

**Zoonotic and Public Health Implications of
Campylobacter species and Squamates
(Lizards, Snakes and Amphisbaenians)**

By

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BSc. (Biol. sc.)

*Thesis
Submitted to Flinders University
for the degree of*

Master of Environmental Health

College of Science and Engineering
October 2020

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SUMMARY

Abstract

Campylobacteriosis is one of the most widespread infectious diseases of veterinary and public health significance. In humans, the disease presents as gastroenteritis with diarrhoea, nausea, vomiting, abdominal cramps and fever. However, in rare cases, the disease may enter the bloodstream and cause life-threatening extra-intestinal infections and autoimmune disorders such as Guillain-Barré syndrome and Miller-Fisher syndrome. Globally the incidence of campylobacteriosis has increased over the last two decades in both developing and developed countries. While the disease is mainly foodborne transmitted, other environmental reservoirs of its causative agent, *Campylobacter* spp., include animals. Squamates (lizards, snakes and amphisbaenians) are a potential reservoir and source of transmission of campylobacteriosis to humans. More people are now keeping lizards and snakes as pets, a trend that has zoonotic and public health implications.

A systematic search of literature was carried out to examine studies from the last 20 years that have reported human campylobacteriosis linked to squamates globally. The literature review examined six case reports and eight environmental surveillance studies that identified lizards and snake species, and the associated *Campylobacter* species that they were shown to carry and potentially spread to humans. The review demonstrated the need to carry out further investigation of *Campylobacter* associated with lizard faeces. Therefore, faeces collected from Australian sleepy lizards (*Tiliqua rugosa*) from South Australia were examined by extracting DNA from all the samples and conducting quantitative PCR to detect presence of *Campylobacter jejuni*.

Of the 60 lizard faecal samples examined, none were positive for *C. jejuni*. This is in contrast with other studies, where the presence of *C. jejuni* in lizards' faecal samples confirms potential zoonotic and public health implications of *Campylobacter* spp. in squamates. It is hypothesized that the wild

sleepy lizards' faecal samples were collected far from areas of human habitation and that might be the reason for there being no detection of *C. jejuni*.

From the systematic literature review, it was found that *C. fetus* subsp. *testudinum* and *C. fetus* subsp. *fetus* were the most frequently isolated species in squamates and the predominant cause of human campylobacteriosis from a squamate host. *C. jejuni* and *C. iguaniorum* were also isolated from lizard faecal samples and reported to pose potential health risks to humans. The common squamate hosts identified included bearded dragons (*Pogona vitticeps*), green iguana (*Iguana iguana*), western beaked gecko (*Rhynchoedura ornate*) and botched blue-tongued skink (*Tiliqua nigrolutea*). One environmental surveillance study reported presence of *Campylobacter jejuni* in lizard faeces collected from Central Australia.

People with underlying chronic illnesses, young children below the age of five years, the immunocompromised and the elderly were identified as the most vulnerable populations. Exposure to pet squamates, wild animals, consumption of reptilian cuisines and cross contamination with untreated water were risk factors associated with campylobacteriosis. Proper hand hygiene practices, responsible pet ownership, and 'One Health' education and awareness on zoonotic diseases, will help reduce the public health risks arising from *Campylobacter* exposure through squamates. Continued surveillance using molecular diagnostic methods will also enhance detection and response to squamate-linked campylobacteriosis.

DECLARATION

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in 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.

Signed: NMM

Date: 18th October 2020

ACKNOWLEDGEMENTS

I sincerely acknowledge Australia Awards for granting me a scholarship for my postgraduate study in Flinders University, without which I wouldn't have made it.

I would like to thank my principal supervisor, Dr. Kirstin E. Ross for her awesome supervision, guidance and support. I am sincerely indebted to her for putting my career ambitions before the need to just acquire a degree and for conceptualizing a project that exactly offered me the skills that I so much desired to acquire.

I am happy to have had a very supportive second supervisor, Dr. Harriet Whiley who offered immeasurable academic input, guidance and support into my studies and research project and for being the most supportive supervisor that I could have ever asked for.

I am immensely indebted to Dr. Thilini K., Mae White and to Muhammed A. N, for taking a keen interest in my quest to understand DNA extraction and qPCR procedures, and for sparing their valuable time to attend to my questions, with a smile on their faces.

I extend my appreciation to the Environmental Health Department for a warm welcome and for providing me with a peaceful ambience to learn and carry out my laboratory work. I am fortunate to have made great friends within the department whose support made my study a resounding success.

I am sincerely grateful to G. Norval for the collection and safe storage of the samples that I used for this study, and for providing me with the information I needed about the samples and the study site.

A special mention to my family; my beloved wife, mum and sister for their unwavering support especially during my two-year study; and to my beloved sons for bearing with my 'long-distance' parental presence. I dedicate this thesis to my beloved wife for her resilience, encouragement, love and moral support.

Above all, I give thanks to God, for He is good. For His mercy endures forever.

Statement of co-authorship

The following people contributed to the submission for publication of the manuscript undertaken as part of this thesis. The co-authors are listed in the order that the co-authored publication appear in the thesis.

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All above listed contributions equated to no more than 25% of the work necessitated for publication of research manuscripts.

Publications

Masila, NM, Ross, KE, Gardner, MG & Whiley, H 2020, 'Zoonotic and public health implications of *Campylobacter* species and squamates (Lizards, snakes and amphisbaenians)', *MDPI Pathogens*, vol. 9, no. 10, 799.

This thesis is based on the published manuscripts.

1.0. INTRODUCTION

1.1. *Campylobacter* spp.

Globally, *Campylobacter* spp. is a common zoonotic pathogen of significant veterinary and public health concern (Hsieh & Sulaiman 2018; Kaakoush et al. 2015). It is the causative agent of campylobacteriosis, a gastrointestinal disease that has been increasing in incidence over the last two decades (Hsieh et al. 2018; Igwaran & Okoh 2019; Strachan et al. 2009; Wilson et al. 2008). The disease presents as gastroenteritis with fever, nausea, vomiting, abdominal pains and watery or bloody diarrhea (Du et al. 2019). While the disease may generally be a self-limiting enterocolitis, clearing on its own within a week, it may also manifest in serious long-term complications including extra-intestinal infections and autoimmune disorders such as Guillain-Barré syndrome, Miller Fisher syndrome, cholecystitis, inflammatory bowel syndrome and reactive arthritis (Du et al. 2019; Endtz 2020; Pike, Guerry & Poly 2013). In recent years, the incidence of campylobacteriosis has increased in both developed and developing countries (Kaakoush et al. 2015). In the USA, it is estimated that *Campylobacter* spp. causes over 1.3 million cases and approximately 130 deaths per year, with the Foodborne Diseases Active Surveillance Network (FoodNet) reporting an increase in annual incidence rate of human campylobacteriosis from 14.3 in 2012 to 19.5 cases per 100,000 population in 2019 (Acheson & Allos 2001; Kaakoush et al. 2015; Ruiz-Palacios 2007; Scallan et al. 2011; Tack et al. 2020)

Campylobacter spp. present a threat to human and animal health because of their zoonotic potential, wide host range, ability to colonize diverse habitats, and emerging resistance to some of the commonly used antimicrobial drugs (Epps et al. 2013). The virulence of different *Campylobacter* species and severity of the resulting enteritis is dependent on the pathogenesis mechanisms used, including adhesion to intestinal wall, colonization of digestive tract, invasion of target cells and toxin production (Haddad et al. 2010). The infection process involves penetration of the gastrointestinal mucus by the bacteria using its high motility and spiral shape, adherence to the gut

enterocytes and then inducing diarrhea through release of toxins mainly enterotoxins and cytotoxins (Wallis 1994). While *Campylobacter jejuni* is a fastidious bacterial pathogen, its virulence is adversely affected by environmental stresses such as nutrient insufficiency, heat stress, absence of water, partial oxygen tension above 10%, low PH, UVB exposure and hydrostatic pressure (Mihaljevic et al. 2007). However, it is able to develop survival mechanisms which include; persisting in the environment, especially in water, in a viable but non-culturable state (Baffone et al. 2006), transition from rod to coccoid shape (Moran & Uptone 1987) and growth in biofilm (Joshua et al. 2006). By altering gene expression pathways, *C. jejuni* can also adapt to new growth temperatures when exposed to a sudden temperature upshift (Stintzi 2003) and persist and grow intracellularly in non-phagocytic host cells through the use of gene encoding catalase (katA) enzyme (Day et al. 2000). While previous studies have provided useful information on virulence of *Campylobacter* spp., further research is needed to inform interpretation of different virulence associated markers or genes.

