (El-Halfawy and Valvano 2015). A multi-pronged approach was taken to ensure the detection and subsequent confirmation of true MRS present in wallaby staphylococci. Preliminary identification and validation of MRS was achieved using chromogenic media ORSAB and KB-AST against OX and FOX. Subsequent validation was achieved with OX MIC by broth microdilution and confirmatory *mecA, mecC* and SCC*mec* typing protocols.

Globally, MRS carriage rates of up to 42% have been reported from clinically normal horses in Slovenia (Vengust et al. 2006) and 28% of healthy Dutch veal

calves (Weese 2010). In Australia, MRS carriage rates of only 5.8% have been reported from a nation-wide study of 771 veterinarians across various specialisation streams such as primary clinical care, wildlife or zoo medicine clinical care, industry, research and teaching, government laboratory and students (Jordan et al. 2011). An Australian study reported a MRSA carriage rate of 3.7% in horses after retrospective analysis of laboratory culture collections over a 6 year period in addition to swabbing horses admitted to ICU in a 30 day infectious disease surveillance exercise (Axon et al. 2011). MRSA recovered from the retrospective laboratory culture collections originated from wounds, nasal cavities, catheter insertion sites and joints. The MRS carriage rate of 7.1% identified in our study, albeit low by global standards was similar to that of hospitalised horses and higher than wild urban rats (3.5% carriage rate) (Himsworth et al. 2014).

6.3.3.1 Characterisation of methicillin-resistant staphylococci isolated from wallabies

The SCC*mec* element belong to a family of heterogeneous mobile genetic elements which are responsible for the transmission and expression of β-lactam resistance in MRS. As discussed in Chapter 1, the inherent structural variations in SCC*mec* elements provides a basis for the development of a tracking method with increased genotyping power. These methods are known as SCC*mec* genotyping with numerous methodologies currently available for use with each a compromise between the number/type of targets and the resolution achieved.

MPCR is one of the most commonly used methods to characterise the *ccr/mec* complex in addition to SCC*mec* type-specific domains. One of the better MPCR methodologies available requires the amplification and analysis of 27 PCR products nested in a total of six multiplex reactions (Kondo et al. 2007). From this methodology, MPCRs 1, 2 and 3 were used for the characterisation of MRS isolates from wallabies. As discussed in Chapter 4 and Chen et al. (2016a), it was observed that while the *mecA* gene could be characterised for all 11 isolates, the *ccr* complex proved elusive for seven. Alternative simplex and multiplex PCRs were employed for the determination of the unknown *ccr* region. Simplex and multiplex strategies published by Lim et al. (2003) and Zhang et al. (2012), respectively, were used in the course of this work, but failed to yield tangible results. Simplex PCR followed by bi-directional Sanger

sequencing for the *ccrA* and *ccrB* regions (Oliveira et al. 2006b) identified one novel *ccrA1B1* subtype from *S. cohnii* (isolate A31) and the presence of an additional *ccrA4B4* region from MRSE SCC*mec* type III. Therefore, the majority of MRS identified in this study were unable to be assigned to a SCC*mec* type. This is unsurprising given a significant portion of MRS isolated were identified as *S. fleurettii*. The methicillin resistance determinant from *S. fleurettii* has been touted to be the progenitor of all *mecA* currently in circulation (Tsubakishita et al. 2010b) as this particular *mecA* did not contain the *ccr* complex in its current form.

Of particular interest are isolates *S. saprophyticus* M21 and *S. succinus* M54 from YFRW and TMW, respectively. These isolates exhibited resistance towards AMP, PEN, OX and FOX by KB-AST, demonstrated an OX MIC value of 32µg/ml by broth microdilution, were unable to grow on ORSAB or produce β-lactamase and most importantly tested negative for *mecA, mecC* and all SCC*mec* components as per the simplex and MPCRs tested (Chen et al. 2014, 2015b, Chen et al. 2016a). This suggests that not all methicillin-resistance is mediated by *mecA* or *mecC*. These non-typeable strains pose an important challenge in clinical settings and emphasises the molecular evolution of the SCC*mec* element from emerging MRS strains. It also places greater emphasis on the need to characterise these particular isolates by WGS. The accessibility of WGS has increased with continually falling prices. This invariably leads to an increase in whole genome comparisons for the identification of SNPs and the screening of mobile genetic elements. Thus making it entirely possible and feasible for WGS analysis to be carried out on these two particular samples.

6.5 Molecular diversity of *S. aureus* isolates

A common theme in historic and current literature as it relates to *S. aureus* is heavily biased towards the detection and characterisation MRSA, with special focus on LA-MRSA CC398. However, the analysis of MSSA, such as CC398-MSSA, is crucial to furthering our understanding on the evolution, spread and adaptation of this opportunistic pathogen. Comparisons between the transferability of CC398-MRSA against CC398-MSSA in humans showed that the methicillin-susceptible variant outperformed CC398-MRSA and demonstrated an increased ability to adhere to human keratinocytes (Uhlemann et al. 2012). Chlebowicz et al. (2010) demonstrated it was possible to convert

ST398-MRSA isolates to ST398-MSSA strains *in vivo* due to the recombination of the recombinase genes *ccrC1* alleles 8 and 10. Interestingly, no detectable differences in competitive growth or virulence were observed between the methicillin resistant and sensitive variants, thus suggesting the carriage of SCC*mec* type V had no negative bearing on host fitness (Chlebowicz et al. 2010).

A recent review by McCarthy et al. (2012b) summarised the genetic diversity of *S. aureus* in animals and highlights the diversity of both MRSA and MSSA clonal complexes in a global context. It also revealed the significant lack of knowledge concerning MRSA and/or MSSA in wildlife. In the Australian, New Zealand and South-West Pacific context, relatively little is known about the molecular epidemiology of MSSA lineages circulating in these regions. Recent studies in Australia and New Zealand highlight the importance of CC1, CC121 and CC30 strains due to their carriage of the *lukF-PV/lukS-PV* genes (Uhlemann et al. 2012, Williamson et al. 2014b).

It is important to highlight the absence of predominant 'Australian clones' such as CC75, ST93, ST30, ST45, ST78, ST5 and ST1850 (Williamson et al. 2014b, Chen et al. 2016b) from our samples particularly as CC75 has been found to account for approximately 8% of all MSSA and 70% of all MRSA isolates recovered from skin lesions in Australian Aboriginal populations. This particular clone and other *S. aureus* lineages only have 90% sequence identity and are typically non-pigmented which has led to calls for renaming this clone as a separate species, *Staphylococcus argenteus* (Holt et al. 2011, Becker et al. 2014b).

This is the first study of its kind to be performed in marsupials and as such there is limited data which can be drawn upon. However, as previously discussed in Chapter 5 and Appendix H, the finding of *S. aureus* CC15, CC49 and CC692 was highly unusual due to their traditional association with human carriage and/or infection, geographic isolation and apparent avian host preference respectively. The novelty of the findings of this preliminary study points to the need for continual surveillance of our native animal population for the emergence of endemic clones and indicates that an alternate route of *S. aureus* transmission and colonisation may exist for this pristine environment. Alternate routes of transmission could follow the example of *cagA*-negative

Helicobacter pylori into the Indigenous Australian and New Guinea population from its South African ancestor due to human migratory patterns and the distribution of Earths' land mass at the time of the last ice age (Moodley et al. 2009, Lu et al. 2014). A similar case could be argued for the *S. aureus* CC49 and CC692 which have not been isolated outside of the European continent; there is a possibility that as a result of mass migration from Europe to Australia in the early 1900's these CC were introduced into our native animal population.

6.6 CONCLUSION

This study has shown that animal sentinel surveillance can be used to address the basic questions of whether a certain pathogen is present. Sentinel surveillance is an effective mechanism to generate broad-scale pictures of what the next emerging human/livestock pathogen could be and the baseline data obtained from the sentinel population can serve as a trigger to warrant further investigation in the target population.

In summary, the antimicrobial resistances of importance found in staphylococcal species recovered from wallabies in this thesis were to AMP, PEN, OX and CTX (Chapter 2). A variety of BlaZ signature types and naturally occurring mutants were recovered and characterised (Chapter 3). Little association between signature type and the production of β-lactamase was observed. The methicillin-resistance gene, *mecA,* was detected in only a handful of non-*S. aureus* isolates and included the recovery of one novel *SCCmec* subtype (Chapter 4). The majority of *mecA*-positive isolates were *S. fleurettii* and these isolates were unable to be assigned into *SCCmec* type with the range of *SCCmec* typing PCR strategies employed. The recently described *mecC* element was not detected in this study. However, the presence of two isolates which demonstrated the phenotypic profile typical of a MRS but were found to be void of all *bla, mec* and *SCCmec* associated genes by the PCR strategies employed is notable and warrants further investigation by WGS (Chapter 4). A total of three CC of *S. aureus* were identified, interestingly, CC49 and CC692 have not been isolated outside of the European continent. This study also represents the first isolation of CC692 from a mammalian source (Chapter 5). To our knowledge, this is the only study to identify MRS in pure culture by selective culturing, functional assays, PCR screening and sequencing methodologies.

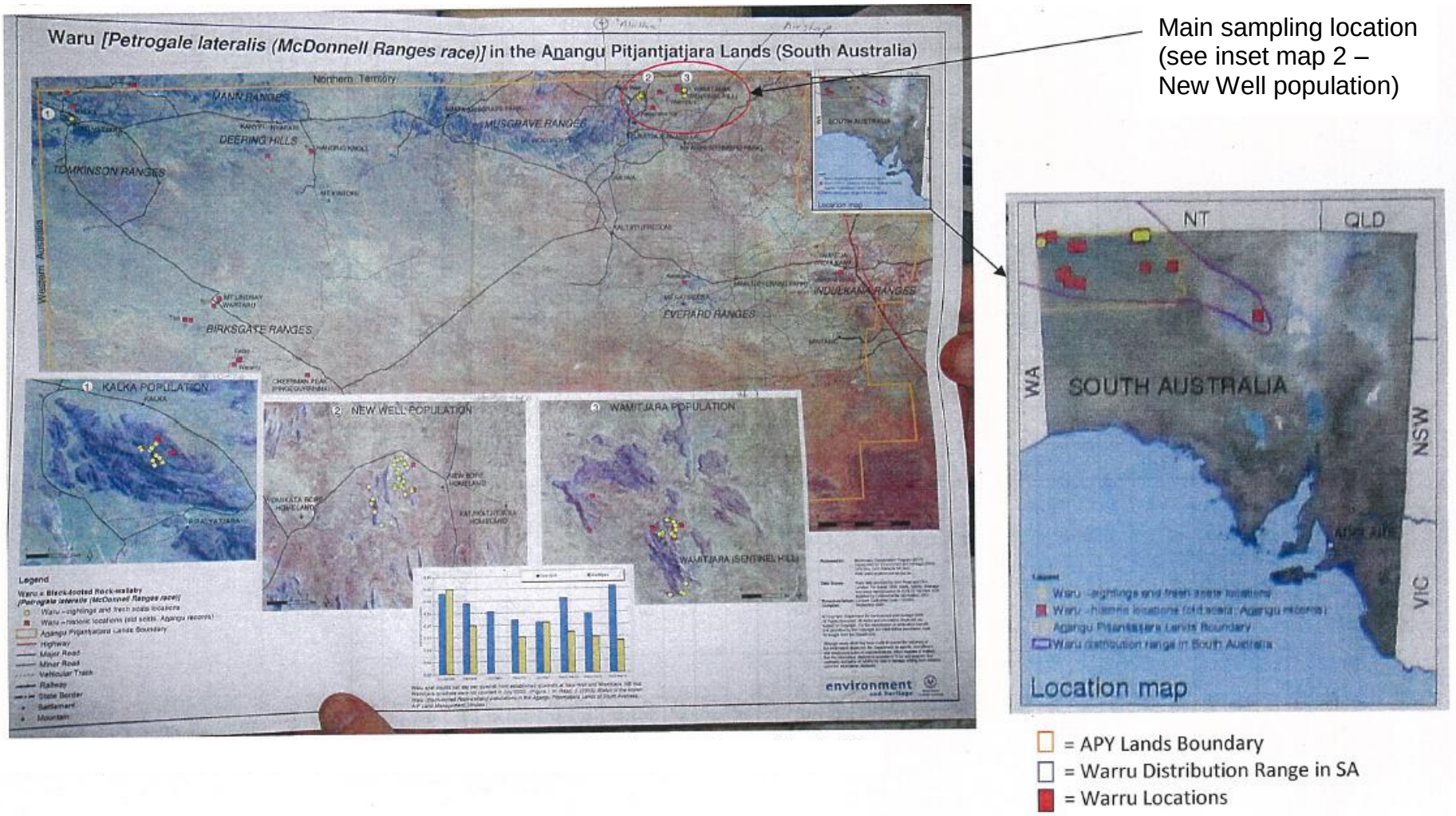
Further work can be undertaken to expand and enhance the scope of this thesis. Such opportunities include longitudinal studies that sample wallabies and other macropods from differing environmental surroundings, multiple body sites (nasal, skin, perianal) and the nasal passages of veterinary staff involved in primary care. This will pave the way forward in finding an answer to whether macropods are persistent, intermittent or non-carriers of staphylococci as commensal organisms. Additionally, the transmission of bacteria from humans to animals and vice versa, a currently contentious issue, can be explored.

In this study staphylococcal isolates M21 and M54 were found to demonstrate resistance towards methicillin in the absence of known methicillin and β-lactam resistance genes. These isolates may therefore possess novel antibiotic resistance mechanisms which could be identified by WGS and confirmed by inactivating the gene of interest followed by resistance assays. Transcriptomic assays on RNA isolated from cells after antibiotic challenge could also be employed to identify genes that have differential expression under these conditions. Analysis of the genome of these strains will also shed light on their overall genetic composition with regard to plasmids, pathogenicity islands and their relative proportion to the core genome.

In the course of this study it became apparent that the diversity of *SCCmec* determinants is larger than first thought. Thus, it is highly likely that due to the plasticity of the genome, including the large number of mobile genetic elements it harbours, and the evolutionary potential of staphylococci that other unique *SCCmec* configurations will be identified in future studies.

APPENDICIES

Appendix A: APY Lands map with wallaby sampling points (July 2009)



Appendix B: Monarto Zoo wallaby health records (ZoosSA MedARKS database, Feb 2013)

Clinical Notes - Individual Specimen Report MONARTO ZOO - page 1 -
==========
PETROGALE XANTHOPUS XANTHOPUS
Sex: Female Acc. #: A19006 YELLOW-FOOTED ROCK WALLABY
Age: 11Y 11M Est Birth: 3.Dec.2000 Chip: 00-05FF-FA73
Tag/Band: Orange ET lost
==========

.....2002...
27.Nov.2002
Problem: corneal opacity - bilateral (Confirmed)
Immobilized with isoflurane for preshipment examination.
Cx: Body condition fair, coat good. Minor scratches due to aggression between individuals in the group. Pouch empty. Some hair missing from forehead.
Chest auscultation - slight bilateral respiratory wheeze (normal). Abdominal palpation negative. Eyes have bilateral corneal opacities in lateral canthus, ears clear. Teeth/gums in good condition, erupting lower right P1. Blood taken.
Tx: Ivermectin 200ug/kg s.c., 0.2 ml Vitamin E and Selenium i.m. (SB)

28.Nov.2002
Problem: corneal opacity - bilateral (Confirmed)
SEROLOGY 02-1006913
Toxoplasma IgG HA <1:80 (negative)
Wallal & Werrago Virus ELISA
Negative for antibody to Wallal virus
Negative for antibody to Werrago virus (SB)

.....2003...
8.Jan.2003
Problem: corneal opacity - bilateral (Confirmed)
Restrained manually for preshipment examination.
Cx: Bilateral corneal opacities have not changed. This animal can not be exported. Faeces collected. Moved back to MBA15B. (SB)

13.Jan.2003
Problem: corneal opacity - bilateral (Confirmed)
MICROBIOLOGY 03-1012109

SPECIMEN:faeces
GRAM:2+ leucocytes
CULTURE:Mixed normal faecal flora. Salmonella sp. not detected from selective cultures.
SENSITIVITY:

PARASITOLOGY
FAECAL FLOTATION - Slight numbers of Strongyles. (SB)

.....2008...
8.Feb.2008
Problem: corneal opacity - bilateral (Confirmed)
Iso for health check. Empty pouch. Teeth all discoloured but no gingivitis or loosening.
bloods taken (MT)

6.Mar.2008
Problem: corneal opacity - bilateral (Confirmed)

11.Aug.2008
Problem: dental disease (Confirmed); lice (Confirmed)
GA (isoflurane by facemask) for health check
left mandibular PM 2 & 3 removed,
cloaca clean, biting lice evident, pouch empty
Rx: OXYTETRACYCLINE (AMPHOTERIC) 140 mg IM q72h for 21 days.
Rx: CARPROFEN 25 mg SQ
Rx: FIPRONIL 39 ml topically
Rx: VITAMIN E/SELENIUM 0.2 ml IM (IS)

1.Sep.2008
Problem: dental disease (Confirmed); lice (Confirmed)
GA (isoflurane by facemask) for health check
tooth root removed from rpevious extraction site, some gingivitis
cloaca clean, pouch empty
plan continue antibiosis for further 2 weeks then can stop without revisit (IS)

15.Sep.2008
Problem: dental disease (Confirmed)

.....2009...
2.Nov.2009
Problem: dental extraction - right maxilla
GA (isoflurane by facemask) for routine health check
part of vit E oral supplementation trial x6 days
teeth poor: left mandible PM2 - M2 missing, right mandible PM1 missing & gongival regression, right maxillary arcade absent but caudal molar root present which was removed
cloaca NSF, abdomen & chest nsf
pelage good
pouch empty & clean
bloods taken
orange ear tag right ear applied to replacee mixed colour tag
single antibiosis given
Rx: OXYTETRACYCLINE (AMPHOTERIC) 160 mg IM

TP 80 (IS)

.....2010...

14.Oct.2010
Rx: OXYTETRACYCLINE (AMPHOTERIC) 140 mg IM q84h for 21 days.
GA (isoflurane by facemask) for health check
part of vit E supplement trial- 200mg/kg pellets POx 6days
teeth: right maxillary arcade absent, left madibular arcadePM1 only remaining, somepus presnet when probing left maxillary arcade caudally,large abcessventrally (slightly left),drain ventrally but left mandibleramusexposed-> packwound & suture in place
other: lice seen but notreatment at this stage
pouch: MT/clean
blood & mouth/nasal swabs collected
plan rv 4 days tocheckwound (IS)

18.Oct.2010
GA (isoflurane by facemask) for health check
remove dressing, granulation tissue is infilling nicely
plan continue treatment as planned, spray cetrigen on wound when handled (IS)

21.Oct.2010
in filling nicely, continue as planned previously (IS)

4.Nov.2010
Proc: GA, recheck asbcess.
Exam: Small 5mm open wound scabbed over, should heal over. Still noticable swelling but likely jsut residual scarring. Mouth good.
Blood and oral swabs taken for PhD student John A.
VitE/Se IM. Final oxytet inj. No need to continue.
Plan: recheck 2 weeks. (DMC)

17.Nov.2010
Exam (manual restraint): wound under mandible has healed over. Still noticable scarring/contracture. No need for further checks. (DMC)

.....2011...

6.Apr.2011
Proc: GA, health check.
1. Mouth: last remaining L lower molar loose - pulled. Drainage hole through the skin present ventrally, though little purulent material. Flushed. Now has no PM/Ms on L lower, R upper arcades. R lower PM/Ms have significant tartar build up - scaled.
2. Pouch: empty
3. Cloaca: clean
4. Exam: NAD
5. Blood sample: In house coulter and chemistries (AZ)
6. Wt: 7.0kg
Rx: VitE/Se 1mL/50kg; OTC LA 300 0.7mL IM (DMC)

15.Apr.2011
Follow-up to a dental procedure

Procedure Anesthesia Iso via facemask worked well
Physical exam mouth looks ok but still have a draining fistula on left mandible tooth about fistula fairly solid.
Procedure Radiograph skull - Old osteo lesions on mandible and decreased bone density
Physical Exam eyes Rt eye mature cateract Left eye corneal scar 8mm toward lateral canthus which takes faint stain
Rx gentocin oint to left eye
Rx OTC 200mg IM
Left ear looks clean
Animal should stay in hospital for a follow-up exam (MB)

27.Apr.2011
Proc: GA, recheck mouth, eyes
1. Mouth: gingiva has healed well, but draining sinus persists L mandible.
Flushed with chlorhex.
Rx: Betamox LA 1mL IM q48h 2wks then recheck
2. Eyes: Ventrolateral corneal opacities on both eyes. Very faint superficial fluorscein stainins dorsal and ventral L cornea, but neither opacity stains positive - trauma from hessian during recovery?
Rx: Solugental drops q48h given she'll be caught for injection anyway.
P: RV 2weeks (DMC)

11.May.2011
Proc: GA, jaw, eyes
1. Jaw: still draining sinus open and plugged with scab material. Swelling around sinus has reduced a lot since last check. Tissue looks healthy, but bone exposed. Debrided contracting scar tissue around drainage hole with scalpel; all appears to be healthy tissue bleeding readily.
2. Eyes: ventrolateral opacities still evident but no fluoroscein uptake.
P: Continue antibiotics for a further 2w, then reassess. Consider closing drainage hole if no apparent infection.
Rx: AMOXICILLIN TRIHYDRATE 150 mg PO q48h for 14 days. (DMC)

25.May.2011
Proc: GA jaw
Mouth: excellent healed nicely
BC: excellent 6.185kg
Sinus on ventral jaw clean and healing. Debride edges and close 3-0 PDS.
Remove in 14 days and send back to section (LJ)

8.Jun.2011
Proc: GA, recheck jaw
1. Exam: sutures have dehisced but excellent granulation bed formed. Skin closed with tissue glue. Mouth continues to look good.
P: RV 2wk (DMC)

22.Jun.2011
Proc: GA, RV jaw
1. Exam: drainage tract has healed over well.
P: return to section, no further rechecks. (DMC)

24.Oct.2011
Rx: TOCOPHEROL, ALPHA 1000 IU PO SID for 4 days. ** group treatment **
(IS)

28.Oct.2011
Proc: Health check annual
Ga via BAM + K trial B 0.4mg/kg, A 0.2mg/kg, med 0.05mg/kg, ket 1mg/kg
Nice stable GA. Reverse naltrexone 2.5 x b and atipam 5 x M
1.) Chest abdo clear
2.) cloaca clear
3.) mouth clear
4.) bloods collected NAD coulter and bicohem. Serum stored.
5.) serum and swab collected for lumpy jaw project
6.) weight: 6.23kg
7.) Vit E/Se 0.2ml IM
8.) Pouch clean and negative
9.) Parameters: temp 35.3 at induction, 5 min later 34.7, RR 28 shallow bpm throughout, HR 80bpm, BP 89/54 (LJ)

.....2012...

19.Sep.2012 Weight: 6.55 Kg (14.4 Lb)
Proc: Annual GA and health check
Exam: good body score; HR 160, RR 20, Temp 36.9C
Cloaca: clean
Pouch: Negative although active nipple
Dental: No teeth in upper arcade, moderate plauqe on lower molars.
Bloods: lateral saphenous EDTA and serum storage
RX: Vit E 0.12mls IM
RX: Ivomec 0.12mls IM (JK)

16.Nov.2012
Found dead in shed. LArge pulmonary abscess L lung and pericardial effusion. (DMC)

Clinical Notes - Individual Specimen Report MONARTO ZOO - page 1 -

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PETROGALE XANTHOPUS XANTHOPUS
Sex: Male Acc. #: A79076 YELLOW-FOOTED ROCK WALLABY
Age: 5Y 8M 22D E Birth: 11.Jan.2007
Name: Brachin Chip: 00-069B-3BA2

==========

.....2007...
30.Sep.2007
scab under chin, possibly due to residual milk after feeding. clean with iodine. Apply otoderm cream twice daily for the next 2-3 days and then re-x. (MT)

.....2008...
14.May.2008
Problem: ascaridiasis (Confirmed); coccidiosis (Confirmed)

1.Sep.2008
Problem: ascaridiasis (Confirmed); coccidiosis (Confirmed)
GA (isoflurane by facemask) for health check
teeth good, cloaca clean
Rx: VITAMIN E/SELENIUM 0.2 ml IM (IS)

.....2009...
6.Oct.2009
Problem: ascaridiasis (Confirmed); coccidiosis (Confirmed)

15.Oct.2009
Rx: TOCOPHEROL, ALPHA 1166.7 mg PO SID for 5 days. ** group treatment ** (IS)

19.Oct.2009
GA (isoflurane by facemask) for health check
teeth good
cloaca clean, pelage good, abrasion on right maxillary labia
?catch-up trauma
nsf chest or abdomen
blood taken from right jugular (IS)

.....2011...
16.Mar.2011
Obs: fights in exhibit bachelor group of late. ONe animal looking very bloodied down front and head, but no obvious wounds. Another individual reported wiht bloody arms. All wallabies looking bright and alert.
P: Monitor. (DMC)

10.Oct.2011
GA (isoflurane by facemask) for health check during quarantine
teeth great, abdo & chest nsf
testes equal

oral swab & bloods collected
Rx: VITAMIN E/SELENIUM 0.2 ml IM (IS)

26.Oct.2011
Rx: Ivermectin 0.2m/kg SC
Lab: 1+ strongyles found on recent faecal float.
P: Give second shot in a week when released from quarantine. (DMC)

30.Nov.2011
Obs: holes chewed in fencing allowing fight with male in adjacent enclosure. Head, forelimbs and cranial torso very bloodied. Left eyelid a little drooped. Appears bright, moving freely, wounds are not visible.
P: monitor for now. Consider intervention if large wounds, infection/abscess, and/or depression become evident. (DMC)

5.Dec.2011
RV skin wounds from fighting
skin has generalised over anterior part of body- head, forearms & upper chest
suspect due to recent fighting
movement of arms & head ok
one off AB & pain releif
rv later in week as had been handling tammars earlier today
Rx: NOROCILLIN LA 1 ml IM
Rx: CARPROFEN 40 mg SQ (IS)

9.Dec.2011
Recheck today, visual only.
Looking good. Moving well.
L forearm has small tag of skin still present
L eye still slight belphorospasm.
Monitor only (LJ)

24.Dec.2011
Found this afternoon by keepers with phalange (P3) of forearm missing. Paw very swollen. Animal aalready v hot and licking forearms. 36deg + and v hot at MZ. Keepers did not want to catch up in this weather. Advise to check first thing in AM, if still bad, catch up and give 0.7ml oxytet LA IM and call MB to have assessed. Leave in vet rom with AC going if req (LJ)

25.Dec.2011
Evaluate wounds on forelegs
Procedure anesthesia iso via face mask
Evidence of bite wounds both forelegs swollen - Rt fore 2nd digit missing P3
proc weight 9.45 KG
Rx Amoxicillin 1 cc sub Q
0.1 cc Metcam Sub Q
Monitor and Rx antibiotic in 3 days (MB)

28.Dec.2011
Exam: Digit healed well with flap of skin/pad covering soft tissues.
Mouth: Good
Rx: Betamox LA 1mL SC
P: No further treatment. (DMC)

.....2012...

24.Feb.2012
Faecal float +ve +4 strongyles.
Warru 1 yard. 4 x YFRW each dose approx 6kg
Feed 5 small piles of pellets, each with 1.2ml panacur 25 (fenbendazole 25mg/ml) once a day for 3 consecutive days. Collect faecals in 10 days for 3 consec days for refloat
Rx: FENBENDAZOLE 30 mg PO for 3 days. (LJ)

29.May.2012
Proc: GA, health check
1. Teeth: teeth are worn but no inflammatory lesions noted.
2. Cloaca: no lesions
3. Exam: no significant findings. Testicles firm and symmetrical. BCS 3/5.
Nail missing on R fore digit 2
4. Blood sample for in house haematology: PCV 41, PCV 59 (DMC)

4.Oct.2012
Found dead in yard; killed and eaten by fox. (IS)

Clinical Notes - Individual Specimen Report MONARTO ZOO - page 1 -
==========
PETROGALE XANTHOPUS XANTHOPUS
Sex: Male Acc. #: A69274 YELLOW-FOOTED ROCK WALLABY
Age: 6Y 6M Est Birth: 1.Jul.2006 Chip: 00-06A3-E931
==========

.....2008...
14.May.2008
Problem: ascaridiasis (Confirmed); coccidiosis (Confirmed)

1.Sep.2008
Problem: ascaridiasis (Confirmed); coccidiosis (Confirmed)
GA (isoflurane by facemask) for health check
teeth good, cloaca clean, new microchip placed
Rx: VITAMIN E/SELENIUM 0.2 ml IM (IS)

.....2009...
6.Oct.2009
Problem: ascaridiasis (Confirmed); coccidiosis (Confirmed)

15.Oct.2009
Rx: TOCOPHEROL, ALPHA 1166.7 mg PO SID for 5 days. ** group treatment ** (IS)

19.Oct.2009
GA (isoflurane by facemask) for health check
teeth good
cloaca clean, pelage good
nsf chest or abdomen
blood taken from right jugular (IS)

.....2011...
5.Oct.2011
GA (isoflurane by facemask) for health check
teeth good, cloaca clean
testes equal, some net catchup trauma around face, split commisure (upper labia), mild generalised fight wounds, otherwise NSF
blood collected & gingival swab taken
Rx: VITAMIN E/SELENIUM 0.2 ml IM (IS)

30.Nov.2011
Obs: holes chewed in fencing allowing fight with male in adjacent enclosure. Head, forelimbs and cranial torso very bloodied. Left eyelid a little drooped. Appears bright, moving freely, wounds are not visible though occasional drip from nose observed.
P: monitor for now. Consider intervention if large wounds, infection/abscess, and/or depression become evident. (DMC)

.....2012...

29.May.2012
Proc: GA, health check
1. Teeth: teeth are worn but no inflammatory lesions noted.
2. Cloaca: no lesions
3. Exam: no significant findings. Testicles firm and symmetrical. BCS 3.5
4. Blood sample for in house haematology: PCV 44, TS 64 (DMC)

4.Oct.2012 Weight: 8.67 Kg (19.1 Lb)
Proc: GA; health check and internal transfer.
1. Teeth: worn but sound
2. Cloaca: clean
3. BCS: 3.5; Weight 8.67kg; Temp 36.7; HR 120.
4 .Exam: R paw missing half P3 (old wound healed completely) and P2 appears to have been broken and set awry.
5. Testicles: symmetrical and firm
6. Blood: lateral tail vein, EDTA and serum storage
RX: Vit E/Sel: 0.2ml IM
RX: Ivomec: 0.18ml IM (JK)

17.Dec.2012
Obs: Keepers seen that both ears are drooped
Exam:visualise from distance, left ear drooping.
DDx: trauma, grass seed, ear infection
Plan: continueto monitor. (JK)

.....2013...

1.Jan.2013
Obs: found dead in enclosure.
PM: significant finds of large necrotic abscess comprising entitity of right lung field. No external findings of trauma. (JK)

Clinical Notes - Individual Specimen Report MONARTO ZOO - page 1 -

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PETROGALE XANTHOPUS XANTHOPUS
Sex: Female Acc. #: A19008 YELLOW-FOOTED ROCK WALLABY
Age: 12Y 8M 2D Birth: 2.Apr.2000 Chip: 00-01D0-FC6A
Tag/Band: Yellow ET R ear

==========

.....2003...

12.Jun.2003
Caught by net for pouch check. Female joey ~5 months removed for handraising. Blood taken for DNA storage. Not enough for serum.
Rx: VITAMIN E/SELENIUM 0.2 ml (SB)

.....2007...
3.Nov.2007
Rx: OXYTETRACYCLINE (AMPHOTERIC) 120 mg IM q84h for 7 doses. (IS)

Caught for removal of pouch young and because saff noticed hypersalivation.
Severe infection in the mouth. SMLNs very enlarged. INfection seems to be concentrated around the lower left incisor. Try to treat inital infection and anorexia before GA to remove tooth. NB I suspect severe osteomyelitis which could result in mandibular fracture when the incisor is removed.
0.4mls rimadyl + 0.6mls oxytetLA (200mg/ml) given. repeat oxytet every 3 days and Rimadyl and SC fluids as required (MT)

26.Nov.2007
GA (isoflurane by facemask) for health check
gingiva less inflammed
continue treatment further 2 weeks then revisit
Rx: OXYTETRACYCLINE (AMPHOTERIC) 120 mg IM q84h for 5 doses. (IS)

19.Dec.2007
GA (isoflurane by facemask) for health check
left lower incisor gingival regression has not improved, exposed bone tissue dorsally.
attempt to remove incisor, using a flattened & blunted 18G needle try to break down periodontal ligament, needs more timeso continue antibiosis and reattempt 27th dec (IS)

26.Dec.2007
Rx: OXYTETRACYCLINE (AMPHOTERIC) 120 mg IM q84h for 5 doses. (IS)

27.Dec.2007
GA (isoflurane by facemask) for health check
left incisor removed, mandibles appear stable

plan continue antibiotics further 2 weeks then revisit (IS)

.....2008...

14.Jan.2008
GA isoflo
bone around extracted incisor still swollen but no obvious active infection. Most other teeth black. gingivitis around 2 teeth therefore manual scaling.
final oxytet LA injection but continue to monitor. (MT)

23.Jan.2008
GA health check. mouth still OK. NO further tx required.
q (NMT)

11.Aug.2008
Problem: dental disease (Confirmed); lice (Confirmed)
GA (isoflurane by facemask) for health check
incisor extraction site looking good but remove PM 1, 2 & 3 from left mandible
biting lice present & pelage poor, cloaca clean, pouch empty & dirty
Rx: OXYTETRACYCLINE (AMPHOTERIC) 140 mg IM q72h for 21 days.
Rx: CARPROFEN 25 mg SQ
Rx: FIPRONIL 36 ml topically
Rx: VITAMIN E/SELENIUM 0.2 ml IM (IS)

1.Sep.2008
Problem: dental disease (Confirmed); lice (Confirmed)
GA (isoflurane by facemask) for health check
no gingivitis at extraction site,
cloaca clean, pouch empty (IS)

.....2009...

3.Nov.2009
GA (isoflurane by facemask) for routine health check
part of vitamin C feeding trial
teeth PM's absent, missing one mandibular incisor, hypersalivation
pelage good, cloaca clean
pouch empty & clean
blood taken

Rx: VITAMIN E/SELENIUM 0.2 ml IM
TP 85 (IS)

.....2010...

14.Oct.2010
Rx: OXYTETRACYCLINE (AMPHOTERIC) 160 mg IM q84h for 21 days.
GA (isoflurane by facemask) for health check
part of vit E supplement trial- 800mg/kg pellets POx 6days
teeth: right mandibular PM1 loose& painful,tannin staining on corresponding wear surfaces indicative of pain,ulceration around upper incisors

other: obvious sinus arrhythmia & haemic murmer that lessened asGA progressed
liceseenbut not treated
pouch:MT clean
blood & mouth/nasal swabs collected
RV 3weeks (IS)

4.Nov.2010
Proc: GA, recheck mouth.
Tooth removal sites have healed well. No further treatment.
Blood sample and oral swabs taken for PhD student John A. (DMC)

.....2011...

6.Apr.2011
Proc: GA, health check.
1. Mouth: gingivitis over upper incisors and lower L incisor (lower R incisor missing) - scaled. Missing quite a few premolars and molars but all healed with no inflammation.
2. Pouch: empty
3. Cloaca: clean
4. Exam: mild systolic heart murmer
5. Blood sample: In house coulter and chemistries (AZ)
6. Wt: 6.8kg
Rx: VitE/Sa 1mL/50kg; OTC LA 0.7mL (DMC)

15.Apr.2011
Follow-up check after dental procedure
Procedure Anesthesia - Iso via face mask worked well
Procedure Physical exam Gingivitis still present with erosions of the mucosa

Rx Otc 200mg IM
Animal to be held in hospital for another check (MB)

27.Apr.2011
Proc: GA, recheck incisors.
1. Mouth: Gingiva generally less inflammated but still some ulceration. Scaled as much plaque/tartar as possible with hand scaler. Significane gum recession and some laxity to R lateral incisor - removed. Significnat bleeding from socket.
Rx: Betamox LA 1mL IM
P: RV 2weeks. (DMC)

11.May.2011
Proc: GA, recheck incisors
1. Exam: No inflammation evident around upper or lower incisors. Tooth removal site has healed well.
P: Return to enclosure. (DMC)

24.Oct.2011
Rx: TOCOPHEROL, ALPHA 1000 IU PO SID for 4 days. ** group treatment ** (IS)

28.Oct.2011
Proc: Health check annual
Ga via BAM + K trial B 0.4mg/kg, A 0.2mg/kg, med 0.05mg/kg, ket 1mg/kg
Nice stable GA. Reverse naltrexone 2.5 x b and atipam 5 x M
1.) Chest abdo clear
2.) cloaca clear
3.) mouth clear
4.) bloods collected NAD coulter and bicohem. Serum stored.
5.) serum and swab collected for lumpy jaw project
6.) weight: 6.23kg
7.) Vit E/Se 0.2ml IM
8.) Pouch clean and negative
9.) Parameters: temp 35.3 at induction, 5 min later 34.7, RR 28 shallow bpm throughout, HR 80bpm, BP 91/49, 60/38 (LJ)

.....2012...

19.Sep.2012 Weight: 6.036 Kg (13.3 Lb)
Proc: GA and annual healthcheck
Exam: Good BCS; HR 124, Temp 36.4
Eyes: moderate bilateral cataracts
Cloaca: clean
Pouch: Negative
Dental: 2x extractions of R upper PM and L upper M3
Bloods: lateral saphenous EDTA and serum storage
RX: Vit E 0.12mls IM
RX: Ivomec 0.12mls IM
RX: Metacam 0.24mls SC
RX: Oxytet 0.6mls IM (JK)

22.Nov.2012 Weight: 5.64 Kg (12.4 Lb)
Proc: GA and health check due to loss of condition
Exam: Poor BCS; HR 128, RR 20, Temp 35.7
Eyes: moderate bilateral cataracts
Cloaca: clean
Pouch: Negative and clean
Dental: 1x extraction of L lower M3, removed withnecrotic piece of bone. Lignocaine spray used and chlorhex gel. Remaining functional teeth are 2x opposing upper and lower molars in right arcade.
Bloods: lateral saphenous EDTA and serum storage
RX: Vit E 0.12mls IM
RX: Ivomec 0.12mls IM
RX: Metacam 0.2mls SC
RX: Oxytet 0.6mls IM
Plan: continue to monitor behaviour and Body condition. If does not improve then to consider euthanasia. (JK)

2.Dec.2012
Obs: DS found in yard and picked up. Appeared flat, listless and unable to use hind quarters.
Exam: conscious exam, MM pink, CRT<2s, mouth healed up, 10% dehydrated
and obvious skin tenting.HR 140, Temp 36.8, delayed proprioception hind-quarters
DDx: underlying pathology or no water access.
Bloods: lateral tail vein, appearsdark and thick.
Urine sample: concentrated USG 1.034
Tx: given hartmanns SC 200mls, Nutrigel 3mls PO and when offered drank 150mls water
RX: Oxytet LA 0.6mls IM
RX: Flunixil 0.12mls IM
RX Vit ADE: 0.12mls IM
RX Vit B Complex 0.25mls IM
Plan: keep in holding yard with water and reassess tomorrow; may require euthanasia. (JK)

3.Dec.2012
Obs: Flat and depressed
Exam: Temp not registering; opt for euthanasia. (JK)

Clinical Notes - Individual Specimen Report MONARTO ZOO - page 1 -

MACROPUS EUGENII EUGENII	Sex: Male Acc. #: A59180
TAMMAR WALLABY	Age: 6Y 6M 20D E Birth: 16.May.2005
Chip: 00-0680-A74B	Tag/Band: White ET L ear

.....2007...

1.Oct.2007
Problem: coccidiosis (Confirmed)

11.Oct.2007
Problem: coccidiosis (Confirmed)
GA (isoflurane by facemask) for health check
teeth good, cloaca clean
pcv 49, tp 56
vit E given (IS)

.....2008...

1.Jul.2008
Problem: coccidiosis (Confirmed)

9.Jul.2008
GA (isoflurane by facemask) for health check
teeth good, cloaca clean
mild fight abrasions ventrally
Rx: VITAMIN E/SELENIUM 0.2 ml IM SID for 1 day. ** group treatment **
(IS)

.....2009...

26.Aug.2009
GA (isoflurane by facemask) for health check
Sex: Male, two large but symmetrical testicles present.
Dental exam: Teeth stained but otherwise in good condition.
Coat in good condition
No sign of cloacal infection
No other abnormalities detected.
Recovered without issues.
Rx: VITAMIN E/SELENIUM 0.2 ml IM (IS)

1.Nov.2009
Jumped into neighbouring 1.0's (A39187) exhibit yday; aggression and fight bt two.
Injuries v superficial loss of fur and epidermis over lateral aspect of cervical region and trauma to lateral canthus of r eye.
Rx: last night 0.2ml vit E se
2ml dex IM
HEld in pet packs in AC'd room onight (v hot 36 deg)
Examine this morn. BAR.
Observe moving back out into exhibit. NAD normal gait no signs of stiffness. (LJ)

.....2010...

24.Mar.2010
GA and health check today.
Iso open mask induction. Nice stable GA and recovery.

Eyes and ears NAD
Mouth excellent
testes normal
Abdo and chest NAD
cloaca clear
weight 6.27kg
ear tag blue right
blood collected

Rx: Vit E 0.1ml IM (LJ)

11.Aug.2010
Problem: coccidiosis (Confirmed)

4.Oct.2010
Problem: coccidiosis (Confirmed); lumpy jaw (Confirmed)
Obs: Drooly mouth noticed yesterday afternoon. Moved to wooden AHC yard.
Advised overphone from AZ to treat with Alamycin. GA tomorrow.
Rx: OXYTETRACYCLINE LA 300 - 0.5mL (150 mg) IM q72h for 5 doses. (DMC)

5.Oct.2010
Problem: coccidiosis (Confirmed); lumpy jaw (Confirmed)
Proc: GA,check mouth
1. Mouth: large submandibular abscess lanced and drained. Necrotic gingiva over proximal R mandible debrided to expose root of R lower incisor; tooth feels stable. Appears to be communication between this lesion and the abscess. Cheek teeth look OK at this stage.
2. Exam: Quite thin and dehydrated. Several patches of alopoecia on chest and flanks from fighting. Quite a few fleas.
Rx: sprayed with frontline. 120mL NaCl SC. 0.1mL VitE/Se. Antibiotics given yesterday
Plan: Reassess in a week. (DMC)

7.Oct.2010
Problem: coccidiosis (Confirmed); lumpy jaw (Confirmed)
Rx: 100mL NaCl SC with oxytet treatment. (DMC)

13.Oct.2010
Problem: coccidiosis (Confirmed); lumpy jaw (Confirmed)
GA (isoflurane by facemask) for revisit
ventral wound still open & communicating with right mandibular diastema, particularly medially
other teeth still appear ok
plan continue AB for 2 more weeks then revisit (IS)

28.Oct.2010
Problem: coccidiosis (Confirmed); lumpy jaw (Confirmed)
GA (isoflurane by facemask) for health check
teeth: worn on RHS
cloaca:normal
testes equal
healed grass seed abscess ventral jaw, also alopecia on left rump from fighting, ear tag removed
weighed, blood sampled & nasal/dental swabs collected
vit E given
Rx: VITAMIN E/SELENIUM 0.2 ml IM (IS)

18.Dec.2010
Problem: coccidiosis (Confirmed)

.....2011...
18.May.2011
GA (facemask Isofluorane) health check
Weight: 6.4 kg
Dental NAD, Staining RHS
HR 144, Temp 36.4
Cloaca: clean
Ear tag: white tag left ear
Bloods: tail vein EDTA and Serum
Vit E/Sel: 0.2mls IM, Ivomec 0.1ml IM (JK)

22.Aug.2011
Problem: coccidiosis (Confirmed)

18.Nov.2011
Problem: coccidiosis (Confirmed)
Proc: GA, health check
Problem: coccidiosis (Confirmed)
1. Teeth: L upper molars 2,3 slightly loose with significant gum recession. No purulent exudate, roots of both teeth black and cavitated. Sockets flushed with chlorhex. Chlorhex gel applied.
2. Exam: Otherwise in good body condition. Testicles symmetrical.
3. Azaperone 8mg (0.2mL) to guage effect for move next week.
Rx: Oxytetracycline 1mL IM
P: reassess at move next week. May require ongoing treatment at Adelaide. (DMC)

Clinical Notes - Individual Specimen Report MONARTO ZOO - page 1 -

MACROPUS EUGENII EUGENII	Sex: Male Acc. #: A89034
TAMMAR WALLABY	Age: 3Y 8M 18D E Birth: 18.Mar.2008
Chip: 00-069B-296D	Tag/Band: White ET L ear

.....2009...

26.Aug.2009

GA (isoflurane by facemask) for health check
Sex: Male, two symmetrical testicles present.
Dental exam: Teeth in good condition.
Abrasions to rostrum/nose and below L eye.
Coat in good condition
No sign of cloacal infection
No other abnormalities detected.
Recovered without issues.
Rx: VITAMIN E/SELENIUM 0.2 ml IM (IS)

.....2010...

14.Apr.2010

Proc: GA, health check
1. Teeth: good
2. Cloaca: clean
3. Pouch: N/A
4. Exam: pelage a bit patchy and unkempt but no wounds or parasites noted.
5. Cloacal temp: 35.5C
6. Blood sample - EDTA, serum archive
Rx: VitE/Se mL IM.
Rx: IVERMECTIN 2 mg IM SID for 1 day. (DMC)

11.Aug.2010

Problem: coccidiosis (Confirmed)

13.Oct.2010

Problem: coccidiosis (Confirmed); corneal ulcer - right eye (Confirmed); laceration - EYELIDS (LEFT) (Confirmed)
keeper report of blephraspasm
GA (isoflurane by facemask) for health check
left thrid eyelid laceration-> trim free edge
right eye abrasions to lateral & medial of eye, superficial corneal ulceration
blood in mouth but all teeth appear ok
also referred chest sounds on induction which settled at sugical anaesthetic depth
PDx traumatic events
plan 2 weeks ABs (im & palpebral) then RV
Rx: OXYTETRACYCLINE (AMPHOTERIC) 160 mg IM q72h for 14 days.
Rx: VITAMIN E/SELENIUM IM
Rx: CARPROFEN 25 mg SQ (IS)

28.Oct.2010
Problem: coccidiosis (Confirmed); corneal ulcer - right eye (Confirmed); laceration - EYELIDS (LEFT) (Confirmed)
GA (isoflurane by facemask) for health check
teeth: mild tarter staining but otherwise OK
cloaca: normal
testes equal
left 3rd eyelid mild scarring & hyperaemia, right eye normal, patches of linear alopecia over left chest (fighting), superficial wound medial R hock
weighed, blood sampled & nasal/dental swabs collected
vit E given
Rx: VITAMIN E/SELENIUM 0.2 ml IM
Rx: OXYTETRACYCLINE (AMPHOTERIC) 180 mg IM (IS)

.....2011...
18.May.2011
Problem: coccidiosis (Confirmed); corneal ulcer - right eye (Confirmed); laceration - EYELIDS (LEFT) (Confirmed)
GA (facemask Isofluorane) health check
Weight: 6.63kg
Dental: NAD mnild plaque
HR 116, Temp 37.2
Cloaca: clean
Ear tag: white tag left ear
Bloods: tail vein EDTA and Serum
Vit E/Sel: 0.2mls; Ivomec 0.1ml IM (JK)

5.Dec.2011
Problem: coccidiosis (Confirmed); corneal ulcer - right eye (Confirmed); laceration - EYELIDS (LEFT) (Confirmed)
GA (isoflurane by facemask) for health check prior to export
teeth ok but blood in mouth- suspect catching trauma
cloaca clean
testicles equal in size
blood taken for pcv/tp & archive
Rx: VITAMIN E/SELENIUM 0.2 ml IM (IS)

Clinical Notes - Individual Specimen Report MONARTO ZOO - page 1 -

PETROGALE LATERALIS Sex: Male Acc. #: A79179
BLACK-FOOTED ROCK WALLABY Age: 3Y 11M 9D Est Birth: 10.Jul.2007
Name: Alalka 3 Chip: 00-06A2-ACCC

.....2008...
29.Feb.2008
Ear puch taken for biopsy (MT)

14.May.2008
Problem: ascaridiasis (Confirmed); coccidiosis (Confirmed)

8.Sep.2008
Problem: ascaridiasis (Confirmed); coccidiosis (Confirmed)
examine conscious: small tail wound, few days old & granulating well, no treatment (IS)

.....2009...
5.Aug.2009
Problem: ascaridiasis (Confirmed); coccidiosis (Confirmed)
GA (isoflurane by facemask) for health check
teeth: nsf
cloaca clean
male
small wound on right ear tip, bleeding slightly
bloods collected, nasal swab collected

Rx: VITAMIN E/SELENIUM 0.1 ml IM (IS)

.....2010...
23.Apr.2010
Problem: coccidiosis (Confirmed)

9.Dec.2010
Problem: coccidiosis (Confirmed); trauma - tail (Confirmed)
Problem: tail injury
Exam: full thickness transverse laceration just proximal to tail tip. Bone not exposed at this stage. Distal tail alopoecic and a bit scabby. Typical of bite wounds but he's on his own at the moment - self trauma? or from mallee fowl in same enclosure?
Rx: neocort topically; duplocillin 0.5mL IM
P: revisit 3 weeks. (DMC)

.....2011...
7.Jan.2011
Problem: coccidiosis (Confirmed); trauma - tail (Confirmed)
Exam: tail tip healing but very slowly. Slightly moist cracked scab over deep healing wound lateral to tail tip.
P: no treatment. COntinue to monitor. Recheck again in 1 month (3 Feb)

(DMC)

18.Feb.2011
Problem: coccidiosis (Confirmed); trauma - tail (Confirmed)
Exam: wound on lateral tail tip looks to be contracting down still.
Suspect it keeps getting knocked.
P: continue to monitor. Recheck again 6-8w. (DMC)

Clinical Notes - Individual Specimen Report MONARTO ZOO - page 1 -

PETROGALE LATERALIS Sex: Female Acc. #: A79180
BLACK-FOOTED ROCK WALLABY Age: 4Y 4M Est Birth: 31.Mar.2007
Name: Alalka 4 Chip: 00-069B-407E

.....2007...
30.Oct.2007
hair loss and scabbing/matting around mouth
no fungal elements seen on hair under microscope
PDx food matting fur
plan oticleans then revisit as required (IS)

.....2008...
16.May.2008
Problem: coccidiosis (Confirmed)

.....2009...
5.Aug.2009
Problem: coccidiosis (Confirmed)
GA (isoflurane by facemask) for health check
teeth: tartered
cloaca clean
pouch MT & clean
bloods collected, nasal swab collected

Rx: VITAMIN E/SELENIUM 0.1 ml IM (IS)

.....2010...
29.Apr.2010
GA and routine Health check today
GA via open mask iso 5% induction, 2% maintenance. Nice induction, maintenance and recovery
Eyes and Ears NAD
Mouth excellent
Chest NAD, abdo NAD
Cloaca clear
Pouch clean and negative
Skin and pelage good
Weight: 2.5kg (LJ)

8.Dec.2010
Still losing weight and further bites on tail from conspecifics.
Advise move to another yard (LJ)

.....2011...
8.Jul.2011
GA for annual health check today and pre-release export
NSF
All clear.

Virginal pouch

0.15ml Vit E/Se IM
Blood collected for coulter and storage
weight 2.59kg
Fitted with radio collar as too small for GPS
Top black bottom white (LJ)

Clinical Notes - Individual Specimen Report MONARTO ZOO - page 1 -

PETROGALE LATERALIS Sex: Female Acc. #: A79174
BLACK-FOOTED ROCK WALLABY Age: 5Y 7M 27D Est Birth: 13.Jun.2007
Name: New Well 6 Chip: 00-06A2-8F18

.....2008...

29.Feb.2008
punch biopsy for DNA. (MT)

14.May.2008
Problem: ascaridiasis (Confirmed); coccidiosis (Confirmed)

12.Sep.2008
Problem: ascaridiasis (Confirmed); coccidiosis (Confirmed); ARTHROPOD BITES (Confirmed)
Mosquito bites over distal areas of pinnae. Discuss different topical options. Most require daily application and difficult to catch up animals.
Can apply one off permethrin equine product 'Swift'. Unsure of safety in these animals which are grooming? Monitor (LJ)

.....2009...

5.Aug.2009
Problem: ascaridiasis (Confirmed); coccidiosis (Confirmed); ARTHROPOD BITES (Confirmed)
GA (isoflurane by facemask) for health check
teeth: good
right nasal bleed due to swab, otherwise ok
cloaca clean
pouch empty & clean
bloods collected, nasal swab collected

Rx: VITAMIN E/SELENIUM 0.1 ml IM (IS)

.....2010...

29.Apr.2010
GA and routine Health check today
GA via open mask iso 5% induction, 2% maintenance. Nice induction, maintenance and recovery
Eyes and Ears NAD R pupil has lateral defect in margin
Mouth excellent-
Chest NAD, abdo NAD
Cloaca clear
Pouch clean and negative
Skin and pelage good
Weight: 3.535kg (LJ)

.....2011...

14.Jan.2011
Problem: obesity (Confirmed)
Obs: overweight. very chubby. In with 2 others so difficult to ration food. Hand reared animal, will cope fine with housing in smaller yard for weight loss.
P: isolate, feed set ration, reducing pellets first. Weigh weekly to monitor and adjust feed accordingly to achieve a downward trend. (DMC)

11.Feb.2011
Problem: obesity (Confirmed)
Obs: looking a lot better, but still has some weight to lose. Most recent weight around 3.9kg (down 400-500g from a month ago). Probably the same again wouldn't go astray.
P: Lucern chaff to be increased a bit to slow rate of weight loss. (DMC)

2.Mar.2011
Problem: obesity (Confirmed)
Exam: weight continues to reduce. Looking much better but still more to go. Another 200-300g likely good. Heidi will increase food ration again to slow weight loss.
P: management plan is to put her in a breeding situation in about a month. (DMC)

23.Mar.2011
Problem: obesity (Confirmed)
Obs: Reported to be looking good. Ration increased again to help stabilise weight. Will be returned to breeding situation soon. (DMC)

27.May.2011
Problem: obesity (Confirmed)
Rx: OXYTETRACYCLINE (AMPHOTERIC) 0.4 ml IM q72h for 4 days. (LJ)

24.Jun.2011
Problem: obesity (Confirmed)
GA for annual health check today.
NSF
All clear.
Virginal pouch
0.15ml Vit E/Se IM
Blood collected for coulter and storage
weight 3.26kg (LJ)

.....2012...