The *Campylobacter* genus displays wide taxonomic diversity currently comprising 32 species and nine subspecies (Iraola & Costa 2019). *Campylobacter* spp. is responsible for 9% of all foodborne illnesses in the United States (Scallan et al. 2011) and molecular typing techniques suggest that up to 80% of human infections are caused by *Campylobacter* strains associated with a poultry host (Newell et al. 2011). *Campylobacter jejuni* is the most common *Campylobacter* species isolated from human cases with campylobacteriosis (Kaakoush et al. 2015; Kirkpatrick & Tribble 2011; Taheri et al. 2019). Additionally, *C. jejuni* causes over 80% of human campylobacteriosis cases with 50-80% of the cases attributed to the chicken reservoir (both broilers and laying hens) (BIOHAZ 2011; Navarro-Gonzalez et al. 2016).

The disease is not only a foodborne illness but is also transmitted through environmental reservoirs including animals (Rukambile et al. 2019; Whiley et al. 2013). Changes in land use, habitat loss, urbanization, encroachment of people into wildlife habitats and community composition are reported to influence wildlife health (Murray et al. 2019). With human-wildlife interactions

becoming more common, the likelihood of zoonotic spread of campylobacteriosis is increasing (Bjelland et al. 2020; Navarro-Gonzalez et al. 2016; Wang et al. 2013). However, information about horizontal transmission of *Campylobacter* spp. through non-foodborne routes is limited, and the zoonotic nature of the disease is often overlooked (Wang et al. 2013; Whiley, McLean & Ross 2017). One potentially overlooked host is squamates (Whiley, McLean & Ross 2017).

Squamata is the largest order of reptiles comprising of three suborders; lizards (suborder: *Lacertilia/Sauria*), snakes (suborder: *Serpentes/Ophidia*) and worm lizards (suborder: *Amphisbaenia*) (Cogger 1993). The suborder, lizards, includes skinks (family: *Scincidae*), dragons (family: *Agamidae*), monitor lizards/goannas (family: *Varanidae*), geckos (family: *Gekkonidae*) and flat-footed lizards (family: *Pygopodidae*) which are all adapted to diverse environments (Cogger 1993). The squamates have been implicated in potentially aiding horizontal transmission of *Campylobacter* spp. either by cross-contamination through their faeces, pet handling or generally as a result of close interaction with human habitats (Whiley, McLean & Ross 2017).

There is also a possibility of *Campylobacter* spp. transmission through squamates, mammals and birds' faeces contaminating rainwater in tanks especially those with no first-flush diverter installed to prevent initial flow of contaminant-laden water from the roof entering the tank when it rains (Ahmed et al. 2012). For example, studies done in South east Queensland showed that 21% of rainwater tank samples contained *Campylobacter* spp. from birds and possum faeces (Ahmed et al. 2016). Though limited research has been done on lizards unlike in food-producing animals, studies point to enhanced risk of animal-associated campylobacteriosis to humans (Horrocks et al. 2009; Dinç, Doğanay & İzgür 2015).

With the propensity to keep reptiles, including squamates, as pets, increasing globally (Alves et al. 2019; Benn, McLelland & Whittaker 2019; Schuppli, Fraser & Bacon 2014) zoonotic disease transfer to humans continues to pose a serious challenge to the public and environmental health sector. This thesis examines the literature evidence pertaining to squamate-linked campylobacteriosis in humans. It also entails environmental surveillance of lizard faecal samples

through DNA extraction and detection of *C. jejuni* by qPCR. Studies describing human campylobacteriosis cases linked to the handling of captive and wild squamates or cross-contamination through their faeces are surveyed. Further, trends in emerging *Campylobacter* subspecies, the lizard and snake species involved in transmission, and possible exposure routes, were also explored. This information will inform more effective management strategies to reduce the risk of zoonotic transfer of *Campylobacter* spp. from captive and wild squamates to humans.

1.2. *Campylobacter* species and squamates worldwide

A systematic literature review (presented below) was carried out to determine the current state of knowledge of human campylobacteriosis associated with squamates exposure globally. The review followed the PRISMA guidelines for a systematic literature review (Moher et al. 2009). All published studies that identified zoonotic and public health implications of *Campylobacter* spp. associated with pet and wild squamates were analyzed. This manuscript was published in the peer-reviewed journal MDPI *Pathogens*, ranked Q1 in Scopus.

**Zoonotic and Public health implications of *Campylobacter* species and squamates
(Lizards, snakes and amphisbaenians)**

This article has been published in:

Pathogens 2020, 9(10), 799;

DOI: <https://doi.org/10.3390/pathogens9100799>

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1.3. *Campylobacter* spp. in Australia

Campylobacter jejuni is a causative pathogen of campylobacteriosis, which is a common gastrointestinal disease of public health significance worldwide (Whiley et al. 2013; Hsieh et al. 2019; Epps et al. 2013; Costa & Iraola 2019). *Campylobacter* spp. is the most frequently notified enteric pathogen in Australia (OzFoodNet 2011). Despite Australia having robust food safety standards, foodborne campylobacteriosis presents a significant public health burden (Moffatt et al. 2020). While *Campylobacter* infections may occur at any time of the year, they are more common in warmer months. Australia's *Campylobacter* cases are among the highest in the high-income countries (Varrone et al. 2018). Despite this high incidence, outbreaks of campylobacteriosis are infrequently detected and reported.

Campylobacteriosis is a notifiable disease in all states and territories. The public health response involves notification of *Campylobacter* infections and suspected outbreaks of foodborne gastroenteritis (OzFoodNet 2018). For example, in the case of an outbreak in New South Wales, a local public health unit investigates the outbreak to identify common exposure to a food source. If a common food is identified, the NSW Health Authority carries out further environmental investigation and initiates control measures (NSW Health 2017). While foodborne transmission is predominant, direct zoonotic transmission can occur through animal contact or indirectly through cross-contamination of water and other environments via animal faecal material (Varrone et al. 2018; Whiley, McLean & Ross 2017). Food-producing farm animals and pets are the most commonly studied transmission vehicles of campylobacteriosis spread, while exotic pets such as squamates are less studied.

1.4. *Campylobacter* spp. and Squamates in Australia

Campylobacter spp. is predominantly considered a foodborne pathogen (Skarp et al. 2016; Whiley, McLean & Ross 2017). However, current research provides new insights into environmental sources that act either as reservoirs or vehicles of transmission of campylobacteriosis to humans or other animals (Whiley et al. 2013, Masila et al. 2020; Moffatt et al. 2020). Reptiles have been implicated for playing a role in the spread of pathogens of zoonotic significance. There is evidence that campylobacteriosis is a zoonotic disease whose transmission through wildlife could be increasing as a result of their domestication as pets and human encroachment into wildlife habitats (Djelland et al. 2020), although, limited research has been carried out on reptiles as reservoirs of *Campylobacter jejuni* (Wang et al. 2013). Chelonians, lizards, crocodiles and snakes are among the reptiles confirmed to harbour *Salmonella* spp., *Escherichia coli*, ticks, nematodes, cestodes, trematodes, protozoans and viruses, although not all are pathogenic to their hosts (Norval et al. 2019). While the majority of reported cases of campylobacteriosis are caused by *C. jejuni*, and *C. coli* to a lesser extent, 43% (13) of the other remaining 30 *Campylobacter* species are implicated in sporadically causing disease in humans and other animals (Costa & Iraola 2019).

There is also a possibility of *Campylobacter* spp. transmission through reptiles, mammals and birds' faeces contaminating rainwater in tanks especially those with no first-flush diverter installed to prevent initial flow of contaminant-laden water from the roof entering the tank when it rains (Ahmed et al. 2012). For example, studies in south east Queensland reported that 21% of rainwater tank samples contained *Campylobacter* spp. from birds and possum faeces (Ahmed et al. 2012; Ahmed et al. 2016). Though limited research has been undertaken on lizards, studies point to enhanced risk of animal-associated campylobacteriosis to humans (Horrocks et al. 2009; Dinç, Doğanay & İzgür 2015).

A common lizard endemic in Australia, and one of the best studied skink species belonging to *Scincidae* family of the squamates, is the sleepy lizard (*Tiliqua rugosa*) (Norval et al. 2019). The sleepy lizards are relatively large, blue-tongued, short-tailed, slow-moving, omnivorous, viviparous

and widely distributed in southern and eastern Australia (Cooper, Bull & Gardner 1997). A review study by Norval et al. (2019) evaluated known parasites of *T. rugosa* and assessed bacteria, viruses, cestodes, trematodes, nematodes, protozoans and ticks that infest sleepy lizards. However, the review did not identify *Campylobacter* spp. as one of the bacteria that was present in the sleepy lizards. Other studies have reported *C. jejuni* in sleepy lizards and other lizard species. Whiley, McLean and Ross (2017) investigated the presence of *C. jejuni* in lizards from Central Australia using quantitative polymerase chain reaction (qPCR). Results confirmed *C. jejuni* in 33% (17/51) of the lizards which included captive bearded dragons (*Pogona vitticeps*), western beaked gecko (*Rhynchoedura ornate*) and unidentified wild lizards. The presence of *C. jejuni* in these common lizard species has zoonotic and public health implications for humans. This informs the need to identify mechanisms and approaches that reduce public health risks that may arise due to close human interaction with pet and wild squamates. One of the emerging global health approaches that promote transdisciplinary collaboration of experts in human, veterinary and ecosystem health to tackle food safety, zoonotic diseases and environmental health challenges is the ‘One Health’ approach.

1.5. One Health Approach

1.5.1. Definition and scope

The World Health Organization (WHO) defines One Health as an “approach to designing and implementing programs, policies, legislation and research in which multiple sectors communicate and work together to achieve better public health outcomes” (WHO 2017 p. 1). The approach is particularly relevant in food safety, control of zoonotic disease risks and antimicrobial resistance (WHO 2017). There are different perceptions advanced by various people, organizations and initiatives about the One Health approach. Many people understand One Health as cooperation only between veterinary and human health in the control of zoonotic diseases (Mackenzie, McKinnon & Jeggo 2014; Johnson, Hansen & Bi 2017), however, the One Health approach focuses on shared

understanding that encompasses public health, veterinary, ecosystem health sectors as well as other relevant fields working together through a structured communication, collaboration and coordination framework at the human-animal-environmental health interface to enhance health outcomes (Hinchliffe, 2015; Johnson, Hansen & Bi, 2017).

The definition of One Health that is most frequently used and widely accepted is given by the Centers for Disease Control and Prevention (CDC) and the American Veterinary Medical Association (AVMA). The CDC defines One Health as “a collaborative, multisectoral and transdisciplinary approach - working at the local, regional, national, and global levels – with the goal of achieving optimal health outcomes recognizing the interconnection between people, animals, plants and their shared environment” (CDC 2018 p. 1). AVMA defines One Health similarly and is a strong promoter of the One Health approach in the United States (AVMA 2020, p. 1). One key aspect of One Health that would be considered appropriate for Australia is the need for interdisciplinary collaboration amongst environmental health, food safety, socio-economic, animal health, ecology and public health sectors, to improve public and environmental health outcomes (Johnson, Hansen & Bi, 2017). Lerner & Berg (2015) argues for a wide approach using the ‘umbrella’ depiction developed by One Health Sweden and the One Health Initiative (Figure 1.2). As the One Health approach is being implemented, it is suggested that societal and cultural elements should also be considered (Mackenzie et al. 2014).

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Campylobacteriosis in pet and wild squamates is thus a One Health issue due to its relevance to food safety, zoonoses, international trade, health security, human-animal bond and antimicrobial resistance; which are health issues of concern to the tripartite organizations WHO, OIE and the FAO (WHO 2019; Wielinga & Schlundt 2012; Lubroth 2012; Lerner & Berg 2015). In this regard, implementation of a coordinated One Health approach would foster interdisciplinary collaboration, communication and sharing of resources and expertise to develop effective surveillance techniques,

molecular diagnostic and therapeutic interventions that enhance health outcomes at the human-wildlife-livestock-environment interface.

To enhance global health security against zoonotic diseases, the US Centers for Disease Control and Prevention (CDC) developed a One Health Zoonotic Disease Prioritization (OHZDP) tool which brings together representatives from human, animal, wildlife and environment health sectors to prioritize the endemic and emerging zoonoses of greatest national concern in a country or region (Salyer et al. 2017). The OHZDP tool has successfully been utilized in prioritizing zoonoses such as salmonellosis, rabies and zoonotic influenza in 25 countries including China and the United States (CDC 2020; Salyer et al. 2017).

The One Health approach had previously been applied in the UK leading to successful decrease in the incidence of *Salmonella* infections in the 1990s (Cogan & Humphrey 2003; Brown et al. 2020). The interventions involved multi-agency coordination, surveillance, improved biosecurity in chicken farms and public health programs. While the measures implemented at that time were not referred to as One Health, it is clear that the approach was multi-sectoral, collaborative, well-coordinated and involved human, veterinary and environmental health experts. This approach may therefore find relevance and application in campylobacteriosis prevention, detection and response, as there are potential implications for horizontal transmission of *C. jejuni* to food production farms via squamates (Whiley, McLean & Ross 2017).

1.6. Aims and Objectives of the Project

1.6.1. Aims

1. Review the evidence in the literature of human campylobacteriosis associated with exposure to squamates (lizards, snakes and amphisbaenians) globally.
2. Assess the incidence of *C. jejuni* in lizard faeces using real-time quantitative PCR (qPCR).

1.6.2. Objectives

- i. To examine all published studies from the last 20 years that have reported squamate-associated human campylobacteriosis in order to establish whether pet and wild squamates are reservoirs for, or aid in, the spread of campylobacteriosis.
- ii. To use established and validated DNA extraction and qPCR methods to determine the prevalence of *C. jejuni* in lizard faeces sampled from sleepy lizard (*T. rugosa*) species in South Australia.
- iii. To identify potential mechanisms and approaches that may help in reducing public health risks arising from zoonotic *Campylobacter* infections linked to squamate exposure.