17.Apr.2012
Problem: obesity (Confirmed)
Proc: GA (iso) health check

1. Mouth: good

2. Pouch Left caudal teat long (young at foot)
3. Cloaca: clean
4. Exam: NSF
5. Blood: smear, PCV 46, TS 62, in house chemistries

Rx: VitE/Se 0.07mL IM (DMC)

19.Sep.2012

Problem: obesity (Confirmed)

Obs: Keepers observed some scabbing on distal pinnae. Only mild scabs likely from mosquitoes/midges. monitor atthis stage (JK)

Clinical Notes - Individual Specimen Report MONARTO ZOO - page 1 -

PETROGALE LATERALIS Sex: Male Acc. #: A79200
BLACK-FOOTED ROCK WALLABY Age: 4Y 4M 6D Est Birth: 19.Mar.2007
Name: New Well 10 Chip: 00-069B-43AE

.....2008...
14.May.2008
Problem: coccidiosis (Confirmed)

.....2009...
5.Aug.2009
Problem: coccidiosis (Confirmed)
GA (isoflurane by facemask) for health check
teeth: nsf
cloaca clean, male
bloods collected, nasal swab collected

Rx: VITAMIN E/SELENIUM 0.1 ml IM (IS)

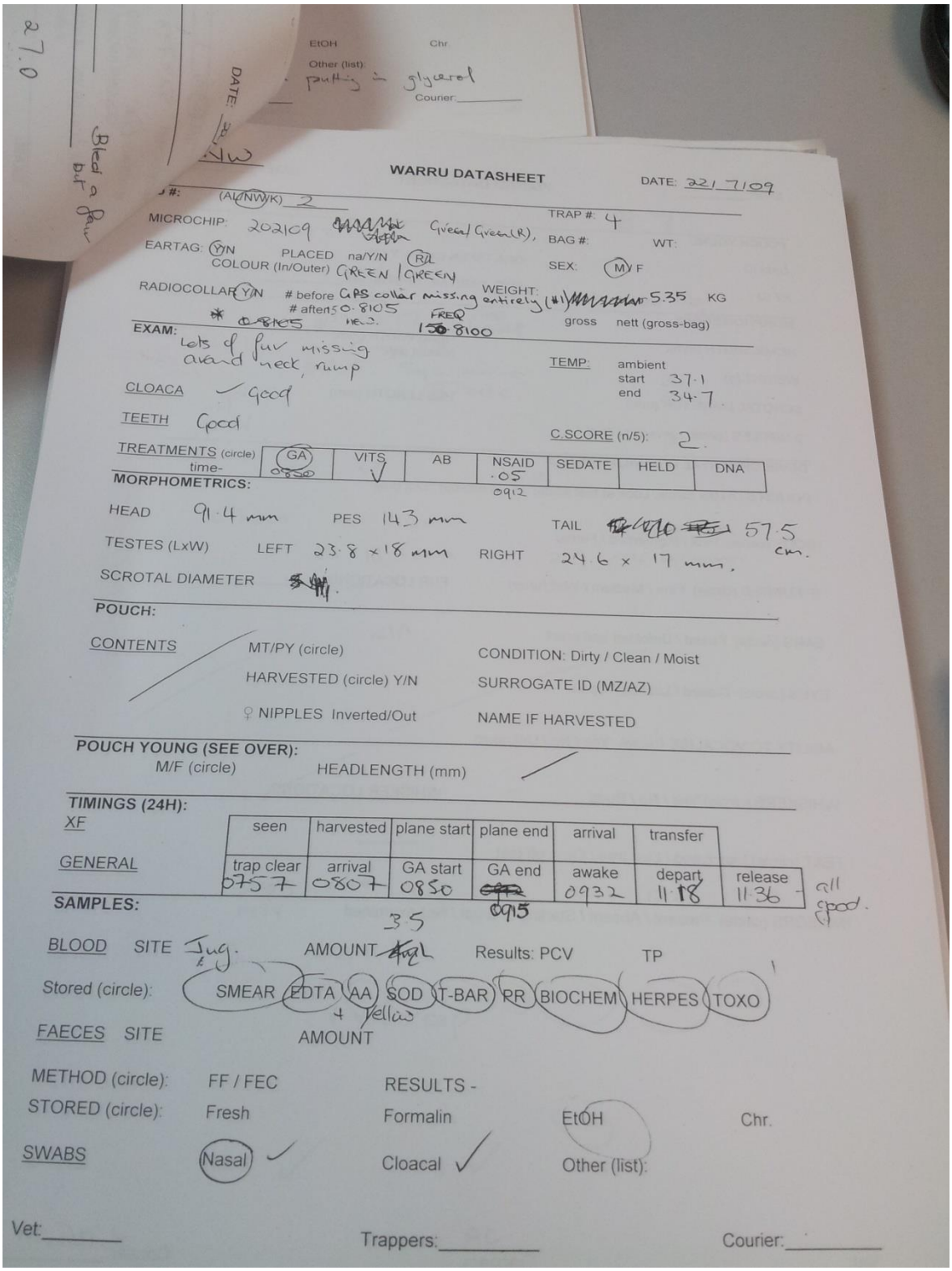
.....2010...
12.Aug.2010
Problem: coccidiosis (Confirmed)

.....2011...
8.Jul.2011
Problem: coccidiosis (Confirmed)
GA for annual health check today and pre-release export
NSF
All clear.
Testes symmetrical
0.15ml Vit E/Se IM
Blood collected for coulter and storage
weight 3.46kg
Fitted with GPS #8 F 151.238
All black (LJ)

15.Jul.2011
Problem: coccidiosis (Confirmed)
Catch today to reassess collars as have all been chewing them
Collar all ok
weight 3.68 (LJ)

Printed on: 7.Feb.2013 /ISIS/MedARKS/5.54.e

Appendix C: APY Land New Well Black-flanked Rock wallaby health datasheet at nasal swab collection (2009)



WARRU DATASHEET DATE: 22/7/09

TRAP #: 4

MICROCHIP: 202109 Green/Green(R), BAG #: WT:

EARTAG: (Y)N PLACED na/Y/N (R)L SEX: (M)/F

COLOUR (In/Outer) GREEN / GREEN

RADIOCOLLAR (Y)N # before GPS collar missing WEIGHT: 5.35 KG

after 50-8105 FREQ 150.8100 gross nett (gross-bag)

EXAM: lots of fur missing around neck, rump

CLOACA ✓ Good

TEETH Good

TEMP: ambient start 37.1 end 34.7

C.SCORE (n/5): 2.

TREATMENTS (circle) time-	GA 0850	VITS ✓	AB	NSAID .05 0912	SEDATE	HELD	DNA

MORPHOMETRICS:

HEAD 91.4 mm PES 143 mm TAIL 57.5 cm.

TESTES (LxW) LEFT 23.8 x 18 mm RIGHT 24.6 x 17 mm.

SCROTAL DIAMETER

POUCH:

CONTENTS MT/PY (circle) CONDITION: Dirty / Clean / Moist

HARVESTED (circle) Y/N SURROGATE ID (MZ/AZ)

♀ NIPPLES Inverted/Out NAME IF HARVESTED

POUCH YOUNG (SEE OVER):

M/F (circle) HEADLENGTH (mm)

TIMINGS (24H):

XF	seen	harvested	plane start	plane end	arrival	transfer	
GENERAL	trap clear 0757	arrival 0807	GA start 0850	GA end 0915	awake 0932	depart 11.18	release 11.36 - all good.

SAMPLES:

35

BLOOD SITE Jug. AMOUNT Results: PCV TP

Stored (circle): SMEAR EDTA AA SOD T-BAR PR BIOCHEM HERPES TOXO

+ Yellow

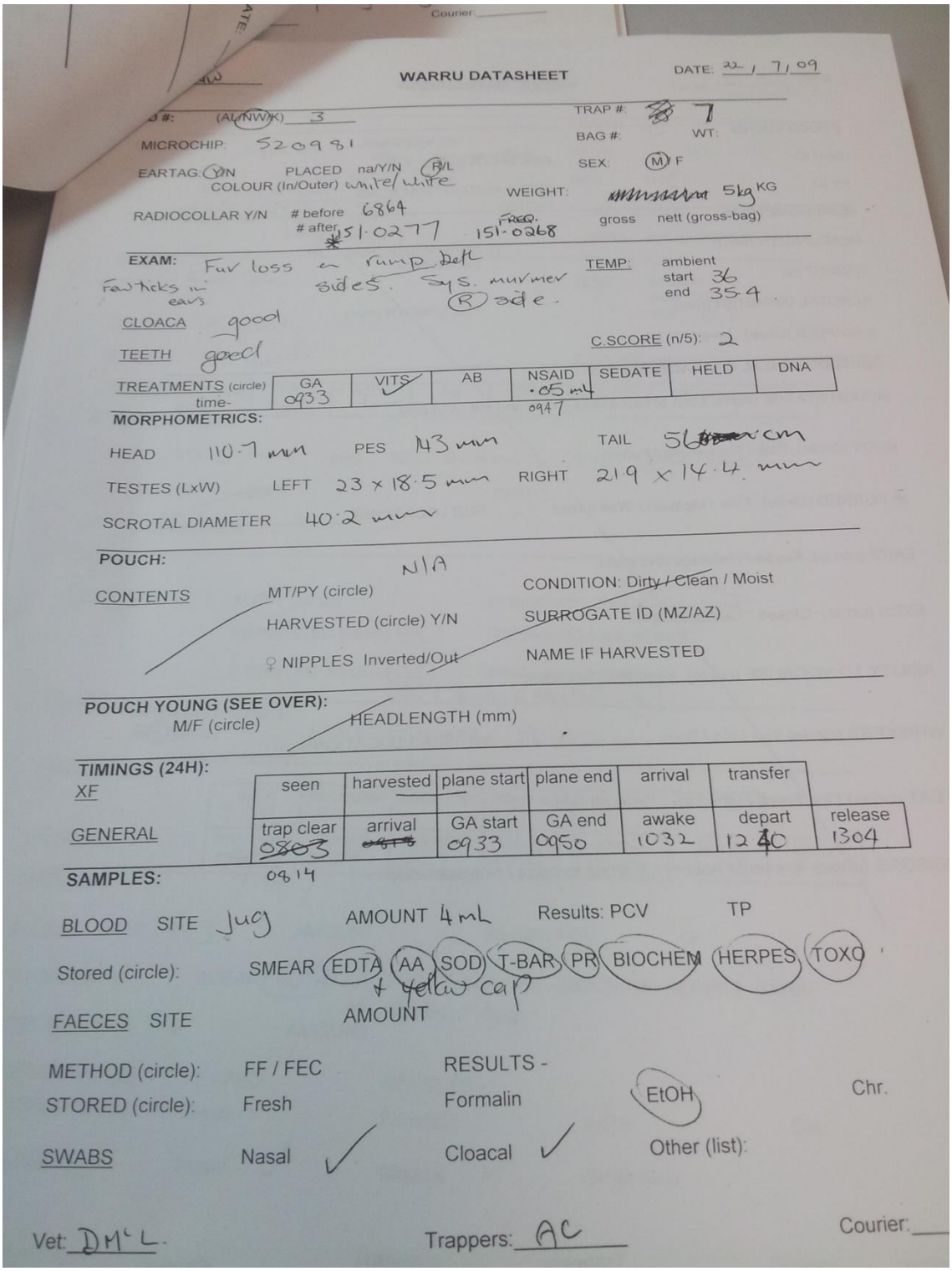
FAECES SITE AMOUNT

METHOD (circle): FF / FEC RESULTS -

STORED (circle): Fresh Formalin EtOH Chr.

SWABS (Nasal) ✓ Cloacal ✓ Other (list):

Vet:_____ Trappers:_____ Courier:_____



WARRU DATASHEET

DATE: 22/7/09

ID #: (AL/NW/K) 3 TRAP #: 7

MICROCHIP: 520981 BAG #: WT:

EARTAG: Y/N PLACED na/Y/N R/L SEX: M/F

COLOUR (In/Outer) white/white

RADIOCOLLAR Y/N # before 6864 WEIGHT: 5kg KG

after 151·0277 FREQ. 151·0268 gross nett (gross-bag)

EXAM: Fur loss on rump both sides. Sys. murmer (R) side. Fat ticks in ears TEMP: ambient start 36 end 35·4

CLOACA good

TEETH good C.SCORE (n/5): 2

TREATMENTS (circle) time-	GA 0933	VITS ✓	AB	NSAID ·05 mL 0947	SEDATE	HELD	DNA

MORPHOMETRICS:

HEAD 110·7 mm PES 143 mm TAIL 56 cm

TESTES (LxW) LEFT 23 x 18·5 mm RIGHT 21·9 x 14·4 mm

SCROTAL DIAMETER 40·2 mm

POUCH: N/A

CONTENTS MT/PY (circle) CONDITION: Dirty / Clean / Moist

HARVESTED (circle) Y/N SURROGATE ID (MZ/AZ)

♀ NIPPLES Inverted/Out NAME IF HARVESTED

POUCH YOUNG (SEE OVER):

M/F (circle) HEADLENGTH (mm)

TIMINGS (24H):

XF	seen	harvested	plane start	plane end	arrival	transfer	
GENERAL	trap clear 0803	arrival ~~0815~~	GA start 0933	GA end 0950	awake 1032	depart 1240	release 1304

SAMPLES: 0814

BLOOD SITE jug AMOUNT 4mL Results: PCV TP

Stored (circle): SMEAR EDTA AA SOD T-BAR PR BIOCHEM HERPES TOXO + yellow cap

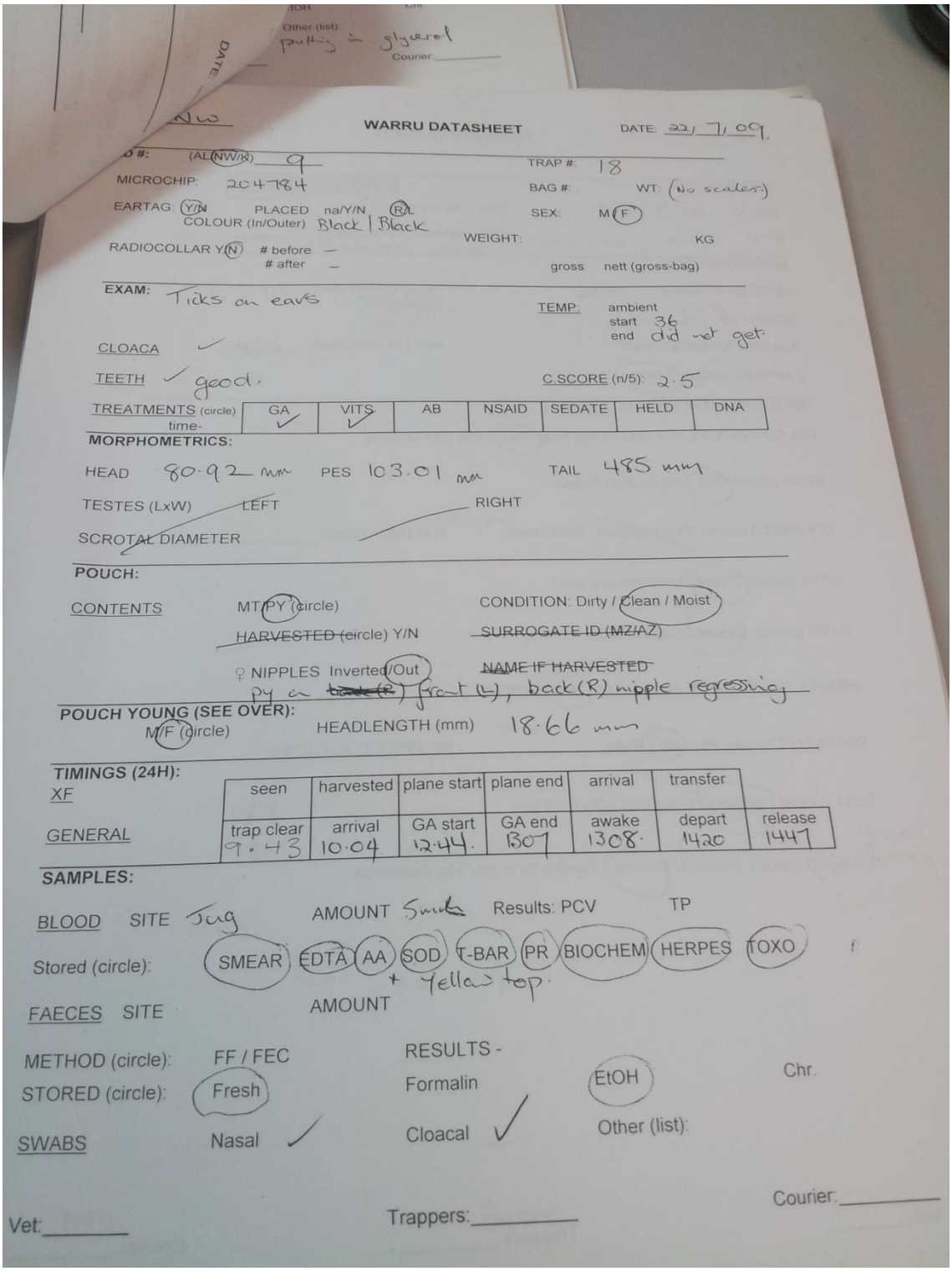
FAECES SITE AMOUNT

METHOD (circle): FF / FEC RESULTS -

STORED (circle): Fresh Formalin EtOH Chr.

SWABS Nasal ✓ Cloacal ✓ Other (list):

Vet: DMcL Trappers: AC Courier:



Other (list): putty + glycerol
Courier:

DATE

NW

WARRU DATASHEET DATE: 22/7/09.

ID #: (AL/NW/K) 9 TRAP #: 18

MICROCHIP: 204784 BAG #: WT: (No scales)

EARTAG: (Y/N) PLACED na/Y/N (R/L) SEX: M (F)

COLOUR (In/Outer) Black | Black

WEIGHT: KG

RADIOCOLLAR Y(N) # before – # after – gross nett (gross-bag)

EXAM: Ticks on ears

TEMP: ambient start 36 end did not get

CLOACA ✓

TEETH ✓ good.

C.SCORE (n/5): 2.5

TREATMENTS (circle) time-	GA ✓	VITS ✓	AB	NSAID	SEDATE	HELD	DNA

MORPHOMETRICS:

HEAD 80.92 mm PES 103.01 mm TAIL 485 mm

TESTES (LxW) LEFT RIGHT

SCROTAL DIAMETER

POUCH:

CONTENTS MT/PY (circle) CONDITION: Dirty / Clean / Moist

~~HARVESTED (circle) Y/N~~ ~~SURROGATE ID (MZ/AZ)~~

♀ NIPPLES Inverted/Out ~~NAME IF HARVESTED~~

py in ~~back (R)~~ front (L), back (R) nipple regressing

POUCH YOUNG (SEE OVER):

M/F (circle) HEADLENGTH (mm) 18.66 mm

TIMINGS (24H):

	seen	harvested	plane start	plane end	arrival	transfer	
XF							
GENERAL	trap clear 9.43	arrival 10.04	GA start 12.44	GA end 1307	awake 1308	depart 1420	release 1447

SAMPLES:

BLOOD SITE Jug AMOUNT Small Results: PCV TP

Stored (circle): SMEAR EDTA AA SOD T-BAR PR BIOCHEM HERPES TOXO + Yellow top.

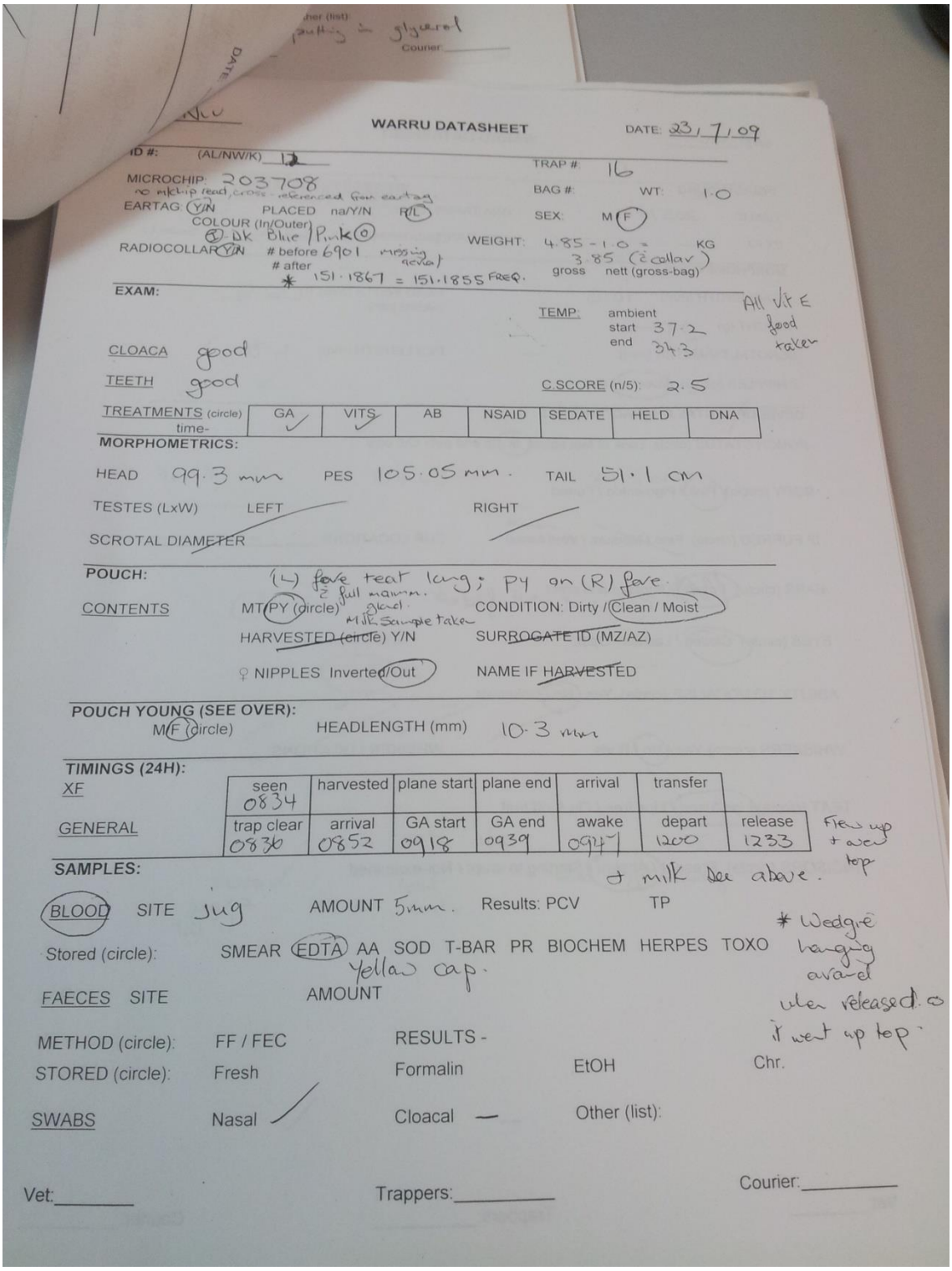
FAECES SITE AMOUNT

METHOD (circle): FF / FEC RESULTS -

STORED (circle): Fresh Formalin EtOH Chr.

SWABS Nasal ✓ Cloacal ✓ Other (list):

Vet:_____ Trappers:_____ Courier:_____



WARRU DATASHEET DATE: 23/7/09

ID #: (AL/NW/K) 12 TRAP #: 16

MICROCHIP: 203708 (no mk1ip read, cross-referenced from eartag) BAG #: WT: 1.0

EARTAG (Y/N) PLACED na/Y/N R/L SEX: M F

COLOUR (In/Outer) DK Blue / Pink

RADIOCOLLAR Y/N # before 6901 missing never found # after * 151.1867 = 151.1855 FREQ. WEIGHT: 4.85 - 1.0 = 3.85 (& collar) KG gross nett (gross-bag)

EXAM:

All Vit E food taken

TEMP: ambient start 37.2 end 34.3

CLOACA good

TEETH good

C.SCORE (n/5): 2.5

TREATMENTS (circle) time-	GA ✓	VITS ✓	AB	NSAID	SEDATE	HELD	DNA

MORPHOMETRICS:

HEAD 99.3 mm PES 105.05 mm. TAIL 51.1 cm

TESTES (LxW) LEFT RIGHT

SCROTAL DIAMETER

POUCH: (L) fave teat lang; PY on (R) fave. 2 full mamm. gland.

CONTENTS MT (PY (circle)) Milk Sample taken CONDITION: Dirty / Clean / Moist

HARVESTED (circle) Y/N SURROGATE ID (MZ/AZ)

♀ NIPPLES Inverted/Out NAME IF HARVESTED

POUCH YOUNG (SEE OVER):

M F (circle) HEADLENGTH (mm) 10.3 mm

TIMINGS (24H):

XF	seen 0834	harvested	plane start	plane end	arrival	transfer	
GENERAL	trap clear 0830	arrival 0852	GA start 0918	GA end 0939	awake 0947	depart 1200	release 1233

Flew up + went top

SAMPLES:

+ milk see above.

BLOOD SITE jug AMOUNT 5mm. Results: PCV TP

Stored (circle): SMEAR EDTA yellow cap. AA SOD T-BAR PR BIOCHEM HERPES TOXO

* Wedge hanging around when released. It went up top.

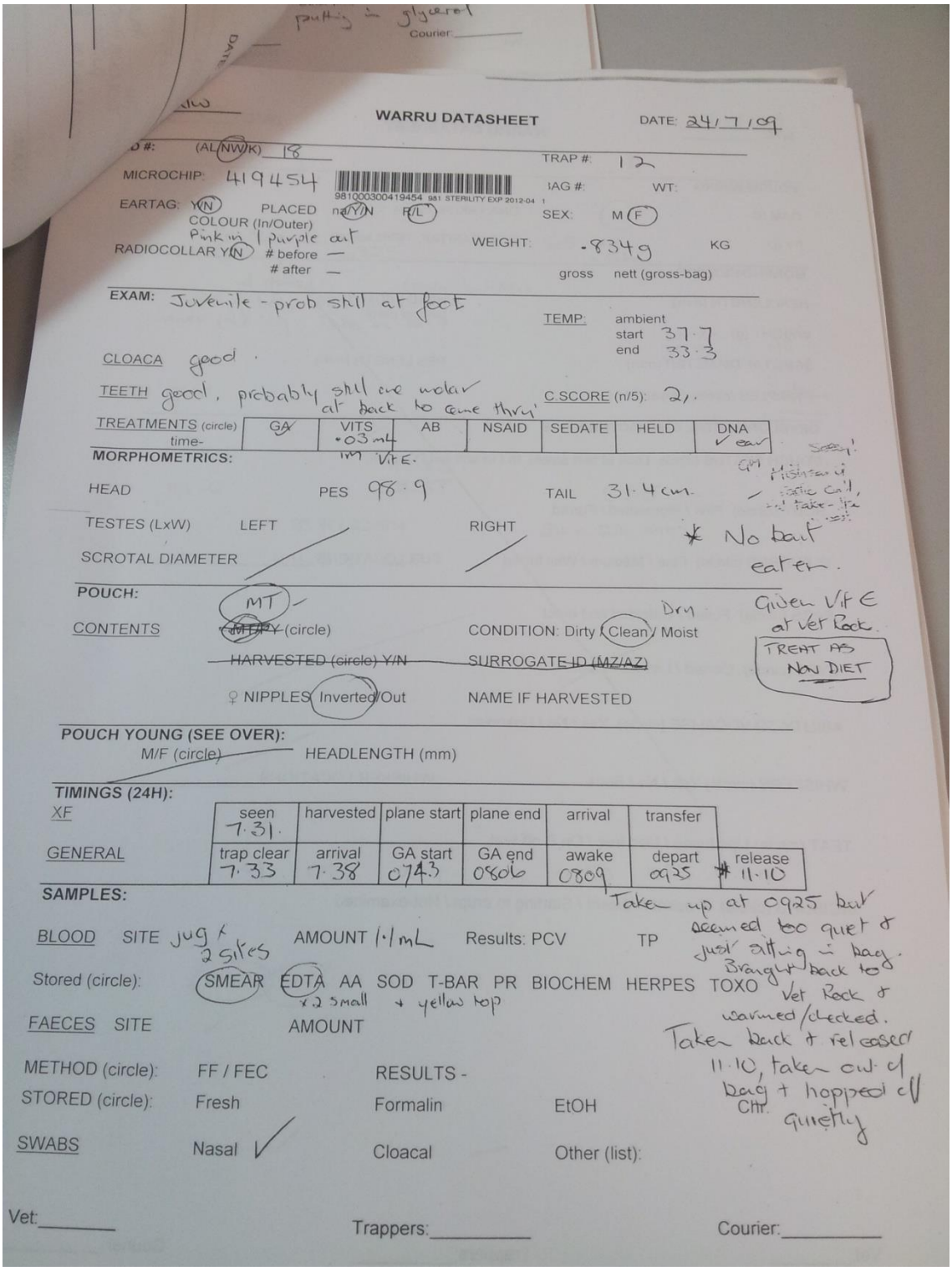
FAECES SITE AMOUNT

METHOD (circle): FF / FEC RESULTS -

STORED (circle): Fresh Formalin EtOH Chr.

SWABS Nasal ✓ Cloacal — Other (list):

Vet:_____ Trappers:_____ Courier:_____



WARRU DATASHEET

DATE: 24/7/09

ID #: (AL/NW/K) 18

TRAP #: 12

MICROCHIP: 419454 981000300419454 981 STERILITY EXP 2012-04

TAG #: WT:

EARTAG: Y/N PLACED n/a/Y/N R/L

SEX: M/F

COLOUR (In/Outer) Pink in | purple ear

RADIOCOLLAR Y/N # before — # after —

WEIGHT: 834g KG

gross nett (gross-bag)

EXAM: Juvenile - prob still at foot

TEMP: ambient start 37.7 end 33.3

CLOACA good.

TEETH good, probably still one molar at back to come thru'

C.SCORE (n/5): 2.

TREATMENTS (circle) time-	GA	VITS	AB	NSAID	SEDATE	HELD	DNA
		0.3 ml IM Vit E.					✓ ear

MORPHOMETRICS:

HEAD PES 98.9 TAIL 31.4 cm.

TESTES (LxW) LEFT RIGHT

SCROTAL DIAMETER

* No bait eaten.

POUCH: MT

CONTENTS ~~YMEAY~~ (circle) CONDITION: Dirty / Clean / Moist Dry

~~HARVESTED (circle) Y/N~~ ~~SURROGATE ID (MZ/AZ)~~

♀ NIPPLES Inverted/Out NAME IF HARVESTED

Given Vit E at Vet Rack.

TREAT AS NON DIET

POUCH YOUNG (SEE OVER):

M/F (circle) HEADLENGTH (mm)

TIMINGS (24H):

XF	seen 7.31.	harvested	plane start	plane end	arrival	transfer	
GENERAL	trap clear 7.33	arrival 7.38	GA start 0743	GA end 0806	awake 0809	depart 0925	release * 11.10

SAMPLES:

Take up at 0925 but seemed too quiet & just sitting in bag. Brought back to Vet Rock & warmed/checked. Taken back & released 11.10, taken out of bag & hopped off chr. quietly

BLOOD SITE jug x 2 sites AMOUNT 1.1mL Results: PCV TP

Stored (circle): SMEAR EDTA AA SOD T-BAR PR BIOCHEM HERPES TOXO x2 small + yellow top

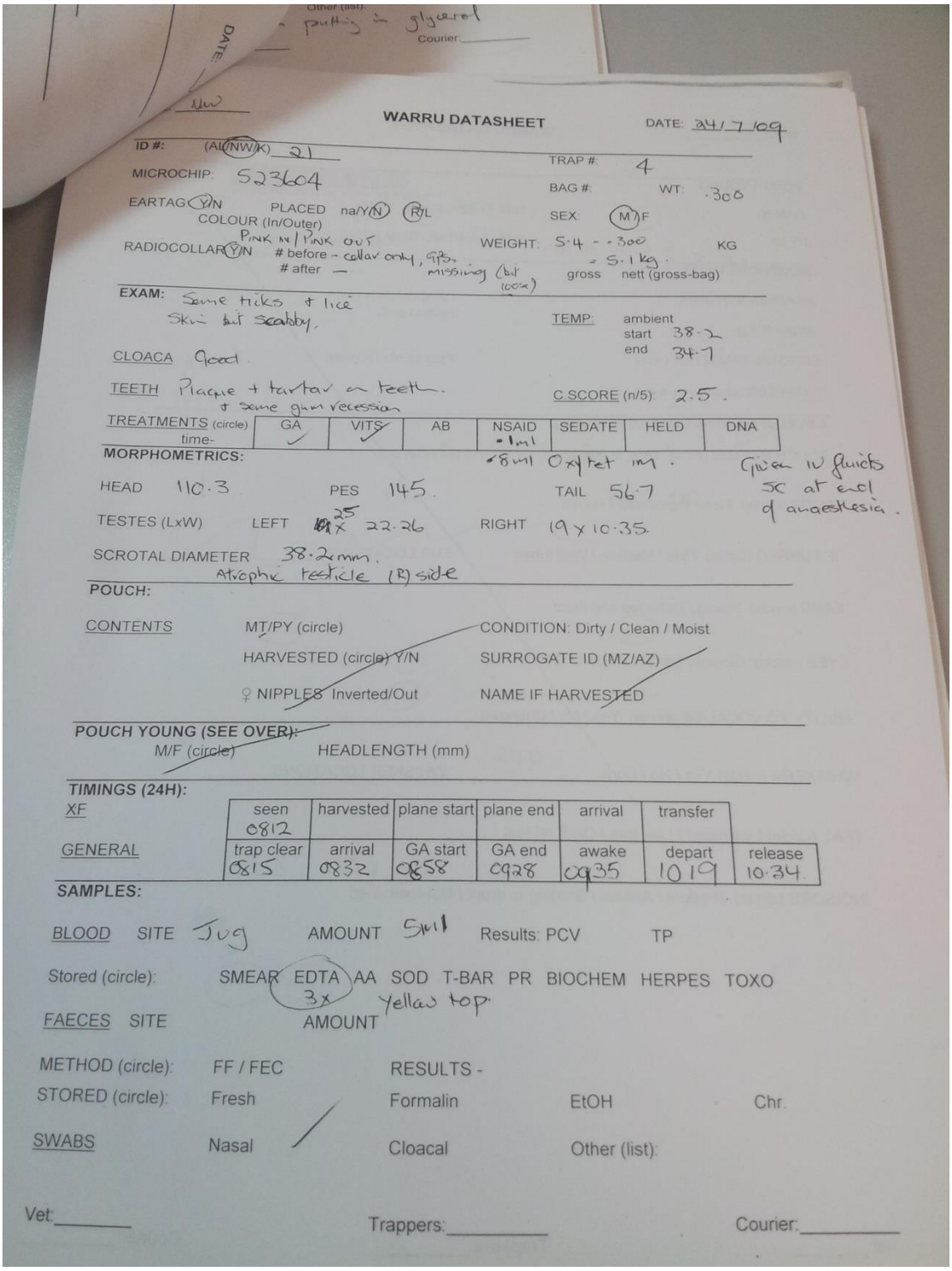
FAECES SITE AMOUNT

METHOD (circle): FF / FEC RESULTS -

STORED (circle): Fresh Formalin EtOH

SWABS Nasal ✓ Cloacal Other (list):

Vet:_____ Trappers:_____ Courier:_____



DATE

putting in glycerol

Courier:

NW

WARRU DATASHEET DATE: 24/7/09

ID #: (AL/NW/K) 21

MICROCHIP: 523604

TRAP #: 4

BAG #: WT: 300

EARTAG (Y)/N PLACED na/Y/(N) (R)/L

COLOUR (In/Outer) PINK IN / PINK OUT

SEX: (M)/F

RADIOCOLLAR (Y)/N # before - collar only, GPS missing (but 100%) # after —

WEIGHT: 5.4 - .300 = 5.1 kg. gross nett (gross-bag) KG

EXAM: Some ticks + lice

Skin but scabby.

TEMP: ambient start 38.2 end 34.7

CLOACA Good.

TEETH Plaque + tartar on teeth. + some gum recession

C.SCORE (n/5): 2.5.

TREATMENTS (circle) time-	GA	VITS	AB	NSAID	SEDATE	HELD	DNA
	✓	✓		1ml	.8ml Oxytet 1ml.		

Given IV fluids SC at end of anaesthesia.

MORPHOMETRICS:

HEAD 110.3 PES 145. TAIL 56.7

TESTES (LxW) LEFT ~~19~~ 25 x 22.26 RIGHT 19 x 10.35.

SCROTAL DIAMETER 38.2mm.

Atrophic testicle (R) side

POUCH:

CONTENTS MT/PY (circle) CONDITION: Dirty / Clean / Moist

HARVESTED (circle) Y/N SURROGATE ID (MZ/AZ)

♀ NIPPLES Inverted/Out NAME IF HARVESTED

POUCH YOUNG (SEE OVER):

M/F (circle) HEADLENGTH (mm)

TIMINGS (24H):

	seen	harvested	plane start	plane end	arrival	transfer	
XF	0812						
GENERAL	trap clear 0815	arrival 0832	GA start 0858	GA end 0928	awake 0935	depart 1019	release 10.34.

SAMPLES:

BLOOD SITE Jug AMOUNT 5ml Results: PCV TP

Stored (circle): SMEAR EDTA 3x AA SOD T-BAR PR BIOCHEM HERPES TOXO Yellow top

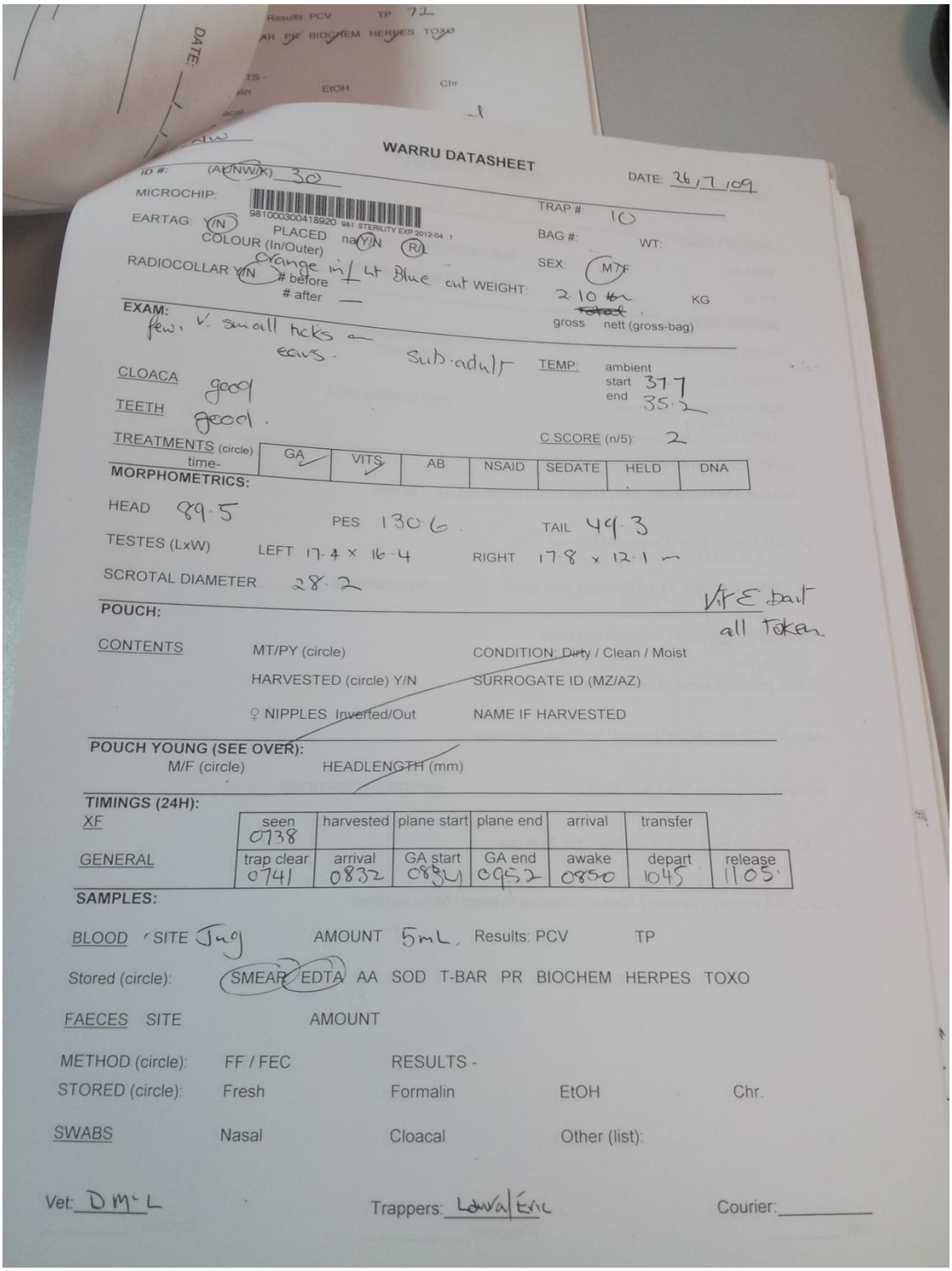
FAECES SITE AMOUNT

METHOD (circle): FF / FEC RESULTS -

STORED (circle): Fresh Formalin EtOH Chr.

SWABS Nasal Cloacal Other (list):

Vet:_____ Trappers:_____ Courier:_____



WARRU DATASHEET

ID #: (A/NW/X) 30 DATE: 26/7/09

MICROCHIP: 981000300418920 981 STERILITY EXP 2012-04 .1

EARTAG: Y/N PLACED na/Y/N COLOUR (In/Outer) R/L

RADIOCOLLAR Y/N # before # after

TRAP #: 10

BAG #: WT:

SEX: M/F

WEIGHT: 2.10 kg KG

gross nett (gross-bag)

EXAM: few v. small ticks on ears. Sub-adult

CLOACA good

TEETH good.

TEMP: ambient start 37.7 end 35.2

C.SCORE (n/5): 2

TREATMENTS (circle) time-

GA	VITS	AB	NSAID	SEDATE	HELD	DNA

MORPHOMETRICS:

HEAD 89.5 PES 130.6 TAIL 49.3

TESTES (LxW) LEFT 17.4 x 16.4 RIGHT 17.8 x 12.1 mm

SCROTAL DIAMETER 28.2

POUCH: VTE Dart all Taken

CONTENTS MT/PY (circle) CONDITION: Dirty / Clean / Moist

HARVESTED (circle) Y/N SURROGATE ID (MZ/AZ)

♀ NIPPLES Inverted/Out NAME IF HARVESTED

POUCH YOUNG (SEE OVER):

M/F (circle) HEADLENGTH (mm)

TIMINGS (24H):

XF	seen 0738	harvested	plane start	plane end	arrival	transfer	
GENERAL	trap clear 0741	arrival 0832	GA start 0854	GA end 0952	awake 0850	depart 1045	release 1105

SAMPLES:

BLOOD SITE Jug AMOUNT 5mL Results: PCV TP

Stored (circle): SMEAR EDTA AA SOD T-BAR PR BIOCHEM HERPES TOXO

FAECES SITE AMOUNT

METHOD (circle): FF / FEC RESULTS -

STORED (circle): Fresh Formalin EtOH Chr.

SWABS Nasal Cloacal Other (list):

Vet: DMcL Trappers: Lowra/Eric Courier:

Appendix D: Staphylococcal isolates recovered from wallabies

Appendix D1 – Staphylococci isolated from Monarto Zoo Yellow-footed Rock (*Petrogale xanthopus*) and Tammar wallabies (*Macropus eugenii*) (Oct 2010)[a]

Original ID	Renamed	Wallaby Specie	Staphylococcal ID
B1.1.2.2	M2	YFRW	*S. warneri*
B1.6.2.2	M3	YFRW	*S. vitulinus*
B1.11.1	M6	YFRW	*S. sciuri*
B1.12.1	M7	YFRW	*S. aureus*
B1.13.1	M8	YFRW	*S. succinus*
B1.14.1	M9	YFRW	*S. aureus*
B1.17.1	M10	YFRW	*S. simulans*
B2.3.3.2	**M11**	**YFRW**	***S. fleurettii***
B2.5.2.1	M14	YFRW	*S. xylosus*
B2.6.2.2	M15	YFRW	*S. succinus*
B2.10.2.2	M17	YFRW	*S. saprophyticus*
B2.11.3.3	M21	YFRW	*S. saprophyticus*
B1.5.1	M26	YFRW	*S. simulans*
B1.16.2.1	M27	YFRW	*S. succinus*
B2.2.3.1	M28	YFRW	*S. xylosus*
B2.2.3.2	M29	YFRW	*S. xylosus*
B2.2.3.3	M30	YFRW	*S. succinus*
B2.3.2.2a	**M31**	**YFRW**	***S. fleurettii***
B2.4.1	M32	YFRW	*S. delphini*
B2.6.2.1	M33	YFRW	*S. delphini*
B2.8.1	M36	YFRW	*S. xylosus*
B2.9.1	M37	YFRW	*S. succinus*
B1.2.3.1	M38	YFRW	*S. vitulinus*
B1.2.3.2	M39	YFRW	*S .vitulinus*
B1.2.3.3	M40	YFRW	*S. succinus*
B1.3.2.2	M41	YFRW	*S. succinus*
B2.3.2.1a	**M47**	**YFRW**	***S. fleurettii***
B2.3.3.1	M48	YFRW	*S. delphini*
B3.10.3.1	M53	TMW	*S. delphini*
B3.10.3.2	M54	TMW	*S. succinus*
B3.11.3.1	M56	TMW	*S. delphini*
B3.11.3.2	M57	TMW	*S. succinus*
B3.11.3.3	M58	TMW	*S. delphini*
B4.5.3.1	M65	TMW	*S .epidermidis*
B4.5.3.2	M66	TMW	*S. epidermidis*
B4.6.1	M68	TMW	*S. epidermidis*
B4.8.1	M69	TMW	*S. hominis*
B4.11.2.1	**M72**	**TMW**	***S. epidermidis***

[a] *mecA*-positive isolates highlighted in bold text.

Appendix D2 – Staphylococci isolated from Monarto Zoo Black-flanked Rock wallabies (*Petrogale lateralis*) (July 2009)[a]

Original ID	Renamed	Wallaby Specie	Staphylococcal ID
51	AK.51	BFRW	*S. saprophyticus*
55	AK.55	BFRW	*S. xylosus*
58	AK.58	BFRW	*S. sciuri*
59	**AK.59**	**BFRW**	***S. fleurettii***
61	**AK.61**	**BFRW**	***S. fleurettii***
64	AK.64	BFRW	*S. carnosus*
66	AK.66	BFRW	*S. simulans*
67	AK.67	BFRW	*S. succinus*
68	AK.68	BFRW	*S. succinus*
69	**AK.69**	**BFRW**	***S. fleurettii***
70	AK.70	BFRW	*S. aureus*
71	AK.71	BFRW	*S. delphini*
72	**AK.72**	**BFRW**	***S. fleurettii***
73	AK.73	BFRW	*S. aureus*
74	AK.74	BFRW	*S. aureus*
75	AK.75	BFRW	*S. succinus*
76	AK.76	BFRW	*S. delphini*
78	AK.78	BFRW	*S. aureus*
79	AK.79	BFRW	*S. xylosus*
81	AK.81	BFRW	*S. sciuri*

[a] *mecA*-positive isolates highlighted in bold text.

Appendix D3 – Staphylococci isolated from APY Land Black-flanked Rock wallabies (*Petrogale lateralis*) (July 2009)[a]

Original ID	Renamed	Wallaby Specie	Staphylococcal ID
1	AK.1	BFRW	*S. warneri*
2	AK.2	BFRW	*S. epidermidis*
4	AK.4	BFRW	*S. delphini*
5	AK.5	BFRW	*S. delphini*
7	AK.7	BFRW	*S. aureus*
8	AK.8	BFRW	*S. aureus*
10	AK.10	BFRW	*S. delphini*
11	AK.11	BFRW	*S. delphini*
14	AK.14	BFRW	*S. warneri*
15	AK.15	BFRW	*S. warneri*
16	**AK.16**	**BFRW**	***S. warneri***
17	**AK.17**	**BFRW**	***S. warneri***
18	AK.18	BFRW	*S. epidermidis*
19	AK.19	BFRW	*S. delphini*
24	AK.24	BFRW	*S. warneri*
25	AK.25	BFRW	*S. simulans*
30	AK.30	BFRW	*S. delphini*
31	**AK.31**	**BFRW**	***S. cohnii***
32	AK.32	BFRW	*S. epidermidis*
33	AK.33	BFRW	*S. delphini*
34	AK.34	BFRW	*S. warneri*
35	AK.35	BFRW	*S. delphini*
37	AK.37	BFRW	*S. succinus*
38	AK.38	BFRW	*S. succinus*
40	AK.40	BFRW	*S. sciuri*
41	AK.41	BFRW	*S. xylosus*
44	AK.44	BFRW	*S. xylosus*
45	AK.45	BFRW	*S. simulans*
46	AK.46	BFRW	*S. succinus*
48	AK.48	BFRW	*S. xylosus*
50	AK.50	BFRW	*S. saprophyticus*

[a] *mecA*-positive isolates highlighted in bold text.

Appendix E: Methicillin-sensitive *Staphylococcus aureus* clonal complex determination by DNA microarray

Strain		A7 F-BFRW	A8 F-BFRW	A78 C-BFRW	A70 C-BFRW	A73 C-BFRW	M9 C-YFRW	M7 C-YFRW
Clonal Complex		CC15	CC15	CC15	CC49	CC49	CC49	CC692
SPECIES MARKER	*rrnD1*	●	●	●	●	●	●	●
	gapA	●	●	●	●	●	●	●
	katA	●	●	●	●	●	●	●
	coA	●	●	●	●	●	●	●
	nuc1	●	●	●	●	●	●	●
	spa	●	●	●	●	●	●	●
	sbi	●	●	●	●	●	●	●
REGULATORY GENES	*sarA*	●	●	●	●	●	●	●
	saeS	●	●	●	●	●	●	●
	vraS	●	●	●	●	●	●	●
	agrI (total)	-	-	-	-	-	-	●
	agrB-I	-	-	-	-	-	-	●
	agrC-I	-	-	-	-	-	-	●
	agrD-I	-	-	-	-	-	-	●
	agrII (total)	●	●	●	●	●	●	-
	agrB-II	●	●	●	●	●	●	-
	agrC-II	●	●	●	●	●	●	-
	agrD-II	●	●	●	●	●	●	-
	agrIII (total)	-	-	-	-	-	-	-
	agrB-III	-	-	-	-	-	-	-
	agrC-III	-	-	-	-	-	-	-
	agrD-III	-	-	-	-	-	-	-
	agrIV (total)	-	-	-	-	-	-	-
	agrB-IV	-	-	-	-	-	-	●
	agrC-IV	-	-	-	-	-	-	-
	hld	●	●	●	●	●	●	●
METHICILLIN RESISTANCE AND SCC*mec* TYPING	*mecA*	-	-	-	-	-	-	-
	Δ*mecR*	-	-	-	-	-	-	-
	ugpQ	-	-	-	-	-	-	-
	ccrA-1	-	-	-	-	-	-	-
	ccrB-1	-	-	-	-	-	-	-
	plsSCC (COL)	-	-	-	-	-	-	-
	Q9XB68-dcs	-	-	-	-	-	-	-
	ccrA-2	-	-	-	-	-	-	-
	ccrB-2	-	-	-	-	-	-	-
	kdpA -SCC	-	-	-	-	-	-	-
	kdpB -SCC	-	-	-	-	-	-	-
	kdpC -SCC	-	-	-	-	-	-	-
	kdpD -SCC	-	-	-	-	-	-	-
	kdpE -SCC	-	-	-	-	-	-	-

Strain		A7 F-BFRW	A8 F-BFRW	A78 C-BFRW	A70 C-BFRW	A73 C-BFRW	M9 C-YFRW	M7 C-YFRW
Clonal Complex		CC15	CC15	CC15	CC49	CC49	CC49	CC692
METHICILLIN RESISTANCE AND SCC*mec* TYPING	***mecI***	-	-	-	-	-	-	-
	mecR	-	-	-	-	-	-	-
	xylR	-	-	-	-	-	-	-
	ccrA-3	-	-	-	-	-	-	-
	ccrB-3	-	-	-	-	-	-	-
	merA	-	-	-	-	-	-	-
	merB	-	-	-	-	-	-	-
	ccrA-4	-	-	-	-	-	-	-
	ccrB-4	-	-	-	-	-	-	-
	***ccrAA* (MRSAZH47)-Probe 1**	-	-	-	-	-	-	-
	***ccrAA* (MRSAZH47)-Probe 2**	-	-	-	-	-	-	-
	***ccrC* (85-2082)**	-	-	-	-	-	-	-
	mecC	-	-	-	-	-	-	-
	***blaZ* -SCC*mec* XI**	-	-	-	-	-	-	-
RESISTANCE: β-LACTAMASE	***blaZ***	●	●	●	-	-	-	-
	blaI	●	●	●	-	-	-	-
	blaR	●	●	●	-	-	-	-
RESISTANCE : MLS-ANTIBIOTICS	***erm(A)***	-	-	-	-	-	-	-
	erm(B)	-	-	-	-	-	-	-
	erm(C)	-	-	-	-	-	-	-
	lnu(A)	-	-	-	-	-	-	-
	msr(A)	-	-	-	-	-	-	-
	mef(A)	-	-	-	-	-	-	-
	mph(C)	-	-	-	-	-	-	-
	vat(A)	-	-	-	-	-	-	-
	vat(B)	-	-	-	-	-	-	-
	vga(A)	-	-	-	-	-	-	-
	***vga(A)* (BM 3327)**	-	-	-	-	-	-	-
	vgB(A)	-	-	-	-	-	-	-
RESISTANCE : AMINOGLYSOSIDES	***aacA-aphD***	-	-	-	-	-	-	-
	aadD	-	-	-	-	-	-	-
	aphA3	-	-	-	-	-	-	-

Strain		A7 F-BFRW	A8 F-BFRW	A78 C-BFRW	A70 C-BFRW	A73 C-BFRW	M9 C-YFRW	M7 C-YFRW
Clonal Complex		CC15	CC15	CC15	CC49	CC49	CC49	CC692
RESISTANCE : GLYCOPEPTIDES	*vanA*	-	-	-	-	-	-	-
	vanB	-	-	-	-	-	-	-
	vanZ	-	-	-	-	-	-	-
RESISTANCE : MISCELLANEOUS GENES	*sat*	-	-	-	-	-	-	-
	dfrS1	-	-	-	-	-	-	-
	far1	-	-	-	-	-	-	-
	fusC (Q6GD50)	-	-	-	-	-	-	-
	mupA	-	-	-	-	-	-	-
	tet(K)	-	-	-	-	-	-	-
	tet(M)	-	-	-	-	-	-	-
	cat (total)	-	-	-	-	-	-	-
	cat (pC221)	-	-	-	-	-	-	-
	cat (pC223)	-	-	-	-	-	-	-
	cat (pMC524/ pC194)	-	-	-	-	-	-	-
	cat (pSBK203R)	-	-	-	-	-	-	-
	cfr	-	-	-	-	-	-	-
	fexA	-	-	-	-	-	-	-
	fosB	●	●	●	-	-	-	●
	fosB (plasmid)	-	-	-	-	-	-	-
RESISTANCE : EFFLUX SYSTEMS	*qacA*	-	-	-	-	-	-	-
	qacC (total)	-	-	-	-	-	-	-
	qacC (consensus)	-	-	-	-	-	-	-
	qacC (equine)	-	-	-	-	-	-	-
	qacC (SA5)	-	-	-	-	-	-	-
	qacC (Ssap)	-	-	-	-	-	-	-
	qacC (ST94)	-	-	-	-	-	-	-
	sdrM	●	●	●	●	●	●	●
VIRULENCE : TOX.SHOCK.TOXIN	*tst1* (consensus)	-	-	-	-	-	-	-
	tst1 (human)	-	-	-	-	-	-	-
	tst1 (bovine)	-	-	-	-	-	-	-