2.0. MATERIALS AND METHODS

2.1. Sample Collection

2.1.1. Ethics

The lizard faecal samples had already been collected by a PhD student for a different research project. The animal ethics approval number from the Flinders University Animal Ethics Committee for the research is E454-17. The lizard faecal samples were collected under the permits for undertaking scientific research (numbers A23436-25 [2017], A23436-26 [2018] and A23436-27 [2019]) that were issued by the South Australian Department of Water, Environment and Natural Resources.

2.1.2. Study area

The study area where the lizard faecal samples were collected (Bundey Bore station) is a semi-arid, flat area in the rain shadow of the Mount Lofty ranges in South Australia. There are human-inhabited homesteads at the intersection of three roads; Salford Road, Bundey-Church Road and Bower Boundary Road (Figure 2.2). The original hypothesis was that the prevalence of *Campylobacter* species in the lizard faecal samples would differ across the ecological gradient at the Bundey Bore station. It was hypothesized that pathogen communities in the lizards decreased as one moves away from the human habitation. Human-wildlife disease interaction is a growing concern because as humans interact with animals, spillover of disease pathogens is inevitable (Gilbert et al. 2014). Active disease and parasite surveillance are therefore extremely important in establishing prevalence and incidence of zoonotic diseases and/or pathogens of veterinary and public health significance in human-wildlife interfaces. The current study investigated the presence of *C. jejuni* in sleepy lizard (*T. rugosa*) faeces from South Australia using quantitative PCR.

“Figure 1.1: Removed due to copyright restriction”

Map of the study area, Bunday Bore station in the northeast of Adelaide, South Australia

(Source: <https://www.google.com/maps/place/33%C2%B053'11.4%22S+139%C2%B021'18.6%22E/@-33.8834657,139.3520053,16z/data=!3m1!1e3?hl=en&gl=US>)

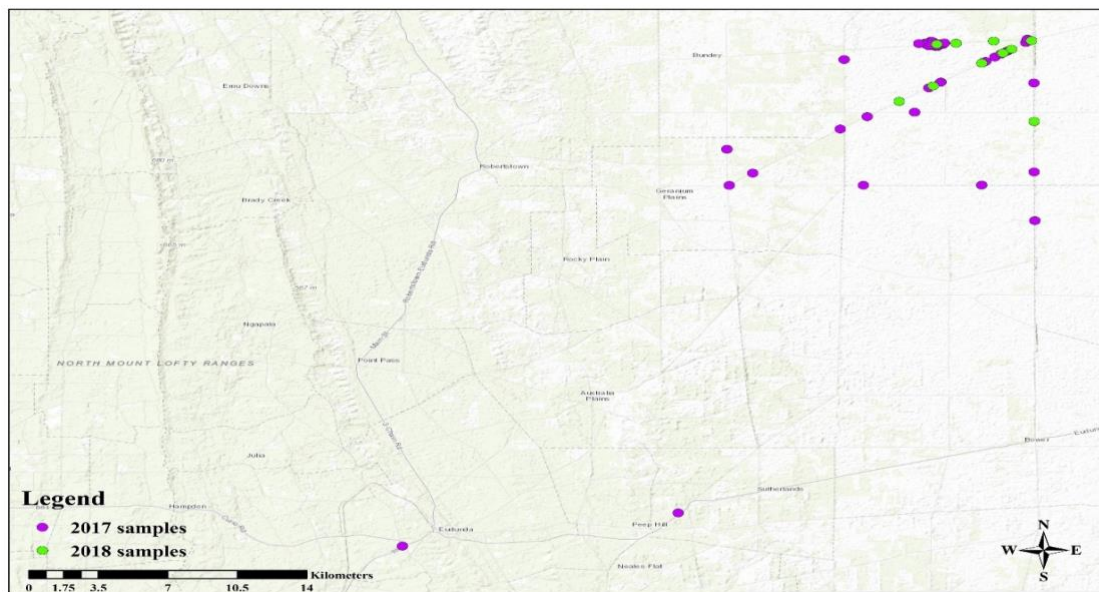


Figure 2.2: Map of the study site, Bunday Bore station showing the actual locations where the lizard faecal samples were collected (Source: G Norval 2020, personal communication, 13 October)

2.1.3. Collection of samples and preservation

A total of 60 sleepy lizard faecal samples (scats) had been collected from Bunday Bore station, South Australia in 2017 and 2018 and appropriately preserved until DNA extraction and screening for *C. jejuni* using quantitative PCR was done. All samples were collected using sterile tweezers, put into sterile Eppendorf® tubes, appropriately preserved in DESS (Dimethyl sulfoxide, disodium EDTA, and saturated NaCl) (Beknazarova et al. 2017) and stored at -20°C prior to DNA extraction.

2.2. Extraction of DNA from lizard faecal samples

Genomic DNA was extracted from approximately 0.25 g of the lizard faeces, after aseptically crushing and mixing it well using mortar and pestle (Whiley, McLean & Ross 2017). DNA extraction was carried out using DNeasy® PowerSoil® (Qiagen, Hilden, Germany) following the manufacturer's instructions. The choice to use DNeasy® PowerSoil® kit was made because it produces greater yields of extracted DNA than other kits available in the market (Ariefdjohan, Savaiano & Nakatsu 2010). Briefly, 0.25 g of each lizard faecal sample as prepared above was added to a Power-bead® tube with lysing buffer and centrifuged at 10,000 g for 30 s using an Eppendorf centrifuge. Four hundred microlitres of the supernatant was transferred to a clean 2 mL collection tube, into which 250 µL of C1® solution was added, the tube inverted several times then vortexed for 10 min and thereafter incubated at 4°C for 5 min. The tubes were centrifuged again at 10,000 g for one min. From the supernatant, 600 µL was transferred to a clean 2 mL collection tube where 200 µL of solution C3® was added, the tube vortexed briefly and incubated again at 4°C for 5 min. The tubes were then centrifuged for 1 min 10,000 g. While avoiding the pellet, 750 µL of the supernatant was transferred to a 2 mL collection tube. One thousand two hundred microlitres of solution C4® was added to the supernatant, vortexed for 5 s, then 675 µL loaded into an MB spin® column, and centrifuged for 1 min at 10,000 g. The flow-through was discarded and the step repeated twice. Five hundred microlitres of solution C5® was added to the column, centrifuged for

30 s at 10,000 g, the flow-through discarded, then the MB spin column centrifuged again for 1 minute at 10,000 g. Finally, the MB spin column was carefully placed into a clean 2 mL collection tube and the DNA eluted from the column by adding 100 µL of solution C6 (10 Mm Tris-HCL) onto centre of the white filter membrane, and then centrifuged for 30 s at 10, 000 g. The MB spin column was discarded. The flow-through was the extracted DNA which was stored at -20°C until it was used for the qPCR.

2.2.1. Positive Control

The DNA extraction methodology was validated by using a *Campylobacter*-negative lizard faecal sample, spiked with a *C. jejuni* positive chicken faecal sample DNA, which was provided by the Flinders University Environmental Health laboratory. The lizard faecal sample, to be used as a matrix for the positive control, was sourced from the animal facility at Flinders University. The *C. jejuni* positive chicken sample DNA was added to a 2 mL collection tube, which contained lysis buffer and the crushed lizard faecal sample. Manufacturer's instructions were then followed to extract the DNA using DNeasy® PowerSoil® Kit (Qiagen, Hilden, Germany). The validation was not undertaken for each DNA extraction as the methodology had been previously validated by Whiley, Mclean and Ross (2017).