Strain		A7 F-BFRW	A8 F-BFRW	A78 C-BFRW	A70 C-BFRW	A73 C-BFRW	M9 C-YFRW	M7 C-YFRW
Clonal Complex		CC15	CC15	CC15	CC49	CC49	CC49	CC692
VIRULENCE : ENTEROTOXINS	***sea***	-	-	-	-	-	-	-
	***sea* (320E)**	-	-	-	-	-	-	-
	***sea* (N315)**	-	-	-	-	-	-	-
	seb	-	-	-	-	-	-	-
	sec	-	-	-	-	-	-	-
	sed	-	-	-	-	-	-	-
	see	-	-	-	-	-	-	-
	seg	-	-	-	-	-	-	-
	seh	-	-	-	-	-	-	-
	sei	-	-	-	-	-	-	-
	sej	-	-	-	-	-	-	-
	sek	-	-	-	-	-	-	-
	sel	-	-	-	-	-	-	-
	selm	-	-	-	-	-	-	-
	***seln* (consensus)**	-	-	-	-	-	-	-
	***seln* (not RF122)**	-	-	-	-	-	-	-
	selo	-	-	-	-	-	-	-
	***egc* (total)**	-	-	-	-	-	-	-
	seq	-	-	-	-	-	-	-
	ser	-	-	-	-	-	-	-
	selu	-	-	-	-	-	-	-
	ORF CM14_ p1	-	-	-	-	-	-	-
	ORF CM14_p2	-	-	-	-	-	-	-
VIRULENCE : HLG / LEUKOCIDINS	***lukF***	●	●	●	●	●	●	●
	lukS	●	●	●	●	●	●	●
	lukS (ST22/ST45)	Δ	Δ	●	Δ	Δ	Δ	●
	hlgA	●	●	●	●	●	●	●
	lukF-PV	-	-	-	-	-	-	-
	lukS-PV	-	-	-	-	-	-	-
	***lukF-PV* (P83)**	-	-	-	-	-	-	-
	lukM	-	-	-	-	-	-	-
	lukD	●	●	●	●	●	●	●
	lukE	●	●	●	●	●	Δ	●
	lukX (lukB)	●	●	●	●	●	●	●
	lukY (lukA)	●	●	●	●	●	●	●
	***lukY* (ST30/ST45)**	-	-	-	-	-	-	-

Strain		A7 F-BFRW	A8 F-BFRW	A78 C-BFRW	A70 C-BFRW	A73 C-BFRW	M9 C-YFRW	M7 C-YFRW
Clonal Complex		CC15	CC15	CC15	CC49	CC49	CC49	CC692
VIRULENCE : HAEMOLYSINS	***hl***	●	●	●	●	●	●	●
	hla	●	●	●	●	●	●	●
	hlIII **(consensus)**	●	●	●	●	●	●	●
	hlIII **(not RF122)**	●	●	●	-	-	-	●
	hlb **_p1**	-	-	-	●	●	●	●
	hlb **_p2**	-	-	-	●	●	●	●
	hlb **_p3**	-	-	●	●	●	●	●
	hlb	-	Δ	-	Δ	Δ	Δ	●
VIRULENCE : HLB-CONV PHAGES	***sak***	-	-	-	-	-	-	-
	chp	●	●	●	-	-	-	-
	scn	●	●	●	-	-	-	-
VIRULENCE : EXFOL.TOXIN	***etA***	-	-	-	-	-	-	-
	etB	-	-	-	-	-	-	-
	etD	-	-	-	-	-	-	-
VIRULENCE : EPITHEL. DIFF. INHIB	***edinA***	-	-	-	-	-	-	-
	edinB	-	-	-	-	-	-	-
	edinC	-	-	-	-	-	-	-
VIRULENCE : ACME LOCUS	**ACME (total)**	-	-	-	-	-	-	-
	arcA **-SCC**	-	-	-	-	-	-	-
	arcB **-SCC**	-	-	-	-	-	-	-
	arcC **-SCC**	-	-	-	-	-	-	-
	arcD **-SCC**	-	-	-	-	-	-	-
VIRULENCE : PROTEASES	***aur*** **(consensus)**	●	●	●	●	●	●	●
	aur **(non MRSA252)**	●	●	●	●	●	Δ	●
	aur **(MRSA252)**	-	-	-	-	-	-	-
	splA	●	●	●	●	●	●	●
	splB	●	●	●	●	●	●	●
	splE	●	●	●	●	●	●	-
	sspA	●	●	●	●	●	●	●
	sspB	●	●	●	●	●	●	●
	sspP **(consensus)**	●	●	●	●	●	●	●
	sspP **(not ST93)**	●	●	●	●	●	●	●

	Strain	A7 F-BFRW	A8 F-BFRW	A78 C-BFRW	A70 C-BFRW	A73 C-BFRW	M9 C-YFRW	M7 C-YFRW
	Clonal Complex	CC15	CC15	CC15	CC49	CC49	CC49	CC692
VIRULENCE : STAPHYLOCOCCAL SUPERANTIGEN/ENTEROTOXIN-LIKE GENES (SSL/SET)	*setC*	●	●	●	●	●	●	●
	ssl01/set6 Probe1_11	-	-	-	●	●	●	●
	ssl01/set6 Probe2_11	-	Δ	Δ	-	-	-	-
	ssl01/set6 Probe1_12	-	-	-	-	-	-	●
	ssl01/set6 Probe2_12	-	-	-	●	●	●	-
	ssl01/set6 Probe4_11	●	●	●	-	-	-	●
	ssl01/set6 ProbeRF122	-	-	Δ	-	-	-	-
	ssl01/set6 (COL)	-	-	-	-	-	-	●
	ssl01/set6 (Mu50/N315)	-	-	-	-	-	-	Δ
	ssl01/set6 (MW2/MSSA4	-	-	-	-	-	-	-
	ssl01/set6 (MRSA252)	-	-	-	-	-	-	-
	ssl01/set6 (RF122)	-	-	-	-	-	-	-
	ssl01/set6 (other alleles)	●	●	●	●	●	●	-
	ssl02/set7	●	●	●	●	Δ	Δ	●
	ssl02/set7 (MRSA252)	Δ	Δ	-	-	-	-	-
	ssl03/set8 Probe 1	●	●	●	●	●	●	●
	ssl03/set8 Probe 2	●	●	●	●	●	●	●
	ssl03/set8 (MRSA252, SAR0424)	-	-	-	-	-	-	-
	ssl04/set9	●	●	●	●	●	●	●
	ssl04/set9 (MRSA252, SAR0425)	-	-	-	-	-	Δ	Δ
	ssl05/set3 Probe 1	●	●	●	-	-	-	●
	ssl05/set3 (RF122, probe-611)	●	●	●	●	●	●	●
	ssl05/set3 Probe 2 (612)	Δ	Δ	●	●	●	●	●
	ssl05/set3 (MRSA252)	-	-	-	-	-	-	-
	ssl06/set21	●	●	●	●	●	●	-
	ssl06 (NCTC8325/MW2)	●	●	●	●	●	●	Δ

Strain		A7 F-BFRW	A8 F-BFRW	A78 C-BFRW	A70 C-BFRW	A73 C-BFRW	M9 C-YFRW	M7 C-YFRW
Clonal Complex		CC15	CC15	CC15	CC49	CC49	CC49	CC692
VIRULENCE : STAPHYLOCOCCAL SUPERANTIGEN/ENTEROTOXIN-LIKE GENES (SSL/SET)	*ssl07/set1*	●	●	●	●	●	●	●
	ssl07/set1 (MRSA252)	Δ	Δ	●	●	●	●	●
	ssl07/set1 (AF188836)	-	-	-	-	-	-	-
	ssl08/set12 Probe 1	●	Δ	Δ	●	●	●	●
	ssl08/set12 Probe 2	●	●	●	●	●	●	●
	ssl09/set5 Probe 1	●	●	●	●	●	●	●
	ssl09/set5 Probe 2	●	●	●	●	●	●	●
	ssl09/set5 (MRSA252)	-	-	-	-	-	-	-
	ssl10/set4	●	●	●	●	●	●	●
	ssl10 (RF122)	Δ	Δ	●	Δ	●	Δ	-
	ssl10/set4 (MRSA252)	Δ	-	-	Δ	-	-	Δ
	ssl11/set2 (COL)	-	-	-	-	-	-	-
	ssl11/set2 (Mu50/N315)	-	-	-	-	-	-	-
	ssl11/set2 (MW2/ MSSA476)	-	-	-	-	-	-	-
	ssl11/set2 (MRSA252)	●	●	●	-	-	-	Δ
	setB3	●	●	●	●	●	●	●
	setB3 (MRSA252)	-	-	-	-	-	-	-
	setB2	●	●	●	●	●	●	●
	setB2 (MRSA252)	-	-	-	-	-	-	-
	setB1	●	●	●	●	●	●	●
CAPSULE- AND BIOFILM-ASSOCIATED GENES	*cap 1* (total)	-	-	-	-	-	-	-
	capH1	-	-	-	-	-	-	-
	capJ1	-	-	-	-	-	-	-
	capK1	-	-	-	-	-	-	-
	cap 5 (total)	-	-	-	●	●	●	●
	capH5	-	-	-	●	●	●	●
	capJ5	-	-	-	●	●	●	●
	capK5	-	-	-	●	●	●	●
	cap 8 (total)	●	●	●	-	-	-	-
	capH8	●	●	●	-	-	-	-
	capI8	●	●	●	-	-	-	-
	capJ8	●	●	●	-	-	-	-

	Strain	A7 F-BFRW	A8 F-BFRW	A78 C-BFRW	A70 C-BFRW	A73 C-BFRW	M9 C-YFRW	M7 C-YFRW
	Clonal Complex	CC15	CC15	CC15	CC49	CC49	CC49	CC692
CAPSULE- AND BIOFILM-ASSOCIATED GENES	*capK8*	●	●	●	-	-	-	-
	icaA	●	●	●	●	●	●	●
	icaC	●	●	●	●	●	●	●
	icaD	●	●	●	●	●	●	●
	bap	-	-	-	-	-	-	-
ADHESION FACTORS/GENES ENCODING MICROBIAL SURFACE COMPONENTS RECOGNIZING ADHESIVE MATRIX MOLECULES (MSCRAMM GENES)	*bbp* (total)	●	●	●	●	●	●	●
	bbp (consensus)	●	●	●	●	●	●	●
	bbp (COL/MW2)	-	-	-	Δ	-	Δ	Δ
	bbp (MRSA252)	-	-	-	-	-	-	-
	bbp (Mu50)	●	●	●	-	Δ	-	●
	bbp (RF122)	-	-	-	-	-	-	-
	bbp (ST45)	-	-	-	-	-	-	-
	clfA (total)	●	●	●	●	●	●	●
	clfA (consensus)	●	●	●	●	●	●	●
	clfA (COL/RF122)	-	-	-	-	-	-	-
	clfA (MRSA252)	-	-	-	-	-	-	-
	clfA (Mu50/MW2)	●	●	●	●	●	●	●
	clfB (total)	●	●	●	●	●	●	●
	clfB (consensus)	●	●	●	●	●	●	●
	clfB (COL/Mu50)	-	-	-	-	-	-	-
	clfB (MW2)	●	●	●	●	●	●	-
	clfB (RF122)	Δ	Δ	Δ	●	Δ	●	●
	cna	-	-	-	-	-	-	-
	ebh (consensus)	●	●	●	●	●	●	●
	ebpS (total)	●	●	●	●	●	●	●
	ebpS Probe 612	Δ	●	●	Δ	●	Δ	-
	ebpS Probe 614	●	●	●	●	●	●	●
	ebpS (01-1111)	-	-	-	-	-	-	●
	ebpS (COL)	●	●	●	-	-	-	-

Strain		A7 F-BFRW	A8 F-BFRW	A78 C-BFRW	A70 C-BFRW	A73 C-BFRW	M9 C-YFRW	M7 C-YFRW
Clonal Complex		CC15	CC15	CC15	CC49	CC49	CC49	CC692
ADHESION FACTORS/GENES ENCODING MICROBIAL SURFACE COMPONENTS RECOGNIZING ADHESIVE MATRIX MOLECULES (MSCRAMM GENES)	*fnbA* (total)	●	●	●	●	●	●	●
	fnbA (consensus)	●	●	●	●	●	●	●
	fnbA (COL)	●	●	●	-	-	-	-
	fnbA (MRSA252)	-	-	-	●	●	●	-
	fnbA (Mu50/MW2)	-	-	-	-	-	-	-
	fnbA (RF122)	-	-	-	-	-	-	-
	fnbB (total)	●	●	●	●	●	●	●
	fnbB (COL)	-	-	-	-	-	-	-
	fnbB (COL/Mu50/MW2)	Δ	Δ	●	●	●	●	●
	fnbB (Mu50)	Δ	Δ	Δ	●	●	●	-
	fnbB (MW2)	-	-	-	-	-	-	-
	fnbB (ST15)	●	●	●	-	-	-	●
	fnbB (ST45-2)	-	-	-	-	-	-	-
	eno	●	●	●	●	●	●	●
	fib	●	●	●	●	●	●	●
	fib (MRSA252)	-	-	-	-	-	-	-
	map (total)	●	●	●	●	●	●	Δ
	map (COL)	●	●	●	●	●	●	Δ
	map (MRSA252)	-	-	-	-	-	-	-
	map (Mu50/MW2)	●	●	●	-	-	Δ	Δ
	sasG (total)	●	●	●	●	●	●	-
	sasG (COL/Mu50)	-	-	-	Δ	●	Δ	-
	sasG (MW2)	●	●	●	-	-	-	-
	sasG (not MRSA252/ RF122)	●	●	●	●	●	●	-
	sdrC (total)	●	●	●	●	●	●	●
	sdrC (consensus)	●	●	●	●	●	●	●
	sdrC (B1)	-	-	-	-	-	-	-
	sdrC (COL)	●	●	●	●	●	●	●
	sdrC (Mu50)	-	-	-	-	-	-	-

Strain		A7 F-BFRW	A8 F-BFRW	A78 C-BFRW	A70 C-BFRW	A73 C-BFRW	M9 C-YFRW	M7 C-YFRW
Clonal Complex		CC15	CC15	CC15	CC49	CC49	CC49	CC692
ADHESION FACTORS/GENES ENCODING MICROBIAL SURFACE COMPONENTS RECOGNIZING ADHESIVE MATRIX MOLECULES (MSCRAMM GENES)	*sdrC* (MW2/ MRSA252/ RF122)	-	-	-	-	-	-	-
	sdrC (not MRSA252/ RF122)	●	●	●	-	-	-	●
	sdrD (total)	●	●	●	●	●	●	-
	sdrD (consensus)	●	●	●	●	●	●	-
	sdrD (COL/MW2)	-	-	-	-	-	-	-
	sdrD (Mu50)	●	●	●	-	-	-	-
	sdrD (other)	-	-	-	●	●	●	-
	vwb (total)	●	●	●	●	●	●	●
	vwb (consensus)	●	●	●	●	●	●	●
	vwb (COL/MW2)	-	-	-	-	-	-	-
	vwb (MRSA252)	-	-	-	-	-	-	-
	vwb (Mu50)	-	-	-	Δ	Δ	Δ	-
	vwb (RF122)	-	-	-	-	-	-	-
IMMUNOD.AG.B	*isaB*	●	●	●	●	●	●	●
	isaB (MRSA252)	Δ	Δ	●	-	-	-	Δ
DEFENSIN RESIST.	*mprF* (COL/MW2)	●	●	●	●	●	●	●
	mprF (Mu50/ MRSA252)	Δ	Δ	Δ	●	●	●	Δ
TRANSFERRIN BINDING PROT.	*isdA* (consensus)	●	●	●	●	●	●	●
	isdA (MRSA252)	●	●	●	-	-	-	-
	isdA (not MRSA252)	Δ	-	-	●	●	●	●

Strain		A7 F-BFRW	A8 F-BFRW	A78 C-BFRW	A70 C-BFRW	A73 C-BFRW	M9 C-YFRW	M7 C-YFRW
Clonal Complex		CC15	CC15	CC15	CC49	CC49	CC49	CC692
PUTATIVE TRANSPORTER	*lmrP* (not RF122) Probe1	●	●	●	-	-	-	●
	lmrP (not RF122) Probe2	●	●	●	-	-	-	●
	lmrP (RF122) Probe1	-	-	-	●	●	●	-
	lmrP (RF122) Probe2	-	-	-	●	●	●	-
TYPE I RESTRICTION-MODIFICATION SYSTEM, SINGLE SEQUENCE SPECIFICITY PROTEIN	*hsdS1* (RF122)	-	-	-	-	-	-	-
	hsdS2 (Mu50/N315/COL/USA300/NCTC8325)	-	-	-	-	-	-	-
	hsdS2 (MW2/MSSA476)	-	Δ	-	-	-	Δ	Δ
	hsdS2 (RF122)	-	-	-	-	-	-	-
	hsdS2 (MRSA252)	-	-	-	-	-	-	-
	hsdS3 (not RF122/ MRSA252)	Δ	●	Δ	Δ	Δ	Δ	-
	hsdS3 (COL/USA300/ NCTC8325/MW2/MSSA476/ RF122)	-	-	-	-	-	-	-
	hsdS3 (Mu50/N315)	-	-	-	-	-	-	-
	hsdS3 (CC51/ MRSA252)	-	-	-	●	●	●	-
	hsdS3 (MRSA252)	-	-	-	-	-	-	-
	hsdSx (CC25)	●	●	●	●	●	●	●
	hsdSx (CC15)	●	●	●	●	●	●	Δ
	hsdSx (etd)	-	-	-	●	●	●	-
MISC. GENES	Q2FXC0	-	-	-	-	-	-	-
	Q2YUB3	-	-	-	-	-	-	Δ
	Q7A4X2	-	-	-	-	-	-	●

Appendix E: Staphylococcus aureus clonal complex determination

Strain		A7 F-BFRW	A8 F-BFRW	A78 C-BFRW	A70 C-BFRW	A73 C-BFRW	M9 C-YFRW	M7 C-YFRW
Clonal Complex		CC15	CC15	CC15	CC49	CC49	CC49	CC692
HYALURONATE LYASE	***hysA1* (MRSA252)**	-	-	-	●	●	●	●
	***hysA1* (MRSA252/RF122) and/or *hysA2* (consensus)**	●	●	●	●	●	●	●
	***hysA1* (MRSA252/RF122) and/or *hysA2* (COL/USA300)**	●	●	●	-	-	-	●
	***hysA2* (not MRSA252)**	●	●	●	-	-	-	-
	***hysA2* (COL/USA300/ NCTC8325)**	●	●	●	-	-	-	●
	***hysA2* (not COL/USA300/ NCTC8325)**	●	●	●	●	●	●	●
	***hysA2* (not COL/USA300/ NCTC8325)**	●	●	●	●	●	●	●
	***hysA2* (MRSA252)**	-	-	-	-	-	-	-

Abbreviations: ●, positive signal; Δ, ambiguous signal; -, negative signal; F-BFRW, Black-flanked Rock wallaby (*P. lateralis*) from a free-ranging environment; C-BFRW, Black-flanked Rock wallaby (*P. lateralis*) from captivity; C-TMW, Mainland Tammar wallaby (M*. eugenii*) from captivity; C-YFRW, Yellow-footed Rock wallaby (*P. xanthopus*) from captivity

Appendix F: Chen *et al*. 2014; Journal of Veterinary Science & Medical Diagnosis

Chen MM et al., J Vet Sci Med Diagn 2014, 3:2
http://dx.doi.org/10.4172/2325-9590.1000136

Journal of Veterinary Science & Medical Diagnosis

Research Article A SCITECHNOL JOURNAL

Nasal Colonization of *Staphylococcus* Spp among Captive and Free-Ranging Wallabies in South Australia

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Rec date: Mar 14, 2014, **Acc date:** Apr 29, 2014, **Pub date:** May 05, 2014

Abstract

Staphylococcal species diversity has been well studied with regard to antibiotic resistance in humans and animals of commercial or social value. However, studies of free-ranging wildlife and animals of conservation value are limited. In this study, multidrug resistant staphylococci were found exclusively in free-ranging wallabies indicating human activity and prior antibiotic exposure may not be significant contributing factors to the development of antibiotic resistance in staphylococci in animal reservoirs. Eight isolates of *Staphylococcus aureus* were identified; one was resistant to ampicillin, penicillin and cefotaxime, one to ampicillin and penicillin and one to oxacillin, while the remaining five isolates were susceptible to all antimicrobial agents tested. Resistance to the β-lactam antibiotic family was the most prevalent with 37% of all isolates being resistant to one or more β-lactams. Fourteen species of *Staphylococcus* were identified from 89 strains isolatedfrom 98 South Australian captive and free-ranging native wallabies. Among the identified isolates, *Staphylococcus delphini* (18%) and *Staphylococcus succinus* (17%) were the dominant species with single isolates of *Staphylococcus cohnii*, *Staphylococcus carnosus* and *Staphylococcus hominis*. To our knowledge, this is the first to study to reportthe presence and diversity of commensal staphylococcal species in any member of the *Macropodidae* family and thus provides baseline data for future work on the prevalence and diversity of common microbial pathogens in macropods.

Keywords: Staphylococcus; Wallaby; Commensal; Multidrug resistance; Wildlife; Macropod

Introduction

The staphylococcus genus comprises 45 species and 21 subspecies [1] and is widely distributed across a variety of environments and hosts including soil, water, skin and mucosal membranes of humans and other animals. A large portion of the literature regarding staphylococcal host colonization and species diversity in animals is concerned predominantly with cattle, pigs, horses, cats and dogs [2-4]. Moreover, staphylococcal species diversity is also grossly under represented because investigations have focused on the detection of common pathogenic species such as *Staphylococcus aureus* [5]. Studies involving free-ranging animals or animals of conservation value are scarce with the exception of a few which have focused on the prevalence of antibiotic resistance genes in various zoo animals and wildlife [6-8].

Zoonotic infections resulting from wildlife reservoirs may become more prevalent as urbanization causes changes in animal behavior and movement [9-11]. In order to begin to detail our understanding of infectious disease in humans we need to gather knowledge about infections in other species with which we share our space and natural resources. It has been estimated that almost 75% of emerging human pathogens originate from animals [12]. The One World, One Health concept encourages healthcare workers from human and veterinary medicine to share scientific data as there is growing evidence to show human and animal health can be negatively impacted by the same microorganisms [13].

In this study, Yellow-footed Rock-wallaby (*Petrogale xanthopus*) (YFRW), Black-flanked Rock-wallaby (*Petrogale lateralis*) (BFRW) and the mainland Tammar wallaby (*Macropus eugenii*) (TMW) were chosen to study the distribution of staphylococci in captive and free-ranging South Australian wallabies. Staphylococci diversity was examined using traditional biochemical methods in conjunction with partial 16S rRNA sequencing. We also determined the antibiograms for each staphylococcal isolate using Kirby-Bauer disc diffusion assay with 10 compounds across seven antimicrobial families and determined the presence of multidrug resistant (MDR) staphylococci in these wallabies.

Materials and Methods

Sampling

A total of 30 free-ranging BFRW from the Anangu Pitjantjatjara Yankunytjatjara (APY) Lands (-26.08, 132.21) and 68 captive wallabies (16 BFRW, 28 YFRW, 24 TMW) from Monarto Zoological Park in South Australia, Australia were sampled between July 2009 and October 2010 during health examinations. The APY Lands, located in the far north-west corner of South Australia, covers 102,650 km^2 which is equivalent to approximately 10 percent of the state's area. It is home to 2,230 people across 33 communities and outstations [14]. All wallabies were classified as apparently healthy at the time of sampling. Sterile single-use nasopharyngeal minitip swabs were used to swab both anterior nostrils and stored in 1 ml liquid Amies medium with 90% glycerol at -80°C until laboratory analysis. Ethical consent was obtained from the Flinders University Animal Welfare Committee.

Isolation and genus identification of *Staphylococcus* species

Swabs were grown in nutrient broth or tryptone soy broth and incubated at 37°C for 24 hr. A 100 µl inoculum was spread onto two selective media; staphylococcus number 110 (S110) and Oxacillin Resistance Screening Agar Base (ORSAB) and plates incubated for 24-48 hr. The S110 medium facilitates and favors the growth of

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doi:http://dx.doi.org/10.4172/2325-9590.1000136

staphylococci due to a high sodium chloride concentration. Sodium chloride concentrations up to 10% have been shown to have a protective effect for staphylococci and aid in the preservation of specific characteristic traits of different staphylococcal strains [15]. Chromogenic ORSAB medium facilitates the selection of methicillin resistant staphylococci (MRS) due to the presence of polymixin B and oxacillin in the medium. At the end of the incubation period, distinct colonies were purified and subjected to Gram-staining, catalase and coagulase tests for discrimination purposes. In order to concentrate on the *Staphylococcus* genus, isolates which were not Gram-positive and catalase positive were not analyzed further.

Species-specific identification of Staphylococcus species

Samples received in 2009 were subjected to the Microbact 12S kit to determine the Staphylococcus species present; results were confirmed by partial sequencing of the 16S rRNA gene. Staphylococcal species were identified from samples obtained in 2010 only by partial sequencing of the 16S rRNA gene using universal primers V3V6F 5'-CCAGACTCCTACGGGAGGCAG-3' and V3V6R 5' ACATTTCACAACACGAGCTGACGA-3' to give a 752 bp product [16]. Nucleotide sequences were determined by the Australian Genome Research Facility, University of Adelaide or First Base Laboratories, Malaysia. Sequences obtained were analyzed with Clustal W, PhyML and TreeDyn 198.3 and then compared with published sequences in the National Center of Biotechnology Information database (accessed August 26 2013).

Staphylococcal species were determined on the basis of 97% or higher similarity to a type strain [17]. Genomic DNA was extracted using the HiYield™ Genomic Mini kit (Bio-Deal, New Zealand) according to the manufacturer's instructions for Gram-positive bacteria.

We tested the hypothesis that staphylococcal species diversity will not be impacted by the wallaby breed or captivity status. The significance of these data was determined by using chi-square analysis. A p-value ≤0.05 was regarded as being statistically significant. The p-values are shown only for results that were statistically significant [18].

Antimicrobial susceptibility testing

The standard agar disc diffusion technique was performed according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [19], to determine the antibiotic susceptibility profile of all isolates. Briefly, overnight cultures were adjusted to 0.5 McFarland standard with 0.085% NaCl and 3 ml was spread onto a Mueller-Hinton agar plate in triplicate. The antibiotics used included β-lactams penicillin G (PEN) (10 units), ampicillin (AMP) (10 µg), oxacillin (OX) (1 µg); aminoglycosides gentamicin (CN) (30 µg), streptomycin (S) (10 µg); vancomycin(VA) (30 µg), cefotaxime (CTX) (30 µg), tetracycline (TE) (30 µg), erythromycin (E) (15 µg) and chloramphenicol (C) (30 µg). Multidrug resistance was defined as resistance to three or more antimicrobial families. A clinical methicillin-resistant *S. aureus* (MRSA) from the Flinders Medical Centre, *S. aureus* ATCC 6538 and *S. epidermidis* ATCC 12228 were used as reference strains.

We tested the hypothesis that antibiotic resistant staphylococci would be recovered more readily from captive wallabies compared to free-ranging wallabies. Raw disc diffusion diameter measurements from the Kirby-Bauer disc diffusion assay were averaged and assigned a qualitative value of R, iR and S according to the CLSI guidelines [19]. The significance of these data was determined using chi-square. A p-value ≤0.05 was regarded as being statistically significant [18].