2.2.2. Negative Control

A *Campylobacter*-negative chicken faecal sample DNA was provided by the Flinders University Environmental Health laboratory for use as a negative control. The negative control contained DNA extracted from poultry faecal sample collected from a farm where routine testing is carried out hence known to be negative for *Campylobacter* spp.

It is necessary to use appropriate PCR controls (positive, negative and non-template control) to ascertain extraction failures and reaction inhibition which may be caused by malfunctioning of the

PCR thermocycler, incorrect PCR mixture or primer, contamination, poor activity of the DNA polymerase enzyme or presence of PCR inhibitory substances in the samples (Lear et al. 2018).

2.3. Polymerase Chain Reaction (PCR)

2.3.1. Primers and Probe

The primer and probe mix were ordered from Bio-Rad as a PrimePCR Assay. They are designed to target *C. jejuni* gene *MapA* (X80135) (Whiley, Mclean & Ross 2017). SsoAdvanced universal probe supermix comes ready-to-use and is optimised for real-time PCR. The probe supermix contains antibody-mediated Sso7d-fusion polymerase, deoxyribonucleotide triphosphate (dnTPs), Magnesium chloride, enhancers, stabilizers and passive reference dyes such as ROX and fluorescein (Bio-Rad Laboratories, CA, USA).

2.3.2. Positive Control (PC) and Negative Control (NC)

The positive control of *C. jejuni* and the negative control that were used for the PCR were provided by the Flinders University Environmental health laboratory, and were prepared as outlined in 2.2.1 and 2.2.2 above respectively. A positive control that has previously been shown to amplify consistently but weakly within an acceptable range should be included in the PCR replicates so as to ascertain the ability of the target DNA to be amplified by the PCR (Lear et al. 2018). A negative control should also be used to confirm the absence of DNA contaminants in the reagent mix (Lear et al. 2018).

2.3.3. *Non-template control (NTC)*

Double sterilized water was used as a non-template control. It was used in place of *Campylobacter* DNA to monitor formation of primer-dimer and control for extraneous nucleic acid contamination or contamination of the mastermix (Qiagen, Hilden Germany 2013).

2.3.4. *PCR Methodology*

The PCR assay was adapted from the Bio-Rad real-time PCR (Bio-Rad laboratories, CA, USA). Previously described primers and probe were used for the qPCR workflow targeting *C. jejuni* gene *MapA* (X80135) (Flekna et al. 2007; Best et al. 2003). All qPCR reactions were prepared by adding all required components as per manufacturer's recommendations (Table 2.1). The assay mastermix was thoroughly mixed then equal aliquots of 15 μ L were dispensed into each PCR vial ensuring good pipetting practices for assay precision and accuracy. These two steps were undertaken in the biosafety cabinet.

Table 2.1: Reagents used for PCR assays (Adapted from the Bio-Rad real-time PCR workflow)

Component	Volume per reaction	Final concentration
PrimerPCR custom assay	1 μ L	1x
1x SsoAdvanced universal probes supermix	10 μ L	1x
cDNA sample	5 μ L	-----
Nuclease free water	4 μ L	-----
Total reaction volume	20 μ L	-----

All PCR vials, except the non-template control, into which 5 µL of double sterilized water had been added, were removed from the cabinet onto the bench. The other preparations included adding to and mixing the mastermix (15 µL) with 5 µL of each of the respective positive control, negative control and extracted DNA samples and were carried out on the bench. Triplicate qPCR was done to detect *Campylobacter jejuni* in the lizard faecal samples. It was demonstrated that there is usually no amplification for other unrelated *Campylobacter* strains and only a very small percentage (0.1%) of samples has been reported to be positive for *C. jejuni* and *C. coli* (Best et al. 2003).

DNA amplifications via PCR were performed at 20 µL reaction volume containing 1 x SsoAdvanced universal probes supermix (Bio-Rad, Gladesville, NSW, Australia), a 300 nM forward primer, a 300 nM reverse primer, 100nM probes and the 5 µL of sample DNA as described by Whiley, McLean & Ross (2017). The primers and probes used for the real-time PCR assay for identification of *Campylobacter jejuni* are as shown (Table 2.2).

Table 2.2: Primers and probes used for the real-time PCR assay for the identification of *Campylobacter jejuni*

Species	Primers and probes	Target gene	References
<i>C. jejuni</i>	5'-CTGGTGGTTTTGAAGCAAAGATT-3' 5'-CAATACCAGTGTCTAAAGTGCGTTTAT-3' 5'-FAM TTGAATTCCAACATCGCTAATGTATAAAAAGCC CTTT-3' TAMRA	<i>C. jejuni</i> gene <i>mapA</i> (X80135)	Whiley, McLean & Ross 2017

The cycling conditions were as follows; initial hold was for 3 minutes at 95°C, followed by 40 cycles at 95°C for 15 s and 60°C for 30 s (Table 2.3). There was a non-template control, a negative control and a positive *C. jejuni* control in all the PCR runs.

Table 2.3: Thermal cycling conditions for qPCR (Adapted from Bio-Rad thermal cycling protocol)

Real-time PCR system	Setting/scan mode	Polymerase activation & DNA denaturation	Amplification		
			Denaturation	Annealing/extension + plate read at 60°C	Cycles
Corbett Rotor-Gene 6000 machine	Fast	95°C for 3 min	95°C for 15 s	60°C for 30 s	40

The following chapter discusses the results which were obtained following the triplicate qPCR of the neat DNA extract.

3.0. RESULTS

This chapter identifies and analyses the results obtained from the DNA extraction, test for PCR inhibitors and the quantitative PCR conducted for the sixty lizard faecal samples.

3.1. Detection of *Campylobacter jejuni* in lizard faeces by qPCR

This current study investigated presence of *C. jejuni* in sleepy lizards (*T. rugosa*) faeces in South Australia. A total of 60 lizard faecal samples had their DNA extracted and quantitative PCR conducted to detect *C. jejuni*. There were no DNA samples positive for *C. jejuni* out of all the 60 lizard faecal samples that were tested. The fluorescent charts (Figures 3.1-3.4) show amplification for only the positive control while the negative control, non-template control and the 15 samples in each of PCR runs did not amplify as shown in Figures 3.1-3.4.

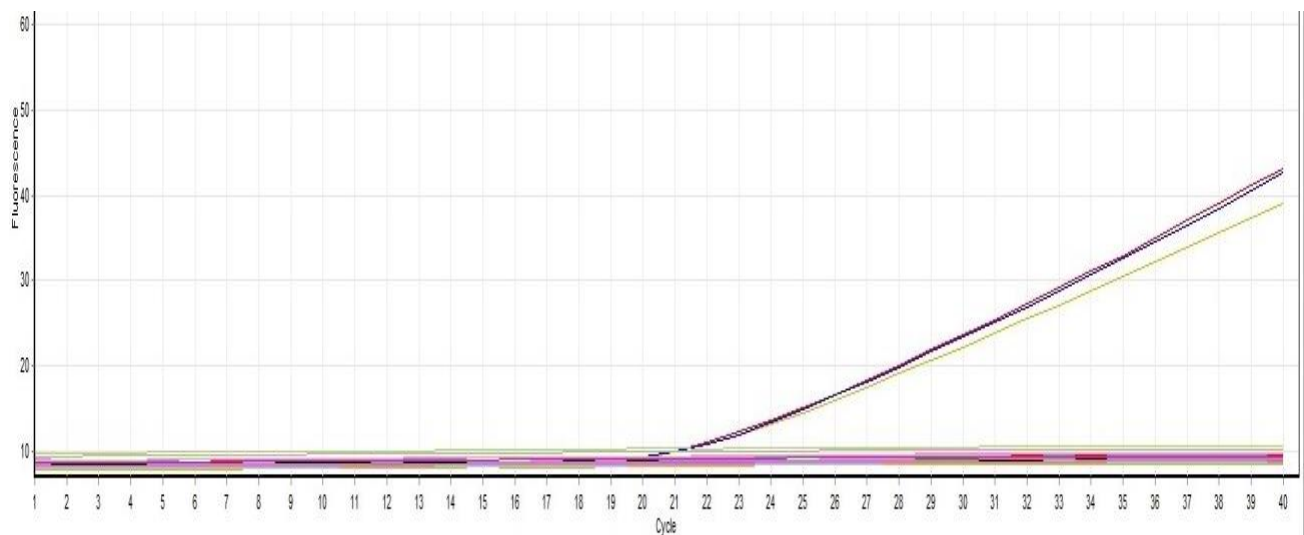


Figure 3.1: Fluorescent chart of 15 lizard faecal DNA (Samples 1-15) (DNA extracted in 2017)

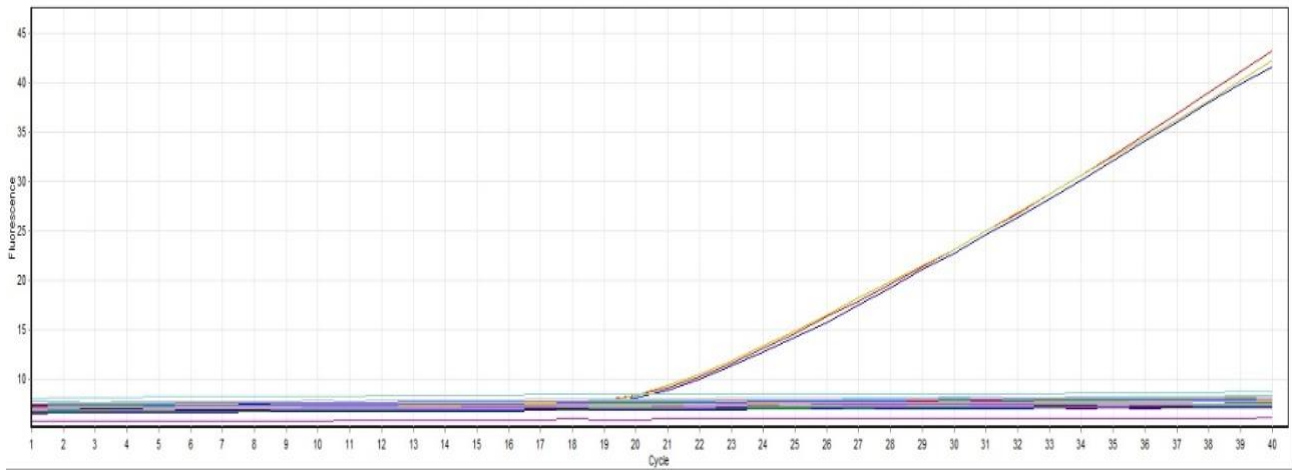


Figure 3.2: Fluorescent chart of 15 lizard faecal DNA (Samples 16-30) (DNA extracted in 2017)

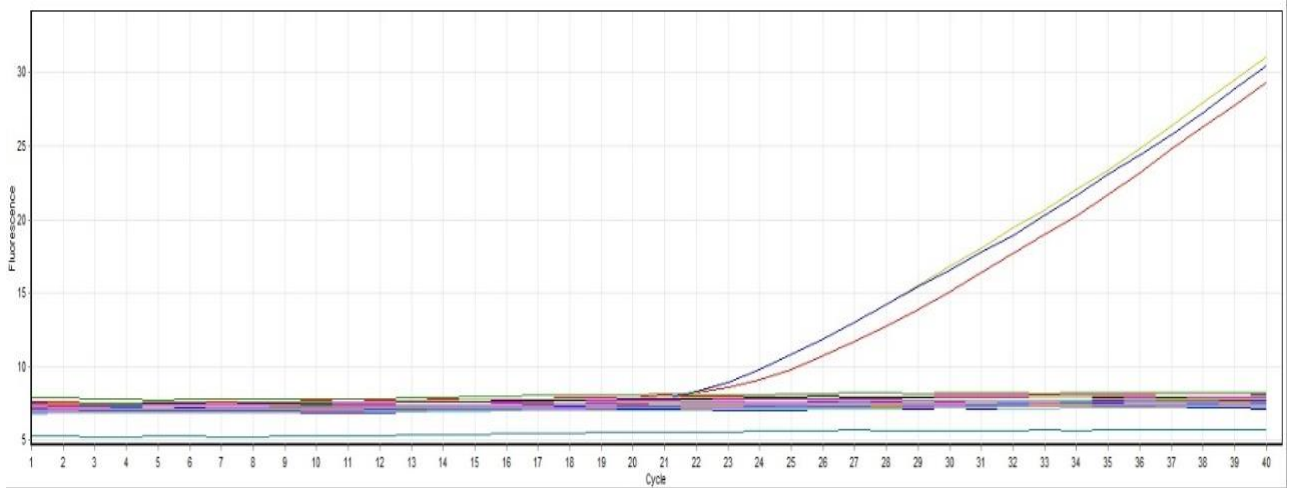


Figure 3.3: Fluorescent chart of 15 lizard faecal DNA (Samples 31-45) (DNA extracted in 2018)

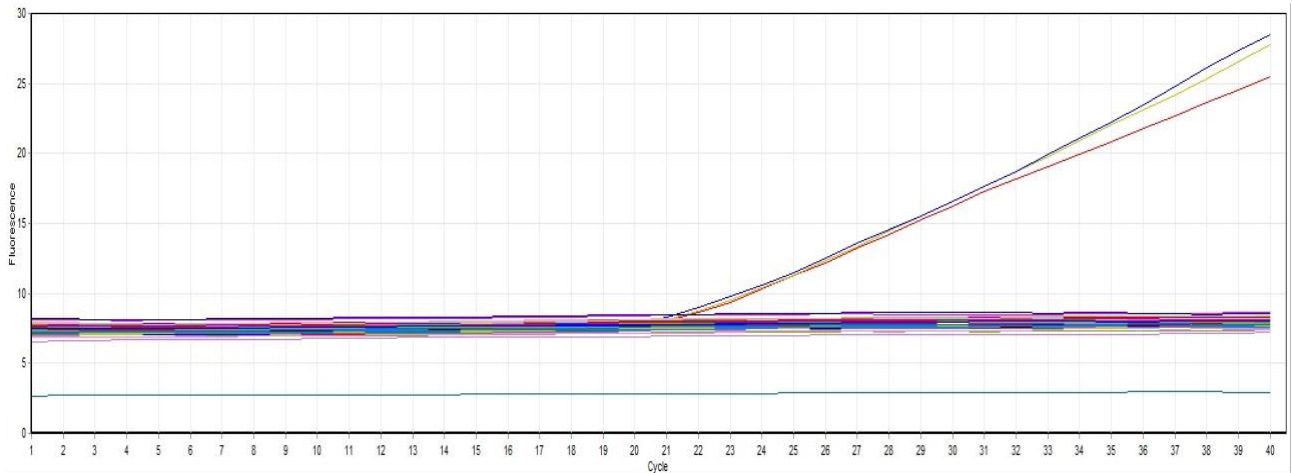


Figure 3.4: Fluorescent chart of 15 lizard faecal DNA (samples 46-60) (DNA extracted in 2020)

3.2. Test for PCR inhibitors

The lizard faecal sample spiked with the *C. jejuni* positive chicken faecal sample had a cycle threshold (Ct) value of 21.0 with undiluted DNA. The Power-bead® tube with C1 solution (10 mM Tris-HCl (PH 8.0), 0.5% SDS, 5mM EDTA) was spiked with the *C. jejuni* chicken faecal DNA without the lizard faecal sample. There were no significant differences observed between the Ct values for the lizard faecal sample spiked with the *C. jejuni* positive chicken faecal sample, and for *C. jejuni* alone thus indicating the tested sample was free of PCR inhibitors. The non-spiked lizard faecal sample did not amplify. Ten-fold and 100-fold dilutions were not carried out. Furthermore, the standard DNA extraction method had previously been validated by Whiley, McLean and Ross (2017).