Results

Isolation and identification of *Staphylococcus* species

A total of 28 YFRW were sampled, from these 19 were positive for bacterial growth resulting in the isolation of 48 Gram-positive cocci; six (12%) isolates were tentatively identified as MRS due to growth on ORSAB medium. From the 48 Gram-positive cocci, 28 were catalase positive and of these 2 were coagulase positive. A total of 10 different staphylococcal species were identified from these 28 catalase positive strains by partial 16S rRNA sequencing.

Species identified included *S. succinus*, *S. warneri*, *S. xylosus*, *S. delphini*, *S. fleurettii*, *S. vitulinus*, *S. saprophyticus*, *S. simulans* and *S. Sciuri* (Table 1). In addition, two *S. aureus* strains were detected in 28 YFRW (carriage rate of 7%). No *S. epidermidis* strains were found.

Staphylococcal species	BFRW[a] (n=30)	BFRW[b] (n=16)	YFRW[b] (n=28)	TMW[b] (n=24)	Animals (n=89)
S. aureus	2	4	2	0	8
S. carnosus	0	1	0	0	1
S. cohnii	1	0	0	0	1
S. delphini	8	2	3	3	16
S. epidermidis	3	0	0	4	7
S. fleurettii	0	4	3	0	7
S. hominis	0	0	0	1	1
S. saprophyticus	0	2	2	0	4
S. sciuri	0	3	1	0	4
S. simulans	1	2	2	0	5
S. succinus	0	6	7	2	15
S. vitulinus	0	0	3	0	3
S. warneri	7	0	1	0	8
S. xylosus	0	5	4	0	9
Total strains	22	29	28	10	89

[a]Wallabies from a free-ranging environment in the APY Lands

[b]Wallabies from a captive environment at Monarto Zoo

Abbreviations: BFRW, Black-flanked Rock wallaby; YFRW, Yellow-footed Rock wallaby; TMW, Tammar wallaby

Table 1: Staphylococcal species recovered from wallaby nasal samples

From 24 TMW sampled, 24 Gram-positive isolates were isolated from the nasal cavities of five animals. Of these 24 isolates, 23 were recovered from S110 medium with one (4%) presumptive MRS isolated from ORSAB medium. Catalase production was detected in 10 isolates; no coagulase positive isolates were identified. These 10 catalase positive isolates were identified as belonging to four staphylococcal species, namely *S. delphini*, *S. epidermidis*, *S. hominis*

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and *S. succinus* (Table 1). TMW were the only captive wallabies to harbor *S. epidermidis* and the only wallaby species to carry *S. hominis*. No *S. aureus* were recovered from the TMW (Table 1).

All 16 captive BFRW were found to carry Gram-positive cocci bacteria. A total of 31 isolates were recovered from S110 medium and 15 (32%) presumptive MRS were isolated from ORSAB plates. Biochemical tests identified 32 catalase positive strains, of which three were identified as *Enterobacter cloacae* and *Aerococcus viridians* (data not shown) and thus not further analyzed. From 29 catalase positive strains, nine staphylococcal species were identified ranging from six isolates of *S. succinus* to a single isolate of *S. carnosus* which was the sole *S. carnosus* strain to be isolated in the study. Other species identified included *S. xylosus*, *S. fleurettii*, *S. sciuri*, *S. simulans*, *S. delphini* and *S. saprophyticus* (Table 1). Coagulase production was detected in 10 isolates of which four were identified as *S. aureus* giving a carriage rate of 12% for *S. aureus* (Table 1). The remaining six coagulase positive species were identified as *S. xylosus*, *S. sciuri* and *S. saprophyticus*.

Of the 30 free-ranging BFRW sampled, 18 harbored cultivable bacteria resulting in the isolation of 24 Gram-positive cocci from S110 medium and 10 (41%) presumptive MRS from ORSAB medium. Catalase production was seen in 22 isolates, of which two were coagulase positive. From these 22 catalase positive isolates, six staphylococcal species were identified which were *S. aureus*, *S. delphini*, *S. warneri*, *S. epidermidis*, *S. cohnii* and *S. simulans* (Table 1). Two isolates of *S. aureus* were recovered giving a carriage rate of 9% (Table 1). Interestingly, despite the prevalence of *S. succinus* in the captive BFRW population, no *S. succinus* isolates were found in the free-ranging population ($p \leq 0.05$). Furthermore, 90% of all *S. warneri* recovered in this study originated from the free-ranging BFRW ($p \leq 0.05$).

Antimicrobial susceptibility testing

Of the 32 isolates recovered from ORSAB medium, nine were identified as staphylococci. These nine strains were identified as *S. aureus* (A74), *S. cohnii* (A31), *S. warneri* (A16 and A17) and *S. fleurettii* (M31, M47, A61, A69 and A72). Of these strains, seven were resistant to AMP, PEN, OX and demonstrated intermediate resistance to CTX, one was resistant only to OX. The *S. aureus* strain A74 was sensitive to all antimicrobial agents tested. See Table 2 and 3.

			Antibiogram									
Strain	**Location**	**16S rRNA**	**AMP**	**OX**	**PEN**	**CN**	**S**	**VA**	**CTX**	**TE**	**E**	**C**
A1	Y	*S. warneri*	R	-	R	-	-	-	-	-	-	-
A2	Y	*S. epidermidis*	R	-	R	-	-	-	-	-	-	-
A4	Y	*S. delphini*	-	-	-	-	-	-	-	-	-	-
A5	Y	*S. delphini*	-	-	-	-	-	-	-	-	-	-
A7	Y	*S. aureus*	R	-	R	-	-	-	-	-	-	-
A8	Y	*S. aureus*	R	-	R	-	-	-	iR	-	-	-
A10	Y	*S. delphini*	-	-	-	-	-	-	-	-	-	-
A11	Y	*S. delphini*	-	-	-	-	-	-	-	-	-	-
A14	Y	*S. warneri*	-	-	-	-	-	-	-	-	-	-
A15	Y	*S. warneri*	-	-	-	-	-	-	-	-	-	-
A16*	Y	*S. warneri*	R	R	R	-	-	-	iR	R	R	-
A17*	Y	*S. warneri*	R	R	R	-	-	-	iR	R	R	-
A18	Y	*S. epidermidis*	-	-	-	-	-	-	-	-	-	-
A19	Y	*S. delphini*	-	-	-	-	-	-	-	-	-	-
A24	Y	*S. warneri*	R	-	R	-	-	-	-	-	-	-
A25	Y	*S. simulans*	-	-	-	-	-	-	-	-	-	-
A30	Y	*S. delphini*	-	-	-	-	-	-	-	-	-	-
A31*	Y	*S. cohnii*	R	R	R	-	-	-	R	-	R	-
A32	Y	*S. epidermidis*	R	-	R	-	-	-	-	-	-	-
A33	Y	*S. delphini*	-	-	-	-	-	-	-	-	-	-
A34	Y	*S. warneri*	-	-	-	-	-	-	-	-	-	-

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A35	Y	*S. delphini*	-	-	-	-	-	-	-	-	-	-
A37	M	*S. succinus*	-	-	R	-	-	-	-	-	-	-
A38	M	*S. succinus*	-	-	-	-	-	-	-	-	-	-
A40	M	*S. sciuri*	R	R	R	-	-	-	-	-	-	-
A41	M	*S. xylosus*	-	-	-	-	-	-	-	-	-	-
A44	M	*S. xylosus*	-	-	-	-	-	-	-	-	-	-
A45	M	*S. simulans*	-	-	-	-	-	-	-	-	-	-
A46	M	*S. succinus*	-	-	-	-	-	-	-	-	-	-
A48	M	*S. xylosus*	-	-	-	-	-	-	-	-	-	-
A50	M	*S. saprophyticus*	-	R	-	-	-	-	-	-	-	-
A51	M	*S. saprophyticus*	-	-	-	-	-	-	-	-	-	-
A55	M	*S. xylosus*	-	-	-	-	-	-	-	-	-	-
A58	M	*S. sciuri*	-	R	-	-	-	-	-	-	-	-
A59	M	*S. fleurettii*	R	R	R	-	-	-	iR	-	-	-
A61	M	*S. fleurettii*	R	R	R	-	-	-	iR	-	-	-
A64	M	*S. carnosus*	-	-	-	-	-	-	-	-	-	-
A66	M	*S. simulans*	-	-	-	-	-	-	-	-	-	-
A67	M	*S. succinus*	-	-	-	-	-	-	-	R	-	-
A68	M	*S. succinus*	-	-	-	-	-	-	-	-	-	-
A69	M	*S. fleurettii*	R	R	R	-	-	-	iR	-	-	-
A70	M	*S. aureus*	-	-	-	-	-	-	-	-	-	-
A71	M	*S. delphini*	-	-	-	-	-	-	-	-	-	-
A72	M	*S. fleurettii*	R	R	R	-	-	-	iR	-	-	-
A73	M	*S. aureus*	-	-	-	-	-	-	-	-	-	-
A74	M	*S. aureus*	-	-	-	-	-	-	-	-	-	-
A75	M	*S. succinus*	-	-	-	-	-	-	-	R	-	-
A76	M	*S. delphini*	-	-	-	-	-	-	-	-	-	-
A78	M	*S. aureus*	-	R	-	-	-	-	-	-	-	-
A79	M	*S. xylosus*	-	-	-	-	-	-	-	-	-	-
A81	M	*S. sciuri*	-	-	-	-	-	-	-	-	-	-

Abbreviations: -: sensitive; R: resistant; iR: intermediate resistant; *: multidrug resistant; AMP: ampicillin; OX: oxacillin; PEN: penicillin; CN: gentamicin; S: streptomycin; VA: vancomycin; CTX: cefotaxime; TE: tetracycline; E: erythromycin; C: chloramphenicol; Y: Isolate from APY Lands, South Australia; M: animal from Monarto Zoological Park, South Australia

Table 2: Source of isolates, resistance profiles and species identification of staphylococci from Black-flanked rock wallabies

In the BFRW and YFRW populations, 30% of staphylococcal strains were resistant to at least one β-lactam antimicrobial compound compared to 60% of strains from TMW (Table 2 and 3). This translated to 33 (37%) out of 89 strains demonstrating β-lactam resistance. Another three TE resistant strains in addition to a single E resistant strain were detected (Table 2 and 3), bringing a total of 37 strains which were resistant to at least one antimicrobial compound. Resistance to CTX was seen in 12 strains with more than half (58%) comprised of *S. fleurettii* strains. Interestingly, from all *S. fleurettii* strains isolated only one was susceptible to CTX.

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		Antibiogram									
Strain	**16S rRNA**	**AMP**	**OX**	**PEN**	**CN**	**S**	**VA**	**CTX**	**TE**	**E**	**C**
M2[a]	*S. warneri*	-	-	-	-	-	-	-	R	-	-
M3[a]	*S. vitulinus*	-	-	-	-	-	-	-	-	-	-
M6[a]	*S sciuri*	-	-	R	-	-	-	R	-	-	-
M7[a]	*S. aureus*	-	-	-	-	-	-	-	-	-	-
M8[a]	*S. succinus*	R	R	R	-	-	-	iR	-	-	-
M9[a]	*S. aureus*	-	-	-	-	-	-	-	-	-	-
M10[a]	*S. simulans*	-	-	-	-	-	-	-	-	-	-
M11[a]	*S. fleurettii*	-	-	-	-	-	-	-	-	-	-
M14[a]	*S. xylosus*	-	-	-	-	-	-	-	-	-	-
M15[a]	*S. succinus*	-	-	-	-	-	-	-	-	-	-
M17[a]	*S. saprophyticus*	-	-	-	-	-	-	-	-	R	-
M21[a]	*S. saprophyticus*	R	R	R	-	-	-	-	-	R	-
M26[a]	*S. simulans*	-	-	-	-	-	-	-	-	-	-
M27[a]	*S. succinus*	R	R	R	-	-	-	-	-	-	-
M28[a]	*S. xylosus*	R	R	R	-	-	-	-	-	-	-
M29[a]	*S. xylosus*	R	R	R	-	-	-	-	-	-	-
M30[a]	*S. succinus*	R	R	R	-	-	-	-	-	-	-
M31[a]	*S. fleurettii*	R	R	R	-	-	-	iR	-	-	-
M32[a]	*S. delphini*	-	-	-	-	-	-	-	-	-	-
M33[a]	*S. delphini*	-	-	-	-	-	-	-	-	-	-
M36[a]	*S. xylosus*	-	-	-	-	-	-	-	-	-	-
M37[a]	*S. succinus*	-	-	-	-	-	-	-	-	-	-
M38[a]	*S. vitulinus*	-	-	-	-	-	-	-	-	-	-
M39[a]	*S. vitulinus*	-	-	-	-	-	-	-	-	-	-
M40[a]	*S. succinus*	-	-	-	-	-	-	-	-	-	-
M41[a]	*S. succinus*	-	-	-	-	-	-	-	-	-	-
M47[a]	*S. fleurettii*	-	R	-	-	-	-	-	-	-	-
M48[a]	*S. delphini*	-	-	-	-	-	-	-	-	-	-
M53[b]	*S. delphini*	-	-	-	-	-	-	-	-	-	-
M54[b]	*S. succinus*	R	R	R	-	-	-	-	-	-	-
M56[b]	*S. delphini*	-	-	-	-	-	-	-	-	-	-
M57[b]	*S. succinus*	R	R	R	-	-	-	-	-	-	-
M65[b]	*S. epidermidis*	R	-	R	-	-	-	-	-	R	-
M66[b]	*S. epidermidis*	R	-	R	-	-	-	-	-	R	-

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doi:http://dx.doi.org/10.4172/2325-9590.1000136

M68[b]	*S. epidermidis*	R	-	R	-	-	-	-	-	-	-
M69[b]	*S. hominis*	-	-	-	-	-	-	-	-	-	-
M72[b]	*S. epidermidis*	R	R	R	-	-	-	iR	-	-	-

[a]Isolate from Yellow-footed Rock wallaby

[b]Isolate from Tammar wallaby

Abbreviations: -, sensitive; R, resistant; iR, intermediate resistant; * multidrug resistant; AMP, ampicillin; OX, oxacillin; PEN, penicillin; CN, gentamicin; S, streptomycin; VA, vancomycin; CTX, cefotaxime; TE, tetracycline; E, erythromycin; C, chloramphenicol

Table 3: Source of isolates, resistance profiles and species identification of staphylococci from captive wallabies from Monarto Zoo

A total of eight *S. aureus* strains were recovered. One was resistant to AMP, PEN and CTX, one to AMP and PEN and one to OX. The remaining five *S. aureus* strains in addition to 47 other non-*S. aureus* staphylococci were susceptible to all compounds tested (Table 2 and 3). Multidrug resistance was identified in *S. cohnii* strain A31 and *S. warneri* strains A16 and A17 (Table 2). These three strains were the only MDR staphylococci isolated in this study and were from free-ranging wallabies. This finding was considered statistically significant ($p \leq 0.05$).

All isolates were sensitive to CN, S, VA and C; sporadic TE and E resistance was observed. Antibiotic resistant staphylococci were equally distributed amongst the wallaby populations studied (p-value: <0.05) which indicates that captivity status is not a major contributor to the development of antibiotic resistance in staphylococci.

Discussion

This is the first study to provide detailed information on commensal staphylococcal carriage in apparently healthy captive and free-ranging wallabies in South Australia. We showed that staphylococcal species prevalence and diversity was higher in captive wallabies compared to their free-ranging counterparts. However, MDR was found only in isolates from free-ranging wallabies.

Bacterial growth on ORSAB medium was unable to definitively predict the OX resistance phenotype; 22 staphylococcal strains demonstrated OX resistance by Kirby-Bauer disc diffusion however only eight grew on ORSAB plates. Furthermore, 23 out of 32 (72%) bacterial strains recovered from ORSAB plates were biochemically identified as non-staphylococcal isolates indicating the limitations of this chromogenic medium when challenged with complex samples as was seen with MRSA select agar [20].

Resistance towards β-lactam antimicrobial agents (AMP, PEN and OX) was found in approximately one third of all isolates irrespective of captivity status and in concordance with studies in sub-clinical mastitis in cattle [21]. This is in contrast to previous findings in healthy and diseased domestic cats and dogs where less than 7% of all staphylococcal isolates were found to be resistant to β-lactam compounds [22]. With the exception of two strains, all antibiotic resistant strains of staphylococci demonstrated resistance to at least one β-lactam compound which demonstrates the prevalence of β-lactam resistant staphylococci in animals that have had limited exposure to β-lactam antimicrobial agents. This limited exposure to β-lactam compounds is supported by the recovery of *S. aureus* strains which were sensitive to all antimicrobial agents tested as almost 90% of all *S. aureus* isolated from humans since 1980 have been described as penicillin resistant [23]. All isolates were susceptible towards CN, S, C and VA, with sporadic cases of TE and E resistance.

The discovery of significant numbers of antibiotic resistant coagulase negative staphylococci and in conjunction with antibiotic susceptible *S. aureus* suggests that coagulase negative staphylococci could serve as reservoirs for antibiotic resistance genes as hypothesized in the case for the emergence of MRSA [24]. Therefore, the roles and pathogenic potential of commensal non-*S. aureus* requires further attention by investigators as the acquisition of genetic elements, such as antibiotic resistance genes, are often methods by which microbes enhance their survivability [25]. Furthermore, these antibiotic resistant staphylococci were recovered in the absence of intensive antimicrobial therapy which highlights the need for research directed at commensal bacteria to further our understanding of the nasal microbiome of animals. The nasal microbiome has been shown to be an important site of infection as the elimination of *S. aureus* from the nasal passages prior to surgery is associated with a reduced risk of a hospital-acquired *S. aureus* infection [26].

We collected and analyzed animal health records for each wallaby from the time they were introduced into the collection (minimum 12 months) at Monarto Zoological Park. We observed approximately equal numbers of staphylococcal isolates which were resistant to the first-line antibiotic TE irrespective of captivity status. In light of these results regarding the prevalence of β-lactam resistant staphylococci and given the importance and dissemination of MRS in human and veterinary medicine, future studies will focus on the detection, isolation and sequence analysis of the β-lactamase and methicillin resistance genes *blaZ* and *mecA*, respectively in the appropriate strains.

The discovery of three MDR staphylococci exclusively of free-ranging wallaby origin was statistically significant ($p \leq 0.05$). These three isolates (A16, A17 and A31) originated from two wallabies residing in separate communities approximately 150 km apart. Strains A16 and A17 were identified as *S. warneri* with identical antibiograms which is indicative of a duplicate isolate. However in the absence of pulse-field gel electrophoresis data, this remains unclear. The presence of MDR bacteria in apparently healthy animals has not been well-studied, however MDR staphylococci isolation rates of 17% in *S. pseudintermedius* in canine pyoderma and otitis externa infections have been reported [27]. Additionally, a 9 year study of canine and feline feces, urine, skin, upper respiratory tract and otitis externa cases were analyzed retrospectively and revealed, on average, an MDR staphylococci incidences of 20% [28]. It was interesting that apparently healthy wallabies, with little to no human interaction, carried antibiotic-resistant or MDR staphylococci. This is of epidemiological significance as it demonstrates prior exposure to antibiotics is not

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doi:http://dx.doi.org/10.4172/2325-9590.1000136

essential to the development of antibiotic resistance in bacteria. Given the remote location of these animals, zoonotic transmission of MDR staphylococci to the general populace is unlikely.

The majority of staphylococci recovered in this study were coagulase-negative species, in agreement with previous reports where animals of non-commercial origin were sampled [6,7,22]. To our knowledge, this is the first study to investigate the commensal staphylococcal species diversity in any member of the *Marsupialia* clade, *Australidelphia* order which includes the *Macropodidae* family [29]. Staphylococci were recovered from 67% of YFRW, 100% of captive BFRW and only 20% of TMW. In contrast, only 46% of free-ranging BFRW were found to carry staphylococci in their nasal passages which could be a reflection on the levels of interaction between animals in their respective environments. Wallabies held in captivity have a higher incidence of interacting with one another as opposed to their free-ranging counterparts. In support of this, data obtained from radio collars fitted to BFRW in the APY Land New Well population indicate intermittent interaction between wallabies in the 15 km granite inselberg environment [30].

The hypothesis that wallabies in captivity are more likely to share a similar nasal microbiome compared to their free-ranging counterparts is supported by this study. We observed that captive wallabies harbored 12 staphylococcal species compared to only five in the free-ranging population ($p \leq 0.05$). In captive wallabies, *S. succinus* was the dominant species recovered and second most prevalent in this study, accounting for 22% (15 isolates) of the total staphylococci from captive wallabies. In contrast, no *S. succinus* was recovered from the free-ranging wallabies ($p \leq 0.05$) indicating the possible presence of an environmental selection pressure. *S. delphini* (8 isolates) and *S. warneri* (7 isolates) accounted for 67% of all staphylococci recovered from free-ranging wallabies. Whilst equal numbers of *S. delphini* was recovered in the captive population; 90% of all *S. warneri* isolates in this study were isolated from the free-ranging population ($p \leq 0.05$).

Interestingly, although staphylococci did not appear abundant in the anterior nares of TMW (10 staphylococci isolated from 27 animals) it was the only wallaby species to harbor *S. hominis* and *S. epidermidis* in captivity. This was a significant finding as we expect strains traditionally associated with humans to be present in captive wallaby populations more readily compared to those from a free-range environment. We did not sample workers at the Animal Health Laboratories at Monarto Zoo or veterinary staff involved in the sampling of APY Land wallabies, so we cannot rule out the possibility of transmission from human carriers. However, veterinarians employed aseptic techniques, including wearing gloves, when obtaining nasal samples thereby reducing the risk of human transmission or contamination.

In our study, *S. delphini* and *S. succinus* were the dominant species recovered, accounting for approximately 35% of all isolates from both captive and free-ranging wallaby origin. Recently Guardabassi et al [31] suggested animals from the order *Carnivora*, family *Mustelidae* (mink, badger and ferrets) be considered the natural hosts of *S. delphini*. However, *S. delphini* has previously been isolated from horses, pigeons, camels and cattle from England, Japan, France and Norway [32,33], respectively. *S. succinus*, a novobiocin-resistant staphylococci which could only be differentiated from other novobiocin-resistant staphylococci on the basis of genomic methods [34] has predominantly been isolated from cheese and sausage production [35,36] after its first isolation from Dominican amber [34] and the occasional human clinical urinary tract infection [37]. Hauschild et al. [7] reported that *S. succinus* accounts for 28% of all staphylococcal isolates recovered from free-living insectivores such as shrews and voles from the *Soricidae* and *Cricetidae* families, respectively. Novobiocin is an important antimicrobial agent and has been shown to have positive activity against MRSA when used in conjunction with rifampicin [38]. *S. succinus* may be ubiquitous in nature therefore, and given its similarities to other human pathogens, its role in the microbial community and pathogenic potential needs to be determined. This study has found both *S. delphini* and *S. succinus* as the dominant staphylococcal species in wallabies (order *Diprotodontia*, family *Macropodidae*). Furthermore, the last common ancestor to be shared by *Macropodidae* and *Mustelidae*, *Soricidae* and *Cricetidae* was approximately 147.7 ± 5.5 million years ago when marsupials separated from placental mammals [39]. We speculate that *S. delphini* has a broad host range and may not have co-evolved with the *Mustelidae* family as previously suggested [31].

Conclusion

Wallabies in South Australia were found to be a significant reservoir of antibiotic resistance in a variety of staphylococcal species. Resistance to the β-lactam family was widespread. Free-ranging wallabies, with little or no contact with humans, carried MDR staphylococci. Wallabies taken into captivity from the wild, or bred in captivity, had an increased diversity of staphylococcal species due to the influence of man. Captive animals are housed in man-made environments which may have been subjected to pre-treatment which could expose wallabies to agricultural, chemical or waste water run-off. Despite this, carriage rates of antibiotic resistance in the staphylococci did not appear to be higher than those of free-ranging animals. *S. aureus* appears to be a member of the wallaby microbial biome but seemed to be relatively susceptible to antibiotics, apart from the β-lactam family.

Acknowledgements

MMSC was supported by a Flinders University Science and Engineering Research Award and thanks the Field Naturalists Society of South Australia for the granting of a Lirabenda Endowment Fund. The authors thank the veterinary nurses at Monarto Zoo, South Australia for their assistance with sample collection. This work was supported by Flinders University.

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Citation: *Chen MM, Boardman WS, Smith I, Goodman AE, Brown MH (2014) Nasal Colonization of Staphylococcus Spp among Captive and Free-Ranging Wallabies in South Australia. J Vet Sci Med Diagn 3:2.*

doi:http://dx.doi.org/10.4172/2325-9590.1000136

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Citation: *Chen MM, Boardman WS, Smith I, Goodman AE, Brown MH (2014) Nasal Colonization of Staphylococcus Spp among Captive and Free-Ranging Wallabies in South Australia. J Vet Sci Med Diagn 3:2.*

doi:http://dx.doi.org/10.4172/2325-9590.1000136

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Appendix G: Chen *et al*. 2015; Journal of Global Antimicrobial Resistance

Journal of Global Antimicrobial Resistance 3 (2015) 184–189

Contents lists available at ScienceDirect

Journal of Global Antimicrobial Resistance

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

Characterisation of β-lactam resistance mediated by *blaZ* in staphylococci recovered from captive and free-ranging wallabies

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ARTICLE INFO

Article history:
Received 30 January 2015
Received in revised form 23 April 2015
Accepted 6 May 2015

Keywords:
Penicillin
blaZ
β-Lactamase
Wildlife
Wallaby
Staphylococcus

ABSTRACT

Staphylococci are commensal organisms of animals, but some species are opportunistic pathogens that are resistant to almost all antimicrobial agents in clinical use. Bacterial resistance to β-lactam antimicrobial agents is widespread and has been investigated in species isolated from humans in addition to food production and companion animals. However, minimal progress has been made towards identifying reservoirs of β-lactam-resistant staphylococci in wildlife. This study was aimed at investigating and characterising β-lactamase resistance from staphylococci of wallaby origin. Staphylococci from free-ranging and captive wallabies were assessed for their phenotypic susceptibility to β-lactam antimicrobial agents prior to sequence analysis of their *blaZ* and *blaR1* genes. Deduced amino acid sequences were classified according to the Ambler molecular characterisation method, assigned a protein signature type and compared with sequences generated from previous studies involving isolates from humans, cattle and companion animals. All BlaZ sequences identified in this study were assignable to a pre-existing β-lactamase class and protein signature type, including the more recently discovered protein signature type 12. Three major phylogenetic groups were resolved upon phylogenetic analysis against published BlaZ sequences. This study has found antibiotic-resistant staphylococci both in free-ranging and captive wallaby populations and these bacteria harbour *blaZ* variants that are different to those recovered from humans, cattle and companion animals. Further studies of staphylococci from non-traditional sources are required in order to enhance our knowledge of the epidemiology of antibiotic resistance genes.

1. Introduction

Staphylococci are commensal organisms that reside in the nasal cavities and on the skin of animals. Under immunocompromised host conditions, certain species such as *Staphylococcus aureus*, *Staphylococcus intermedius* and *Staphylococcus pseudintermedius* can become opportunistic pathogens in humans and other animals [1]. An ecological approach should be applied when furthering our understanding of infectious agents in humans. It has been estimated that almost 75% of emerging human pathogens originated in animals. Whilst there have been numerous studies investigating the incidence of staphylococci, in particular carriage of *S. aureus*, in humans, livestock and pets, the same information is lacking for native wildlife and animals in zoological park collections. Constraining epidemiological studies to animals that have the most direct contact with humans provides us with a narrow snapshot of the overall ecology of these pathogens. This was evident during the 1976 Nigerian brucellosis outbreak in the Ibarapa District, which resulted in >45% of cattle in nomadic herds testing positive for acute bovine brucellosis [2]. In contrast, all government and privately owned farms were *Brucella*-free. However, the flow-on effects of this outbreak, which effectively halved the number of cattle in the area, resulted in acute meat shortages, malnutrition in the general populace and over 100 human cases of brucellosis [2,3].