This chapter has presented results obtained from the quantitative PCR conducted on DNA samples extracted from lizard faeces that were collected from Bundey Bore station. These results are further discussed in the next chapter where they have been put into context using the wider literature.

4.0. DISCUSSION

This chapter presents a short discussion directly relating to the experimental component (section 4.1), and a more general discussion synthesising the review and experiment outcomes (section 4.2-4.9). The general discussion has put the experimental component in the wider context of the molecular techniques applied in *Campylobacter* screening, preservation and storage of lizard faecal samples and the relevance of One Health approach in managing zoonotic diseases such as non-foodborne transmitted campylobacteriosis in Australia.

4.1. Screening of sleepy lizards faecal samples for *C. jejuni* using qPCR

In Australia, only one study (Whiley, McLean & Ross 2017) had previously been done to examine the presence of *C. jejuni* in lizard faeces. In contrast with their study, in the current study, no faecal sample was positive for *C. jejuni*. The confirmed presence of *C. jejuni* in Australian captive and wild lizards in Central Australia by Whiley, McLean and Ross (2017) demonstrates that *Campylobacter* spp. may be spread through environmental sources such as squamates. With lizards being commonly kept as pets in Australia, there are implications for public health risks being spread via pet handling, cross contamination or contact with captive and wild squamate faeces.

The current study involved screening sleepy lizards' faecal samples for *C. jejuni* using quantitative PCR. *Tiliqua rugosa* is a common lizard endemic in Australia and one of the best studied skink species belonging to *Scincidae* family of the squamates (Norval et al. 2019). The known parasites of *T. rugosa* that have already been identified include bacteria, viruses, cestodes, trematodes, nematodes, protozoans and ticks (Norval 2019). *Campylobacter* species has not been reported among the pathogens that were found to have infected the lizard species, however, other studies have reported the *C. jejuni* and *C. fetus* subsp. *testudinum* in other pet and wild lizard species (Whiley, McLean & Ross 2017; Masila et al. 2020). *C. fetus* subsp. *testudinum* was reported in the sleepy lizard in an environmental surveillance study by Gilbert et al. (2016). The non-proximity of

human habitation to the sites where the lizard faecal samples were collected for this current study may explain the absence of *C. jejuni* in sleepy lizards at Bunday Bore station.

4.2. Molecular methods used in testing for *Campylobacter* spp. in squamates

Different molecular methods have been used in testing for *Campylobacter* spp. in reptiles and in determining the genetic relationship between reptilian and mammalian *Campylobacter fetus* strains (Dingle et al. 2010; Hou et al. 2018). Molecular techniques such as quantitative PCR can offer benefits such as speed, sensitivity, greater specificity and reproducibility over culture methods (Al-Habsi et al. 2018). In detection of *C. jejuni*, an added advantage of qPCR over culture is that it can detect viable but non-culturable organisms (Flekna et al. 2007), however culture provides isolates that make it possible to do further analyses on phenotypic characterization or examination of isolate diversity (Gilbert et al. 2014).

Molecular diagnostic techniques currently play a key role in identification of emerging *Campylobacter* subspecies. Some of the methods encountered in the current systematic review of literature included; quantitative PCR for testing lizard faecal samples for *C. jejuni*, 16S ribosomal RNA (rRNA) sequencing, CRISPR (clusters of regularly interspaced short palindromic repeats) typing, Multilocus sequence typing (MLTS), sap insertion PCR, Matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS), serotyping and culturing.

The CRISPR typing tool was successfully used in characterizing isolates of *Campylobacter fetus* subspecies, tracking the source and transmission routes of mammalian *C. fetus* infections (Calleros et al. 2017). CRISPR molecular tool also allowed genotypic differentiation of *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis*. The comparison of *C. fetus* subsp. *fetus* and *C. fetus venerealis* in bovine isolates with *C. fetus* subsp. *testudinum* strains showed striking divergence making it

possible to assess intra-species genetic variability in *C. fetus*. The use of CRISPR sequences therefore enabled determination of the original host of the human infection, which was reptiles.

Secondly, multilocus sequence typing (MLTS) is a useful molecular technique that has been used to characterize closely related strains of reptilian *C. fetus* isolates. In the study by Dingle *et al.* (2010) reptile *C. fetus* isolates were characterized by MLST. It was found that they shared over 90% nucleotide sequence identity with classical mammalian *C. fetus* (*C. fetus* subsp. *venerealis* and *C. fetus* subsp. *fetus*): the reptilian *C. fetus* was thus potentially found to be capable of infecting humans. The MLTS method thus exploits the genetic variation present in several loci to determine the genetic relationship among different *Campylobacter* isolates (Dingle *et al.* 2010).

The 16S rRNA has been found to be better than PCR in identifying species up to species level. However, it cannot be used for differentiating very closely related species such as *C. jejuni* and *C. coli* (Kaakoush *et al.* 2015). 16S rRNA sequencing was successfully used in confirming divergence of *C. iguaniorum* subsp. *nov* from *C. fetus* and *C. hyointestinalis* (Gilbert *et al.* 2015).

Lastly, the Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS), is an emerging diagnostic technology that provides rapid identification of pathogens using intact cells or cell extracts. The process is rapid, sensitive and economical as it does not involve the time-consuming and laborious DNA extraction and purification processes (Singhal *et al.* 2015). It was used in a polyphasic study by Fitzgerald *et al.* 2014 in the USA thus leading to successful isolation of *C. fetus* subsp. *testudinum* subsp. *nov* from a cluster of thirteen *C. fetus*-like strains obtained from humans and reptiles. In another study applying MALDI-TOF MS by Gilbert *et al.* 2015 in Europe, *C. iguaniorum* subsp. *nov* was isolated from five strains of unknown *Campylobacter* genus sampled from lizards and chelonians. A limitation of MALDI-TOF MS is that identification of new isolates of a microbe requires a spectral database that contains peptide mass fingerprints of the type strains of specific genus, species, subspecies or strain being identified (Singhal *et al.* 2015). These molecular diagnostic technologies have not only made it possible to

detect novel subspecies of *Campylobacter* from reptiles but have also enhanced the identification of sources and transmission routes of these emerging *Campylobacter* species in environmental samples.

Molecular diagnostics and improvements in culture media have improved detection of *C. jejuni* and other neglected *Campylobacter* species such as *C. fetus*, *C. lari*, *C. upsaliensis* and *C. concisus* which have been reported to sporadically cause human infections (Patrick et al. 2013; Sheppard et al. 2009). Understanding how these *Campylobacter* species evolve, cause and transmit disease, is vital in developing interventions for control of their spread. This calls for continued application of molecular typing techniques to inform and enhance our understanding of campylobacteriosis epidemiology, transmission routes and intervention strategies (Whiley et al. 2013).