Since its introduction into clinical medicine in 1940, penicillin has been the drug of choice for the treatment of staphylococcal

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http://dx.doi.org/10.1016/j.jgar.2015.05.002
2213-7165/

infections [4]. However, penicillin-resistant staphylococci were reported as early as 1942 [4] and ongoing studies have found the prevalence of penicillin-resistant *S. aureus* to be between 84% and 88% in Australia [5]. Penicillin resistance in staphylococci can be mediated by the production of an altered form of penicillin binding protein 2A (PBP2A), encoded by the *mecA* gene, or as a result of enzymatic inactivation of the drug by the β-lactamase enzyme. This enzyme is encoded by *blaZ*, which is part of the three-membered *bla* operon also containing a repressor (*blaI*) and signal transducer/sensor protein (*blaR1*) [4]. Under the simplest classification system, four classes (A, B, C and D) of the staphylococcal β-lactamase gene product, BlaZ, have been reported based on conserved and distinguishing amino acid motifs in the protein sequence [6]. Classes A, C and D hydrolyse their substrates through a serine active site, whereas class B β-lactamases function through an active-site zinc ion [6,7]. A more detailed typing scheme that groups different BlaZ together on the basis of their amino acid sequence into 1 of 12 signature types has been proposed [7]. Novel protein signature types are formed when three or more deviations from an existing type are detected [7].

As *blaZ* can be carried on the chromosome and on mobile genetic elements [8], to investigate its spread within a bacterial genus and between strains of the same species it is necessary to combine traditional microbiological techniques with bioinformatic tools. Numerous methods for the phenotypic detection of β-lactam resistance in staphylococci have been established; all had a sensitivity of <72%. This has led to the detection of *blaZ* by PCR to be the recommended gold standard [9]. The presence of *blaZ* has been well documented in staphylococci of human and cattle origin [7,10] as well as in cats and dogs [11]. However, to our knowledge, there are no reports describing *blaZ* in staphylococci of wallaby origin. This study aimed to investigate the natural diversity of the *blaZ* gene from staphylococci of captive Black-flanked Rock wallaby (*Petrogale lateralis*) (BFRW), captive Yellow-footed Rock wallaby (*Petrogale xanthopus*) (YFRW) and captive Mainland Tammar wallaby (*Macropus eugenii*) (TMW) in addition to free-ranging BFRW both by traditional and molecular techniques.

2. Materials and methods

2.1. Bacterial isolates

A total of 89 staphylococcal isolates (56 penicillin-susceptible and 33 penicillin-resistant) were used in the first phase of this study to detect the presence of the *blaZ* gene. These strains originated from a previously described collection of staphylococcal strains isolated from anterior nasal swabs obtained from 68 captive and 30 free-ranging wallabies in South Australia [12]. Staphylococci were preliminary identified by Gram staining and catalase and coagulase production. DNA was extracted using a HiYield™ Genomic DNA Mini Kit (Bio-Deal, Auckland, New Zealand) and isolates were identified to species level by 16S rRNA sequencing. All Gram-positive, catalase-positive isolates that were identified as staphylococci by 16S rRNA sequencing were added to the strain collection.

2.2. PCR detection of the bla operon

All 89 isolates were tested using primer pairs 486–488, 487–373 and 487–531 to amplify a 1.16 kb cumulative fragment of the *blaR1–blaZ* genes as previously described [7]. This fragment contained the first 209 bp of *blaR1*, a 106 bp non-coding intergenic region, and the complete 846 bp *blaZ* gene. As a supplement to primer pair 487–531, primers to amplify an 861 bp fragment (B861) of the *bla* region encompassing *blaZ* [11,13] were used on a limited number of isolates. The *blaI* and *blaR1* genes were amplified from strains demonstrating the presence of the *blaZ* gene in addition to selected controls with oligonucleotides *blaI*F (5′-CTAATTTAATAAGAGTCAAGC-3′) and *blaI*R (5′-TGTTTGGACTT-GACCGACAT-3′), and *blaR1*F (5′-TCCATGACATACGTGAATTTT-3′) and *blaR1*R (5′-ATAATCAAGCGCCCACAGTT-3′) to give products of 979 bp and 1033 bp, respectively. These oligonucleotides were designed based on the *bla* operon from *Staphylococcus epidermidis* (GenBank accession no. X52734).

Each PCR reaction contained 12.5 μL of GoTaq® Green Master Mix (Promega, Madison, WI), 25 pmol each of forward and reverse primer, 5 μg of DNA and 9.5 μL of sterile nuclease-free water (Promega); all reactions were performed with 1.5 mM $MgCl_2$. The PCR cycle conditions for these sets of primers included an initial denaturation of 95 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 54 °C for 1 min and 72 °C for 1 min, and a final extension at 72 °C for 5 min. PCR products were separated on 1% agarose gels (Promega) in 0.5 × TAE [Tris–acetate–ethylene diamine tetra-acetic acid (EDTA)] buffer. Resulting gels were stained with ethidium bromide and were visualised under ultraviolet light and the image was analysed on Gel Doc™ EZ Imager (Bio-Rad, Melbourne, VIC, Australia). PCR products resulting from primer pairs 486–488 and 487–531 as well as representative amplicons from *blaI* and *blaR1* were purified from reaction components using a Wizard® SV Gel and the PCR Clean-up System (Promega) by centrifugation according to the manufacturer's instructions and were sequenced by First Base Laboratories (Selangor, Malaysia) using the forward primers.

2.3. Nucleotide analysis of blaZ and protein signature typing

DNA sequences were assembled manually using BioEdit v.7.1.11 [14] (http://www.mbio.ncsu.edu/bioedit/bioedit.html), were subjected to homology analysis on National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/genbank/) and multiple alignments were performed with Clustal X2 with bootstrap 1000 [15] (http://www.clustal.org/clustal2/). The *bla* operon from *S. epidermidis* (GenBank accession no. X52734) was used as a reference in addition to other published *blaZ* sequences available in GenBank. From the 103 *blaZ* sequences available in the public database as of September 2013, 43 sequences with lengths >780 bp were selected for further analysis. Approximate likelihood ratio test analysis was performed by PhyML 3.0 including bootstrap 100 analysis (http://www.phylogeny.fr/one_task.cgi?task_type=phyml) using default settings. Dendrograms were constructed in Newick format using TreeDyn 198.3 (http://www.phylogeny.fr/one_task.cgi?task_type=treedyn). Nucleotide sequences of *blaZ* genes determined in this study were submitted to GenBank under accession nos. KM362524–KM362542 and KM368805–KM368812.

2.4. Antibiotic susceptibility testing

A total of 41 staphylococcal strains ($n = 25$ wallabies), comprising 33 β-lactam (ampicillin, penicillin and oxacillin)-resistant isolates from a previous study and 8 *blaZ*-positive, β-lactam-sensitive isolates identified in Section 2.2, were challenged with the β-lactam antimicrobial agents amoxicillin/clavulanic acid (AMC) (30 μg) to ascertain β-lactamase activity and cefoxitin (30 μg) to confirm oxacillin results by Kirby–Bauer disc diffusion according to Clinical and Laboratory Standards Institute (CLSI) guidelines [16]. *S. aureus* ATCC 6538, *S. epidermidis* ATCC 12228 and *Escherichia coli* DH5α were used as reference strains.

2.5. Iodometric detection of β-lactamase production

Production of β-lactamase was detected using a method based on previous publications [17,18]. Briefly, an overnight bacterial

culture was pelleted, washed twice with 0.5 M phosphate-buffered saline (PBS) and re-suspended in 100 μL of 0.5 M PBS in 96-well microtitre plates (LabSource, Hallam, VIC, Australia). The cell suspension was standardised to an optical density at 595 nm (OD_{595}) of 2 in a 100 μL volume containing 25 μL of 400 μg/mL penicillin (Sigma–Aldrich, SA, Australia) dissolved in distilled water. Cell suspensions were incubated at room temperature for 60 min prior to the addition of 20 μL of 1% starch and 10 μL of iodine (5 g I_2 and 15 g KI dissolved in 100 mL of distilled water, stored in a dark bottle). Microtitre plates were read with a MultiSkan® EX Type 355 (Thermo Fisher Scientific, Norwood, SA, Australia) at 10-min intervals for 70 min at 26 °C. Assays were performed in triplicate. A positive reaction was recorded upon the colour change from purple to colourless indicating that the starch–iodine complex was cleaved by the β-lactamase enzyme [17].

3. Results

3.1. Phenotypic assessment of β-lactam resistance

Of the 33 penicillin-resistant strains from the previously described strain collection [12], 9 were found to be resistant to cefoxitin and all were susceptible to AMC. Furthermore, nine strains demonstrated the ability to produce detectable levels of the β-lactamase enzyme in the presence of starch and iodine upon exposure to penicillin (Table 1).

3.2. Presence of the bla operon in staphylococci of wallaby origin

From the 89 purified strains, 27 demonstrated the presence of the structural *blaZ* gene and signal/transducer *blaR1* gene using PCR; the β-lactamase repressor gene was detected in 11 strains (Table 1).

3.3. Protein signature types from the BlaZ region

A total of seventy 780 bp *blaZ* sequences (24–803 of X52734), comprising 43 from the public database and 27 obtained from this study, were analysed. A dendrogram was constructed using the deduced amino acid sequences of the 70 *blaZ* sequences and revealed three major phylogenetic groups (I, II and III) (Fig. 1) consisting of four protein signature types (3, 5, 6 and 12) (Table 2) encompassing three out of four β-lactamase molecular classes.

Group I strains were composed of protein signature types 3 and 12 from the β-lactamase class C family. A total of 14 BlaZ sequences, all recovered from captive wallabies, were identified as protein signature type 3 making it the most prevalent signature type in this study. These 14 strains were identical to type strain *S. aureus* AP004832, however only strain *S. aureus* A78 demonstrated the ability to produce β-lactamase (Table 1). The more recently identified protein signature type 12 was found in two staphylococcal strains from a captive YFRW and a captive TMW (Table 2). These two strains differed from the type strain by a point mutation resulting in the change of valine-86 to isoleucine (Table 2).

Group II contained strains with β-lactamase classes A and D and multiple protein signature types. Here, however, only protein signature type 5 from β-lactamase class D was identified in this group (Table 2), and these strains were recovered primarily from BFRW. Of these seven strains, two *Staphylococcus warneri* isolates were identical to type strain *Enterococcus faecalis* U43087 and produced β-lactamase (Table 1). Point mutations identified in five isolates of *S. aureus* and *S. warneri* resulted in two different amino

Table 1
Source of isolates, resistance profile and presence of *bla* genes in staphylococci of wallaby origin.

Strain	Location and wallaby species	Resistance profile					β-Lactamase production	Presence of:			Ambler class	Signature type	*blaZ* accession nos.
		AMP	PEN	OXA	FOX	AMC		*blaZ*	*blaR1*	*blaI*			
S. epidermidis ATCC 12228		R	R	S	S	S	Yes	+	+	+	–	–	–
S. aureus ATCC 6538		S	S	S	S	S	No	–	–	–	–	–	–
Escherichia coli DH5α		S	S	S	S	S	No	–	–	–	–	–	–
S. warneri A1	F-BFRW	R	R	S	S	S	No	+	+	+	D	5	KM362524
S. aureus A7	F-BFRW	R	R	S	S	S	Yes	+	+	+	D	5	KM362525
S. aureus A8	F-BFRW	R	R	S	S	S	Yes	+	+	+	D	5	KM362526
S. warneri A16	F-BFRW	R	R	R	R	S	Yes	+	+	+	D	5	KM362527
S. warneri A17	F-BFRW	R	R	R	R	S	Yes	+	+	+	D	5	KM362528
S. warneri A24	F-BFRW	R	R	S	S	S	No	+	+	+	D	5	KM362529
S. aureus A78	C-BFRW	R	R	S	S	S	Yes	+	+	+	C	3	KM362530
S. epidermidis M68	C-TMW	R	R	S	S	S	No	+	+	+	D	5	KM362531
S. epidermidis M72	C-TMW	R	R	R	R	S	No	+	+	+	C	12	KM362532
S. epidermidis A2	F-BFRW	R	R	S	S	S	Yes	+	+	–	B	6	KM362533
S. epidermidis A32	F-BFRW	R	R	S	S	S	Yes	+	+	–	B	6	KM362534
S. saprophyticus M21	C-YFRW	R	R	R	R	S	No	+	+	–	C	3	KM362535
S. succinus M27	C-YFRW	R	R	S	S	S	No	+	+	–	C	3	KM362536
S. xylosus M28	C-YFRW	R	R	S	S	S	No	+	+	–	C	3	KM362537
S. xylosus M29	C-YFRW	R	R	S	S	S	No	+	+	–	C	3	KM362538
S. succinus M30	C-YFRW	R	R	S	S	S	No	+	+	–	C	3	KM362539
S. fleurettii M31	C-YFRW	R	R	R	S	S	No	+	+	–	C	3	KM362540
S. epidermidis M65	C-TMW	R	R	S	S	S	Yes	+	+	–	B	6	KM362541
S. epidermidis M66	C-TMW	R	R	S	S	S	Yes	+	+	–	B	6	KM362542
S. saprophyticus M17	C-YFRW	S	S	S	S	S	No	+	+	–	C	12	KM368805
S. simulans M26	C-YFRW	S	S	S	S	S	No	+	+	–	C	3	KM368806
S. delphini M32	C-YFRW	S	S	S	S	S	No	+	+	–	C	3	KM368807
S. delphini M33	C-YFRW	S	S	S	S	S	No	+	+	+	C	3	KM368808
S. xylosus M36	C-YFRW	S	S	S	S	S	No	+	+	+	C	3	KM368809
S. succinus M37	C-YFRW	S	S	S	S	S	No	+	+	–	C	3	KM368810
S. vitulinus M38	C-YFRW	S	S	S	S	S	No	+	+	–	C	3	KM368811
S. vitulinus M39	C-YFRW	S	S	S	S	S	No	+	+	–	C	3	KM368812

F-BFRW, Black-flanked Rock wallaby from a free-ranging environment; C-BFRW, Black-flanked Rock wallaby from captivity; C-TMW, Mainland Tammar wallaby from captivity; C-YFRW, Yellow-footed Rock wallaby from captivity; AMP, ampicillin; PEN, penicillin; OXA, oxacillin; FOX, cefoxitin; AMC, amoxicillin/clavulanic acid; R, resistant; S, sensitive; +, amplified; –, not amplified.

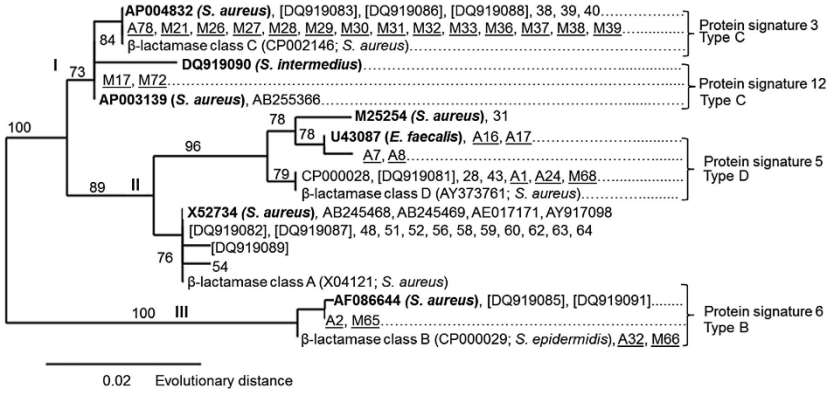

Fig. 1. Phylogenetic analysis of *blaZ* sequences with reference to published sequences. Strains in bold represent the type strain. DNA sequence types in numerals refer to [7]; square brackets refer to [11]; underlined font refers to this study and accession no. of sequences from GenBank.

acid substitutions (Table 2) that appear to have differing effects on β-lactamase production (Table 1).

Group III consisted only of strains with β-lactamase protein signature type 6, belonging to β-lactamase variant class B family (Fig. 1). Signature type 6 was identified in four *S. epidermidis* strains from two captive TMW and a single free-ranging BFRW (Fig. 1; Table 1). Of the four strains, two were identical to type strain *S. aureus* AF086644 and the remaining two strains contained a single point mutation resulting in a change at residue 241 from a valine to isoleucine (Table 2). Irrespective of the mutation, all four strains were able to produce β-lactamase.

4. Discussion

The presence and diversity of the *bla* operon has been studied in staphylococci from humans, cattle, dogs and cats; however this is the first study from commensal staphylococcal in apparently healthy captive and free-ranging wallabies.

This study emphasises the natural diversity of BlaZ amongst wallaby strains of staphylococci and increases our understanding of staphylococci carriage in Australian macropods. The first study to investigate BlaZ diversity in staphylococci reported 69 unique sequences from 105 cattle strains [7]. Of these 69, 60 sequences were represented by a single bacterial isolate, with signature type 6 being the most prevalent. Other studies, such as those by Malik et al. [11] who identified six unique sequences from 13 cat and dog strains, and by Bagcigil et al. [19] who identified 25 unique sequences from 78 cattle, also found BlaZ signature type 3 to be the most prevalent both amongst coagulase-positive and coagulase-negative staphylococci. Here, four unique BlaZ sequences were identified from 27 of 89 wallaby strains studied, with all sequences represented by at least two isolates. From these four sequences, one was unique to free-ranging BFRW and was found in strains

Table 2
Protein signature types of BlaZ.

Signature type[a]	Strains[b]	BlaZ variable position[c]																									
		19	10	02	24	25	57	78	61	21	29	13	91	12	25	34	63	97	37	00	12	40	15	58	18
1	X52734 (*S. aureus*)[c]	V	I	N	S	A	K	S	V	V	A	T	K	V	Q	R	E	E	K	L	S	P	Y	V	G	V	K
3[d]	AP004832 (*S. aureus*) M21[e], A78[f]	V	M	N	S	A	K	S	V	V	E	T	K	V	Q	R	E	K	N	F	N	P	Y	V	G	V	K
5	U43087 (*E. faecalis*) A16[f], A17[f] A7[f], A8[f] A1[f], A24[f], M68[g]	A	I	N	P	A	K	S	V	I	E	T A	K	V	Q	R	G	E	K	L	S	S	C Y	V	G	V	K
6	AF086644 (*S. aureus*) A32[f], M66[g] A2[f], M65[g]	A	I	T	S	A	K	A	V	V	E	K	N	I	K	R	K	K	N	L	N	P	Y	V I	N	I	K
12	DQ919090 (*S. intermedius*) M17[e], M72[g]	V	I	N	S	T	Q	S	V	V I	E	T	K	V	Q	H	E	K	N	F	N	P	Y	V	G	V	K

[a] Signature types are defined based on [7,11].
[b] Strains in bold represent the current type strain.
[c] Variable positions are shown and refer to the deduced protein sequence of X52734.
[d] This is shared by 14 strains from captive wallabies.
[e] Isolate from Yellow-footed Rock wallaby.
[f] Isolate from Black-flanked Rock wallaby.
[g] Isolate from Mainland Tammar wallaby.

S. aureus A7 and A8. These two strains were isolated from two wallabies living independently of one another in two populations ca. 350 km apart. The second sequence, also a variant of signature type 5, was identified in two free-ranging BFRW sampled in 2009 and a captive TMW sampled in 2010. A third sequence that was a variant of signature type 12 was identified from staphylococci isolated from captive TMW and YFRW. Finally the last sequence, a variant of signature type 6, was identified in staphylococci from one free-ranging BFRW and one TMW. Given the spatial and temporal distribution of these wallabies, the discovery of bacteria harbouring these identical signature types is unlikely to be the result of direct transmission and is indicative of the natural diversity of BlaZ. These results support previous studies where very little exchange of *bla* genes between coagulase-positive and coagulase-negative staphylococci was observed [7]. Furthermore, Milheiriço et al. [20] found no evidence of correlation between *bla* allotypes, strain background, β-lactam resistance phenotypes or strain origin of *S. aureus* isolates, indicating that the *bla* genes have evolved independently of *S. aureus*. Whilst the current study is not as detailed or broad as that described above, we also see some indication of signature type clustering with respect to wallaby species. For example, the majority of BlaZ signature type 5 staphylococci were recovered from free-ranging BFRW, and staphylococci from YFRW were associated with either the common BlaZ signature type 3 or the more recently identified BlaZ signature type 12 from pet dogs. However, these observations are preliminary and are based on a small sample size; large-scale typing and epidemiological studies would need to be performed to substantiate these observations.

Production of β-lactamase was tested using two methods. The first used a disc diffusion assay with AMC. However, when all strains returned a sensitive result, indicating that they were susceptible to the actions of clavulanic acid, an iodometric method was employed to detect β-lactamase production. Several observations were made regarding the impact of the identified naturally occurring BlaZ mutants (Table 2). First, β-lactamase production was not observed in *S. warneri* (A1 and A24) and *S. epidermidis* (M68) strains carrying the genes encoding products with protein signature type 5 containing an alanine in place of a threonine at residue 119, whereas modification at residue 220 had no effect on β-lactamase production. Second, all mutants of protein signature type 6 strains were able to produce β-lactamase. Third, residue 86 in protein signature type 12 appears to play a minimal role in conferring β-lactam resistance; the two strains containing this mutation had opposing antibiograms; type strain *S. intermedius* DQ919090 was resistant only to penicillin [11].

Diversity amongst BlaZ sequences was demonstrated by phylogenetic analysis with the emergence of three main groups (Fig. 1). All BlaZ sequences examined in this study belonged to one of the four molecular classes of β-lactamase and to a previously described signature type. Surprisingly, no class A β-lactamase genes were identified in this study, which was unusual given that this group of enzymes are the predominant β-lactamases in Gram-positive cocci [6].

Expression of the β-lactam-resistant phenotype could be a result of the expression of more than one gene. The current results indicating that detection of *blaZ* could not be used to assume the presence of *blaI* and *blaR1* are comparable with those of Milheiriço et al. [20]. Similar to previous authors [11,21], we detected staphylococcal strains that were β-lactam-resistant but were negative for the *blaZ* gene by PCR (data not shown). Conversely, detection of the *blaZ* gene does not guarantee the demonstration of a β-lactam-resistant phenotype, as was seen in eight isolates. Analysis of *blaZ* sequences from these eight β-lactam-susceptible isolates revealed no variations compared with their β-lactam-resistant counterparts. Likewise, other studies by Pereira et al. [9] and Papanicolas et al. [22] have also found strains that exhibited sensitivity to penicillin, did not produce the β-lactamase enzyme and yet were positive for the *blaZ* gene using conventional PCR, sequencing and real-time PCR. Genetic data found no differences in the *blaZ* region between the β-lactam-resistant and -sensitive isolates [9]. This finding could be the result of DNA mutations in the regions encoding the repressor (*blaI*) or promoter (*blaR1*), with data indicating that the promoter region is the primary target for the accumulation of mutations, presumably in response to the different types of β-lactam compounds [20].

5. Conclusion

In summary, this study indicates that *blaZ* plays a role in β-lactam resistance in staphylococci isolated from wallabies and that it is comparable with the *blaZ* genes of human, cattle, dog and cat origin. The finding of phenotypically penicillin-sensitive staphylococcal isolates presenting with *blaZ* gene sequences that were 100% identical to their penicillin-resistant counterparts is corroborated by other authors in the field. In light of the diversity of *blaZ* sequences uncovered from free-ranging BFRW, further longitudinal studies encompassing more native wildlife species are required in order to enhance our studies of the epidemiology of antibiotic resistance genes and how they may relate to their evolution or relationship with certain animal hosts.

Funding

MMSC was supported by a Flinders Science and Engineering Research Award and thanks to the Field Naturalists Society of South Australia for the granting of a Lirabenda Endowment Fund research grant.

Competing interests

None declared.

Ethical approval

Approval was received from the Flinders University Institutional Biosafety Committee (Adelaide, SA, Australia) [approval no. 2009-25].

Acknowledgments

The authors thank the staff at Monarto Zoo (Monarto, SA, Australia) for their assistance with sample collection.

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Appendix H: Chen *et al*. 2016; One Health

One Health 2 (2016) 31–32

Contents lists available at ScienceDirect

One Health

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

Clonal diversity of methicillin-sensitive *Staphylococcus aureus* from South Australian wallabies

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ARTICLE INFO

Article history:
Received 13 April 2015
Received in revised form 4 December 2015
Accepted 10 December 2015
Available online 3 March 2016

Keywords:
MSSA
DNA microarray
StaphyType
Healthy wildlife
Macropods

ABSTRACT

Seven methicillin-sensitive *Staphylococcus aureus* nasal isolates from apparently healthy captive and wild wallabies were characterised by DNA microarray and antibiotic susceptibility assays. Isolates were found to belong to uncommon clonal complexes including those previously associated with birds, pigs and humans.

Staphylococcus aureus is a versatile bacterium which can infect or colonise a variety of mammals, including humans. With the use of molecular techniques, *S. aureus* strains can be differentiated assigning them to clonal complexes (CC) [1]. Clonal complexes group bacterial isolates from the same species based on the genetic variation present in seven housekeeping genes. This variation can be used to infer the relationship of all isolates within a particular CC to a common ancestor [2]. Some CCs have been identified predominantly in specific hosts, such as CC692 in birds whereas others, such as CC15, are less specific [3,4].

Here, anterior nasal swabs were collected from 68 captive and 30 free-ranging wallabies (*Petrogale lateralis*, *Petrogale xanthopus* and *Macropus eugenii*) at two locations in South Australia, the Monarto Zoo (a 1000 ha open range zoo) and the Anangu Pitjantjatjara Yankunytjatjara Lands (a lightly populated remote indigenous land), during routine health examinations between July 2009 and October 2010. From these 98 nasal swabs, a total of seven *S. aureus* isolates were identified from five captive animals and two free-ranging animals (Table 1). Isolates were identified with a combination of biochemical assays and 16S rRNA sequence analysis [5]. Antimicrobial susceptibility tests revealed that four strains were susceptible to every antimicrobial agent tested [5,6]. Antibiotics tested included β-lactams, aminoglycosides, macrolide, glycopeptide, cephalosporin, tetracycline and chloramphenicol. Three strains, A7, A8 and A78, exhibited ampicillin and penicillin resistance with A8 also demonstrating intermediate resistance towards cefotaxime (Table 1).

Molecular characterisation of the *S. aureus* isolates was performed by DNA microarray analysis (StaphyType, Alere Technologies, Jena, Germany) using previously described protocols and modified to include probes for *mecC* and the SCC*mec*-XI-associated *blaZ* allele [7]. The assignment of isolates to a CC was determined by an automated comparison of hybridisation profiles to reference profiles [1]. Analyses identified a single CC692 strain, three CC49 and three CC15 strains. All strains demonstrated the absence of methicillin-resistance genes and were thus classified as methicillin-sensitive *S. aureus* (MSSA). Microarray analysis also revealed that these strains possessed limited antimicrobial resistance determinants confirming the above-mentioned phenotypic analyses, with the *bla* operon being the only one identified (Table 1).

Virulence genes common in staphylococci including those encoding enterotoxins and exfoliative toxins (*eta*, *etb* and *etd*), epidermal cell differentiation inhibitors (*edinA*, *edinB* and *edinC*), toxic shock syndrome toxin (*tst*) and the Panton–Valentine leucocidin toxin (*lukF*-PV and *lukS-PV*), were not found. These data combined with veterinary records which indicated that these animals were in a non-diseased state support the *S. aureus* status as commensal organisms in this population. All isolates carried a variety of genes encoding proteins associated with adherence to host structures. The presence of these genes as well as their assignment to allelic variants depended on CC affiliation and not on host species as there was no difference to previously described isolates of the same CCs from other hosts [1,8,9].

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http://dx.doi.org/10.1016/j.onehlt.2015.12.001

Table 1
Microbiological characteristics of commensal MSSA in the nasal passages of South Australian wallabies [b].

				Antibiotic susceptibility profile[a]												Microarray-based analysis
Isolate	Wallaby species	Captivity status	Clonal complex	AMP	OX	PEN	AMC	FOX	CN	S	VA	CTX	TE	E	C	Resistance genes
A7	BFRW	Free-ranging	CC15	R	-	R	-	-	-	-	-	-	-	-	-	*blaZ, blaI, blaR1*
A8	BFRW	Free-ranging	CC15	R	-	R	-	-	-	-	-	iR	-	-	-	*blaZ, blaI, blaR1*
A78	BFRW	Captive	CC15	R	-	R	-	-	-	-	-	-	-	-	-	*blaZ, blaI, blaR1*
A70	BFRW	Captive	CC49	-	-	-	-	-	-	-	-	-	-	-	-	None detected
A73	BFRW	Captive	CC49	-	-	-	-	-	-	-	-	-	-	-	-	None detected
M9	YFRW	Captive	CC49	-	-	-	-	-	-	-	-	-	-	-	-	None detected
M7	YFRW	Captive	CC692	-	-	-	-	-	-	-	-	-	-	-	-	*fosB*

Abbreviations: BFRW, black-flanked rock wallaby (*Petrogale lateralis*); YFRW, yellow-footed rock wallaby (*Petrogale xanthopus*); –, sensitive; R, resistant; iR, intermediate resistant; AMP, ampicillin; OX, oxacillin; PEN, penicillin; AMC, amoxicillin-clavulanic acid; FOX, cefoxitin; CN, gentamicin; S, streptomycin; VA, vancomycin; CTX, cefotaxime; TE, tetracycline; E, erythromycin; C, chloramphenicol.

[a] Disk diffusion breakpoints were determined as previously described [5].

[b] Data pertaining to antibiotic susceptibility profiles and isolate captivity status have been previously published [5] but have been included here to aid the reader in analysis.

To date, CC692 has been isolated exclusively from various birds and, as such, to the best of our knowledge, this represents the first report of CC692 from any mammalian species [4]. For CC49-MSSA, only a few human and veterinary cases have been reported from Western Europe. CC49-MRSA were observed in pigs and rats (the latter being *mecC*-positive) [9,10]. CC15-MRSA is very common in humans; studies from Europe showed it to be one of the most prevalent lineages [8]. Isolates of CC15 were an unexpected finding in two free-ranging wallabies and a single captive wallaby. These three CC15 strains had very similar hybridisation profiles to the 115 *S. aureus* CC15 strains examined from asymptomatic human carriers in Germany, including the presence of immune evasion genes *chp* and *scn* and the absence of staphylokinase gene *sak*, both considered typical qualities of this lineage [3]. This provides further support to the notion that these strains are commensal organisms and are not involved in disease. Since immune evasion cluster genes are apparently host-specific and their carriage might rapidly change in adaption to a new host, this observation might suggest a recent transmission of CC15 from humans to wallabies.

This study describes the first genotyping data on commensal *S. aureus* from South Australian native wallabies. Results indicate that wallabies did not harbor unique host-specific strains as the three identified CCs have been described in other species. Reassuringly, resistance genes were rare with no MRSA recovered amongst the seven isolates from apparently healthy wallabies. The absence of both common virulence genes in conjunction with resistance genes provides further confirmation to support these strains status as commensal organisms. Additionally, given the *S. aureus* genotypes are typically associated with humans and birds, interspecies transmission from non-macropod hosts cannot be ruled out. These findings also raises questions about which genotypes can be considered the indigenous flora of wallabies and there was no evidence for a zoonotic background of particular "Australian" clones of *S. aureus* such as ST93 and ST1850.

Acknowledgments

We thank veterinarians Dr. Wayne Boardman and Dr. Ian Smith and the veterinary nurses at Monarto Zoological Park for the provision of wallaby nasal swabs as well as Antje Ruppelt and Bettina Stieber for performing the array experiments. We thank Flinders University for the provision of a Science and Engineering Research Award and the Field Naturalists Society of South Australia for the granting of a Lirabenda Endowment Fund to MMSC.

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Appendix I: Chen *et al*. 2016; Infection Ecology and Epidemiology

INFECTION ECOLOGY & EPIDEMIOLOGY
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ORIGINAL RESEARCH ARTICLE

Methicillin resistance gene diversity in staphylococci isolated from captive and free-ranging wallabies

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Background: Infection with methicillin-resistant staphylococci (MRS) can be life-threatening in humans and its presence in animals is a cause for public health concern. The aim of this study was to measure the prevalence of MRS in captive and free-ranging wallabies over a 16-month period in South Australia, Australia.
Materials and methods: Eighty-nine purified staphylococcal isolates recovered from 98 captive and free-ranging wallabies' anterior nasal swabs were used in this study. All isolates were tested for the presence of the *mecA*, *mecA1*, and *mecC* genes. Multiplex PCR-directed SCC*mec*-typing, *ccrB*-typing, and determination of the minimal inhibitory concentration of oxacillin were performed on *mec*-positive isolates.
Results and discussion: In total, 11 non-*Staphylococcus aureus* MRS were isolated from 7 out of 98 animals, corresponding to a 7.1% carriage rate. The SCC*mec* types I, III, and V were identified by multiplex PCR and sequencing of the *ccrB* gene. This is the first report of MRS carriage in both captive and free-ranging wallabies in Australia. These data demonstrate a low prevalence of MRS and no association between wallaby captivity status and MRS carriage could be assigned. These animals may act as a reservoir for the exchange of genetic elements between staphylococci. Furthermore, the *mecA* genes of animal isolates were identical to that found in human MRS strains and thus the possibility of zoonotic transfer must be considered.

Keywords: *macropods*; *wildlife*; Staphylococcus; *methicillin-resistance*; *SCC*mec

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Received: 6 March 2016; Revised: 10 April 2016; Accepted: 13 April 2016; Published: 17 May 2016

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a frequent pathogen of humans and many animal species. In addition, methicillin-resistant coagulase-negative staphylococci (MRCNS) has long been recognised as important human and animal pathogens. Both MRSA and MRCNS are of interest to human and animal medicine and are collectively known as methicillin-resistant staphylococci (MRS). The *mec* genes encoding resistance to methicillin and almost all β-lactam antibiotics are carried by a large mobile genetic element, the staphylococcal cassette chromosome *mec* (SCC*mec*), which is able to integrate into the staphylococcal chromosome at a specific site within the 3′ end of the ribosomal methyltransferase (1, 2). The SCC*mec* element is highly variable in various staphylococcal species. To date, 11 SCC*mec* types (I–XI) and numerous subtypes have been identified in MRSA (2–4). In 2011, a new divergent *mecA* homologue, designated *mecC*, located in a new SCC*mec* cassette–designated SCC*mec* type XI, was described in *S. aureus* (5, 6). This homologue is not detectable using routine *mecA*-specific PCR approaches, and various studies have searched for this new element in different animal hosts (7–10).

The origins of the SCC*mec* elements remain unknown, but it is believed that the *mecA* gene began with a single common ancestor. Homologues of the *mecA* gene have been found in *Staphylococcus sciuri*, *S. vitulinus*, and *S. fleurettii*. Furthermore, the *mecA* gene of *S. fleurettii* has 99 to 100% sequence homology to MRSA strain N315 thus indicating that a direct precursor to the methicillin resistance determinant for MRSA is present in *S. fleurettii* (11–14).

Although studies on MRSA in humans, companion animals, and livestock have been widely documented, there is still a scarcity of information on infections, carriage,

Citation: Infection Ecology and Epidemiology 2016, **6**: 31507 - http://dx.doi.org/10.3402/iee.v6.31507
(page number not for citation purpose)

and the role of this particular pathogen in wildlife. Here, we report the assignation of *mec* types to staphylococcal isolates and from captive and free-ranging wallabies from South Australia, Australia.

Materials and methods

Bacterial isolates

A collection of 89 staphylococcal isolates obtained from captive and free-ranging wallabies in a surveillance study undertaken in 2009–2010 was used in this study (15). Free-ranging wallaby samples were obtained from three colonies living in the Anangu Pitjantjatjara Yankunytjatjara (APY) Lands (102,650 km^2). The APY Lands, classified as a 'managed conservation and natural environment resource' by the Australian Department of Agriculture, are located in the far north-west corner of South Australia, Australia, and are home to approximately 2,230 people across 33 communities and outstations (15, 16). Anterior nasal swabs were collected from 68 captive and 30 free-ranging wallabies during routine health examinations. All animals were assessed as apparently healthy at the time of sampling. Staphylococcal species identification, antibiotic susceptibility profiles, and the presence of the β-lactamase resistance operon from the 89 isolates have been reported previously (15, 17).

Detection of the mecA1, mecA, *and* mecC *elements*

All 89 staphylococcal strains were screened for the presence of *mecA* as previously described (18). Additional PCRs were performed for the detection of the *mecC* gene identified in all cefoxitin-resistant strains (5, 6). Furthermore, all *S. sciuri* isolates were screened for the presence of the ubiquitous *mecA1* homologue (19).

We tested the hypothesis that the carriage of *mecA* and *mecC* in staphylococci would be impacted by the captivity status of the wallaby host. The significance of these data was determined by using chi-square analysis. A p-value ≤ 0.05 was regarded as being statistically significant. The p-values are shown only for results that were statistically significant (20).

Phenotypic screening of cefoxitin resistance

In total, 22 oxacillin-resistant isolates from the 2009–2010 surveillance study and 11 *mecA*-positive isolates identified as described above were assessed for their ability to grow in the presence of cefoxitin by Kirby–Bauer disc diffusion test with a 30-μg cefoxitin disc (Oxoid, Basingstoke, UK). Inoculum preparation, inoculation, and incubation were performed as recommended by the Clinical and Laboratory Standards Institute (CLSI) guidelines (21). Instead of clinical breakpoints, for the present study, the results were evaluated according to the epidemiological cut-off values of the European Committee for Antimicrobial Susceptibility Testing (EUCAST) susceptibility breakpoints for *S. aureus* and coagulase-negative staphylococci. Data from the EUCAST disc diffusion distribution website were last accessed January 2016 (www.eucast.org). *S. aureus* strain ATCC 6538 and a clinical MRSA SCC*mec* II isolate were used as internal quality controls.

Determination of oxacillin minimum inhibitory concentration

Oxacillin minimum inhibitory concentration (MIC) analysis was carried out on 11 *mecA*-positive strains and two isolates that exhibited discordant results for *mecA*-specific PCR and cefoxitin resistance. Bacterial suspensions were adjusted to 0.5 McFarland standard in 0.85% saline. In total, 14 twofold dilutions of oxacillin were made to cover the concentrations 0.03125 to 256 μg/ml in Mueller–Hinton broth supplemented with 2% NaCl. The test was incubated aerobically at 37°C for 24 h and results were read with a MultiSkan® EX Type 355 (Thermo Fisher Scientific, SA, Australia) spectrophotometer at 595 nm. Interpretation of MIC breakpoints for all strains followed the guidelines provided by EUCAST; data from the EUCAST MIC distribution website were last accessed January 2016 (www.eucast.org). A clinical MRSA SCC*mec* II, *S. aureus* ATCC 6538, and *S. epidermidis* ATCC 12228 were used as quality control strains in addition to a sterility control.

*Typing of SCC*mec *and* ccr *elements*

All 11 *mecA*-positive isolates were typed using multiplex PCR methodologies 1 and 2 with 1.5 mM $MgCl_2$ to classify isolates into the main SCC*mec* types I, II, III, IV, and V. SCC*mec* IV subtyping was performed with multiplex PCR 3 (22). Supplementary SCC*mec*- and *ccr*-typing methodologies (23–25) were used in selected *mecA*-positive strains that could not be assigned a SCC*mec* type with the aforementioned multiplex PCR. Appropriate control strains that have been previously assigned to *mec* classes from Malik et al. were included for the SCC*mec*-typing protocols (26).

Nucleotide analysis of mec *and phylogenetic analysis*

DNA sequences were assembled manually using BioEdit v.7.1.11 (27) (available at www.mbio.ncsu.edu/bioedit/bioedit.html), subjected to homology analysis on National Centre for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov/genbank/) and multiple alignments performed with Clustal X2 with bootstrap 1000 (28) (available at www.clustal.org/clustal2/). The *mec* sequences from Ito et al. (29) were used as references. Approximate likelihood ratio test analysis was performed using PhyML 3.0 with bootstrap 100 analysis (www.phylogeny.fr/one_task.cgi?task_type=phyml) using default settings. Dendrograms were constructed in Newick format using TreeDyn 198.3 (www.phylogeny.fr/one_task.cgi?task_type=treedyn). Nucleotide sequences of *mecA* and *ccrB* genes determined in this study were submitted to

Citation: Infection Ecology and Epidemiology 2016, **6**: 31507 - http://dx.doi.org/10.3402/iee.v6.31507

GenBank under accession numbers KT021005–KT021015, KT003668, and KT003669.

Results

Detection and diversity of the staphylococcal mec *genes from wallabies*

Initially, the presence of *mecA* or *mecC* was set as the gold standard to consider an isolate as a MRS irrespective of its phenotype. The *mecA* gene was detected and subsequently sequenced from 11 staphylococcal isolates. These 11 isolates originated from 7 out of the 98 sampled animals, corresponding to a MRS carriage rate of 7.1%. The *mecA1* gene purportedly present in every *S. sciuri* isolate was detected in only 2 out of the 4 *S. sciuri* isolates recovered in this study (data not shown) and the *mecC* gene was not identified in any isolates.

Sequencing and subsequent bioinformatic analysis of the 11 *mecA* and 2 *mecA1* genes revealed that irrespective of resistance phenotype, all 7 methicillin-resistant *S. fleurettii* isolates contained 19 nucleotide variations in the *mecA* gene and that these variations were comparable to those found in the NCBI database. The remaining 4 *mecA* sequences from *S. cohnii*, *S. epidermidis*, and *S. warneri* were comparable with human-derived MRSA strains found in NCBI. Finally, the *S. sciuri mecA1* genes recovered in this study demonstrated 97 to 99% sequence homology to type strains K11 and ATCC 700061 across 1016 bp (data not shown).

Antimicrobial susceptibility testing

All MRS isolates were found to be susceptible to gentamicin, streptomycin, vancomycin, and chloramphenicol (Table 1) as assessed by Kirby–Bauer disc diffusion. Interestingly, two isolates of *S. fleurettii* (M11, M47) were identified to be β-lactam sensitive by disc diffusion and three cefoxitin-sensitive *S. fleurettii* isolates were identified. Despite their phenotypic resistance profiles, all five isolates demonstrated oxacillin MIC values greater than 16 μg/ml (Table 1). Conversely, *mecA*- and *mecC*-negative isolates M21 (*S. saprophyticus*) and M54 (*S. succinus*) which demonstrated resistance toward ampicillin, penicillin, oxacillin, and cefoxitin had MIC values of 32 μg/ml (Table 1). These two isolates were included in subsequent SCC*mec*-typing protocols to aid in the determination of the genetic mechanism behind this irregular phenotypic profile.

*Typing of SCC*mec *elements from MRS*

In total, 4 different staphylococcal species were identified from 11 *mecA*-positive isolates (Table 1) with the majority dominated by *S. fleurettii* from the *S. sciuri* species group. Overall, five different *ccr-mec*-complex combinations were detected in these 11 MRS isolates. These combinations could be broadly separated into three categories based on the method of classification. First, SCC*mec* types III (M72) and V (A16 and A17) were identified by multiplex PCR. Second, two novel variants were identified. Novel variant one (A31) was identified by the unique combination of a novel *ccrAB1* allele 117 and a class A *mec* element. Novel variant two was characterised by the detection of a type 4 *ccrB* element from the aforementioned SCC*mec* III strain M72. Finally, a single non-typeable variant was identified. This non-typeable element accounted for more than half (63.6%) of all SCC*mec* elements identified and all were found in *S. fleurettii* strains (Table 1). No SCC*mec* element genes could be detected for the two *mecA*- and *mecC*-negative cefoxitin-resistant strains (M21 and M54).

Discussion

A systematic review of the literature investigating antimicrobial-resistant bacteria in wildlife populations yielded 210 novel studies up until mid-2015 (30). Studies investigating the presence of *Escherichia coli* (115 studies), *Salmonella* spp (54 studies), and *Enterococcus* spp (43 studies) comprised the bulk of the literature. However, analysis of staphylococcal carriage involving free-ranging animals are scarce as the majority of studies conducted have focused on captive animals which have had regular contact with humans (31–33). However, in recent years, a trend of sampling free-ranging animals for the purpose of determining the prevalence of MRS has emerged (7, 34, 35). Most recently, analysis of faecal pellets from captive and free-ranging brush-tailed rock wallabies revealed the presence of class 1 integrons via PCR amplification (36). However, the present study is the first to investigate the carriage of MRS using nasal swabs collected from apparently healthy, captive, and free-ranging wallabies.

Worldwide, a large range of MRS incidence rates have been reported ranging from 15.4% in Turkish dogs (37), 28.6% in Polish riding horses (38), 29.5% in Belgian pigs (39), and up to 43.0% in Danish goats and sheep (40). Previous studies in Australia have found MRS carriage rates to be 4.0% in South Australian cats and dogs (18), 3.7% in horses admitted to a veterinary intensive care unit in New South Wales (41), and 0.9% in a nationwide study of pigs (42). In the present study, surveillance of healthy wallabies revealed MRS carriage rates of 7.4% for captive wallabies (5 out of 68 animals) and 10.0% for free-ranging animals (3 out of 30 animals). These carriage rates, however, were not considered statistically significant and thus indicate that the carriage of MRS was not affected by the captivity status of the wallabies. This finding is significant as we had expected wallabies with close association with humans to carry MRS more readily compared with those in a low-human environment.

The CLSI now recommends the use of cefoxitin discs as opposed to oxacillin discs for the detection of methicillin resistance in staphylococci by Kirby–Bauer disc diffusion and the amplification of the *mecA* gene by PCR as the

Table 1. Phenotypic and genotypic characterisation of methicillin-resistant staphylococcal isolates from wallabies

		Resistance profile												SCC*mec*-typing				
														ccr complex				
Strain	OX MIC (μg/ml)	AMP	OX	PEN	AMC	FOX	CN	S	VA	CTX	TE	E	C	Multiplex PCR[a]	*ccrB* typing[b,c]	*mec*	SCC *mec*	*mecA* accession number
S. aureus (MRSA)	8	R	R	R	S	R	R	S	S	S	S	S	S	A2B2	ND	A	II	NA
S. aureus ATCC 6538	0.047	S	S	S	S	S	S	S	S	S	S	S	S	–	–	–	NA	NA
S. cohnii (A31)[†]	0.625	R	R	R	S	R	S	S	S	R	S	R	S	–	A1B1 (117)	A	I	KT021007
S. epidermidis (M72)[‡]	1.5	R	R	R	S	R	S	S	S	iR	S	S	S	A3B3 + C	A4B4 (602)	C C	III	KT021015
S. fleurettii (A59)[‡]	16	R	R	R	S	R	S	S	S	iR	S	S	S	–	–	A	NT	KT021008
S. fleurettii (A61)[‡]	32	R	R	R	S	R	S	S	S	iR	S	S	S	–	–	A	NT	KT021009
S. fleurettii (A69)[‡]	32	R	R	R	S	S	S	S	S	iR	S	S	S	–	–	A	NT	KT021010
S. fleurettii (A72)[‡]	32	R	R	R	S	S	S	S	S	iR	S	S	S	–	–	A	NT	KT021011
S. fleurettii (M11)[‡]	16	S	S	S	S	S	S	S	S	iR	S	S	S	–	–	A	NT	KT021012
S. fleurettii (M31)[‡]	16	R	R	R	S	S	S	S	S	iR	S	S	S	–	–	A	NT	KT021013
S. fleurettii (M47)[‡]	16	S	R	S	S	S	S	S	S	S	S	S	S	–	–	A	NT	KT021014
S. warneri (A16)[†]	4.5	R	R	R	S	R	S	S	S	iR	R	R	S	5	–	C	V	KT021005
S. warneri (A17)[†]	4	R	R	R	S	R	S	S	S	iR	R	R	S	5	–	C	V	KT021006
S. saprophyticus (M21)[‡]	32	R	R	R	S	R	S	S	S	S	S	R	S	–	–	–	–	NT
S. succinus (M54)[‡]	32	R	R	R	S	R	S	S	S	S	S	S	S	–	–	–	–	NT

[†]Staphylococci from free-ranging animal.
[‡]Staphylococci from captive animal.
[a]Multiplex PCR performed as recommended by Kondo et al. (22).
[b]*ccrB*-typing performed as recommended by Oliveira et al. (25).
[c]*ccrB* sequence submitted to GenBank under accession numbers: KT003668 and KT003669.
R, resistant; iR, intermediate resistant; S, susceptible; –, no amplification; NA, not available; NT, not typeable; AMP, ampicillin; OX, oxacillin; PEN, penicillin; AMC, amoxicillin-clavulanic acid; FOX, cefoxitin; CN, gentamicin; S, streptomycin; VA, vancomycin; TE, tetracycline; E, erythromycin; C, chloramphenicol.

gold standard (43). However, similar to other authors (44), we detected staphylococcal isolates which contained the *mecA* gene but were susceptible to both oxacillin and cefoxitin by disc diffusion. Our strains had a non-induced oxacillin MIC of 32 μg/ml compared to 1 μg/ml observed by Cuirolo et al. (44). This demonstrates the limitations of using cefoxitin as a predictor of *mecA*-mediated methicillin resistance.

Many SCC*mec*-typing strategies have been developed since the publication of its discovery in 2000 (45). However, the majority of studies have been conducted with human samples and thus most methodologies were designed specifically for the detection of MRSA. As all MRS identified in this study were CNS, a range of multiplex PCR strategies were selected from the literature and trialled in this study. No PCR amplicons could be

Citation: Infection Ecology and Epidemiology 2016, **6**: 31507 - http://dx.doi.org/10.3402/iee.v6.31507

obtained from the 11 MRS isolates using PCR methodologies from Lim et al. (23) or Zhang et al. (24). SCC*mec*-typing by the Kondo multiplex PCRs (22) showed the carriage of known types (III and V). However, the majority of *mec* elements, identified as containing class A *mec* elements, were unable to be classified by this method.

MRCNS are considered to be a source of SCC*mec* elements by horizontal gene transfer to *S. aureus* and the diversity of SCC*mec* element types among CNS is larger than that among *S. aureus* (14). Although *S. aureus* was not found in coexistence with MRCNS in our nasal swabs, native wildlife have already been shown to potentially act as a reservoir for multidrug-resistant staphylococci (15) and could function as a reservoir for the evolution of novel SCC*mec* types. This is extremely worrisome given that *S. epidermidis* and the *S. sciuri* species group are also opportunistic pathogens and their zoonotic potential cannot be discounted (46). In this study, two novel variants of the existing SCC*mec* types were identified by sequencing the *ccrB* gene. Variant one contained a class A *mec* element with a previously undescribed variant of *ccrAB1* thus leading to the formation of a novel SCC*mec* I variant (*S. cohnii* strain A31). Variant two was identified by the discovery of a type 4 *ccrAB* element in *S. epidermidis* SCC*mec* III strain M72. Furthermore, two MRS isolates (*S. warneri* strains A16 and A17) from a single free-ranging wallaby were identified as harbouring community-acquired SCC*mec* type V. This SCC*mec* type was first identified in the Australian indigenous population in 2005 (47), and the discovery of this element in free-ranging wildlife living in indigenous land is further evidence of its community origin.

Conclusions

To be best of our knowledge, this is the first report of MRS in captive and free-ranging wallabies in Australia. Our data demonstrate the absence of MRSA and a low prevalence of MRCNS in both captive and free-ranging wallabies, indicating that MRS occurs naturally even in the absence of human intervention. However, the presence of multidrug-resistant staphylococci carrying the *mecA* gene isolated from indigenous land is worrisome and may have implications for wildlife rehabilitation and subsequent antimicrobial treatment in cases of wound infections, localised inflammation and systemic bacterial infections. This study highlights the need for further longitudinal and environmental studies involving a larger range of native wildlife species in order to increase our understanding regarding the epidemiology of resistance genes.

Acknowledgements

MMSC was supported by a Flinders University Science and Engineering Research Award. The authors thank Dr Ian Smith and veterinary nurses at Monarto Zoo, South Australia, for their assistance with sample collection. This work was supported by Flinders University.

Conflict of interest and funding

The authors received funding from the Field Naturalists Society of South Australia to conduct this study.

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Citation: Infection Ecology and Epidemiology 2016, **6**: 31507 - http://dx.doi.org/10.3402/iee.v6.31507

Appendix J: Abbreviations

ABC	Adenosine triphosphate binding cassette
AMC	Amoxicillin/clavulanic acid
AMP	Ampicillin
AMR	Antimicrobial resistant
APA	Aminopenicillanic acid
APY	Anangu Pitjantjatjara Yankunytjatjara
ATCC	American type culture collection
β	Beta
BFRW	Black-flanked rock wallaby
BNTW	Bridled nail-tail wallaby
bp	Base pair
BURP	Based upon repeat pattern
C	Chloramphenicol
CA-MRSA	Community-acquired methicillin-resistant *Staphylococcus aureus*
CC	Clonal complex
CDC	Centers for Disease Control and Prevention
CLSI	Clinical and Laboratory Standards Institute
CN	Gentamycin
CNS	Coagulase negative staphylococci
CPS	Coagulase positive staphylococci
CTX	Cefotaxime
DNA	Deoxyribonucleic acid
E	Erythromycin
ECDC	European Centre for Disease Prevention and Control
EMRSA	Epidemic methicillin-resistant *Staphylococcus aureus*
ESBL	Extended spectrum β-lactamases
ESCMID	European Society of Clinical Microbiology and Infectious Diseases
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FOX	Cefoxitin
HA-MRSA	Hospital-acquired methicillin-resistant *Staphylococcus aureus*
HGT	Horizontal gene transfer
ICU	Intensive care unit
IS	Insertion sequence
kDa	Kilodaltons

LA-MRSA	Livestock-associated methicillin-resistant *Staphylococcus aureus*
M	Molar
MALDI-TOF	Matrix-assisted laser desorption ionisation-time of flight mass spectrometry
MATE	Multidrug and toxic compound extrusion
MDR	Multidrug resistant
MFS	Major facilitator superfamily
mg	Milligram
MGE	Mobile genetic elements
ml	Millilitre
MLST	Multi locus sequence typing
MSCRAMM	Microbial surface components recognising adhesive matrix molecules
MRCNS	Methicillin-resistant coagulase negative staphylococci
MRSA	Methicillin-resistant *Staphylococcus aureus*
MRS(A)	Methicillin-resistant *Staphylococcus aureus* and/or staphylococci
MRS	Methicillin-resistant staphylococci
NCBI	National Center for Biotechnology Information
nm	Nanometre
OD	Optical density
ORSAB	Oxacillin resistance screening agar base
OX	Oxacillin
p-value	Probability value
PEN	Penicillin
PBP	Penicillin binding protein
PBP2a	Penicillin binding protein 2a
PBS	Phosphate-buffered salinePCE Proteobacterial chlorhexidine efflux
PCR	Polymerase chain reaction
PDR	Pan drug resistance/resistant
PVL	Panton-Valentine leukocidin
PFGE	Pulse field gel electrophoresis
RND	Resistance nodulation cell division
rRNA	Ribosomal ribonucleic acid
S110	Staphylococcus medium no 110

S	Streptomycin
SCC	Staphylococcal cassette chromosome
SCC*mec*	Staphylococcal cassette chromosome *mec*
SMR	Small multidrug resistance
ST	Sequence type
TAE	Tris-base acetic acid ethylenediaminetetracitic acid
TE	Tetracycline
TMW	Mainland Tammar wallaby
Tn	Transposon
µg	microgram
µL	microlitre
USA	United States of America
VA	Vancomycin
WGS	Whole genome sequencing
WHO	World Health Organisation
XDR	Extensive drug resistance/resistant
YFRW	Yellow-footed rock wallaby

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