Real-time PCR was used in the current experimental study to screen sleepy lizard faecal samples for *C. jejuni* while MALDI TOF MS, MLST, 16 rRNA sequence typing, multiplex PCR were the other molecular techniques found to have been commonly used in the case studies and environmental surveillance studies analyzed in the literature review. Due to the need for tracing the sources of emerging strains of *Campylobacter* spp., differentiating them from known subspecies, identifying transmission routes of the pathogens and antimicrobial resistant genes, Whole Genome Sequencing (WGS) is a modern molecular technique recommended for use in the surveillance of squamate-linked Campylobacteriosis.

4.3. Preservation and storage of lizard faecal samples for DNA extraction and PCR

Two common challenges faced in DNA extraction are DNA loss through degradation due to long storage and unsuitability for enzymatic manipulation of extracted DNA by PCR (Deuter et al. 1995). These challenges usually arise due to presence of copurified excrements such as bilirubin and bile salts, plant inhibitors from the lizards' diet, DNA from food remains or gut parasites and cells shed from the intestinal lining of the scatting animal. These inhibitors potentially impede the extraction and amplification processes (Deuter et al. 1995; Ramon-Laca et al. 2015). The sample

age, environmental conditions, prolonged and wrong choice of storage or DNA extraction method may thus contribute to DNA degradation hence affecting its quality and quantity for further analysis (Ramon-Laca et al. 2015). This may be solved by substantially reducing these substances from the purified DNA by use of adsorptive matrices and by 1/10 dilutions of the neat DNA extract (Deuter et al. 1995; Whiley, Mclean & Ross 2017).

This subsection highlights the need for testing for PCR inhibitors during DNA extraction, and the importance of appropriate preservation and storage of lizard faecal samples at temperatures of -20°C or -80°C depending on the duration of samples storage. The possible explanation for no detection of *C. jejuni* in the lizards faeces was due to less shedding of pathogens by the lizards because of less disturbance and stress in the wild, as there was no human habitation where most of the lizard scats were collected. The long storage of up-to three years may have also contributed to potential degradation of any faecal sample DNA present. However there have been limited studies done to establish how long extracted DNA or lizard faecal samples take before DNA degradation occurs and on the survival of *C. jejuni* in lizard faeces .

The sample size in the current study was sufficient, however confidence in detecting *C. jejuni* in the 60 samples could be increased by having more samples and from different lizard species. Only one environmental surveillance study was previously done in Central Australia where 51 samples were screened for *C. jejuni* using qPCR. The overall prevalence for *C. jejuni* was 33% (17/51). This included 30% (14/46) faecal samples from unknown wild lizards and 60% (3/5) samples from captive lizards; bearded dragons and western beaked gecko. This gives an indication that the prevalence of *C. jejuni* in the wild sleepy lizards in Bunday Bore station may be very low.

4.4. Campylobacteriosis incidence in Australia

Campylobacteriosis cases continue to rise globally despite concerted efforts to improve food safety standards in developed countries. While campylobacteriosis is typically a foodborne illness, other environmental sources are evident. Twenty seven percent of these non-foodborne outbreaks in

Australia still have unknown transmission routes (Moffatt et al. 2020): this calls for active surveillance of environmental sources of the *Campylobacter* spp. For example, a total of 84 campylobacter outbreaks were reported in Australia in the period 2001-2016 (Moffatt 2020). Data retrieved from the National Register of Enteric and Foodborne Diseases Outbreaks show that 61% (51/84) of the campylobacteriosis outbreaks were foodborne transmitted, mostly by chicken dishes. A quarter of all the 1042 cases related to the outbreaks, occurred in aged-care centres. With regard to non-food outbreaks, 27% (23/84) had unknown transmission routes. Aged care facilities had the highest number of cases 26% (22/84) and 45% (10/22) of cases whose transmission routes were unknown (Moffatt et al. 2020). There have been increasing cases of campylobacteriosis per 100,000 people from 112.3 in 2010, 139.7 in 2015 to 143.5 in 2019. It is thus vital to enhance environmental surveillance so as to identify other zoonotic and environmental sources of campylobacteriosis.

A probable case of zoonotic transmission of campylobacteriosis from a puppy has been reported in an aged-care facility (Moffatt et al. 2020). Puppies and adolescent dogs' carriage and excretion of *Campylobacter* spp. has previously been well demonstrated (Hald et al. 2004). This affirms the need for people with low immunity and the elderly to ensure proper hand hygiene practices and minimal interaction with pets including exotic pets such as lizards and snakes whose potential to spread *Campylobacter* spp. has been confirmed (Masila et al. 2020). Unpasteurized milk accounted for <2% as a foodborne *Campylobacter* transmission vehicle, while water source estimates showed that 11% of cases were related to water contamination (Moffatt et al. 2020). This confirms the need for more active surveillance for these non-foodborne transmission vehicles such as contaminated water, and animals including pet squamates. Appropriate approaches involving environmental health, veterinary and human health sectors need to be promoted. One such collaborative and multi-sectoral global health approach that can help in addressing issues of food safety and zoonotic diseases at the interface of human health, wildlife and the environment is the 'One Health'.

4.5. Evolution of the One Health Approach Globally

The term ‘One Health’ was conceptualized by physician Rudolf Virchow in the 19th century in reference to zoonoses (Lee 2013; Zinsstag et al. 2011). Later in the 1960s, Dr. Calvin Schwabe came up with ‘One Medicine’ in a bid to call for a unified approach in dealing with zoonoses (Brown et al. 2020). In 2004, the Wildlife Conservation Society’s meeting used the term “One World, One Health” based on the twelve ‘Manhattan Principles’ (<http://www.oneworldonehealth.org/>) or interdisciplinary collaboration in a globalized world (Cook, Karesh & Osofsky 2004; Mackenzie 2012). One of the principles, that is relevant to squamates and their links with *Campylobacter* spp., is that the health of wildlife in their habitat is mutually interdependent on the health, and the interactions they have with the livestock and humans surrounding them (Zinsstag et al. 2011). Thereafter continued cooperation among multi-sector experts led to the formation of the ‘One Health Initiative’ bringing together veterinary and human medicine sectors. This cooperation was evident during the Avian Flu conference in Delhi in 2007 where the term ‘One Health’ was formally recognized and used for the first time (Chien 2013). There has been progress in the One Health collaboration with support from the World Health Organization (WHO), International Organization for Animal Health (OIE) and Food and Agricultural Organization (FAO) through a tripartite agreement (WHO 2019; Lubroth 2012). Technical meetings aimed at operationalizing the concept in 2009 and 2010 led to the formation of the One Health Global Network by the European Union One Health Office.

4.6. Implementation of the One Health Approach in Australia

In Australia, One Health concepts and principles were developed during the First International One Health Congress held in Melbourne in 2011 (Jeggo & Mackenzie 2014). With current enhanced awareness of how wildlife reservoirs continue to negatively impact on human health by acting as pathogenic hosts, implementation of One Health initiatives targeting environmental sources of

Campylobacter may enhance public and environmental health outcomes in Australia. For example, an investigation of an outbreak of Q-fever in a goat farm in Australia where a One Health approach was successfully applied, involved a team of animal, environmental and public health professionals (Bond et al. 2016). The interventions implemented to contain the outbreak included serological and molecular studies, evaluation of farming practices, human vaccination, environmental and biosecurity strategies (Bond et al. 2016). The most recent development concerning One Health in Australia has been the establishment of the One Health Special Interest Group by the Public Health Association of Australia (PHAA) in 2017. The PHAA supports policy and strategy development in the implementation of One Health concepts (PHAA 2017).

The One Health Special Interest Group aims to create awareness about collaboration between environment, animal and human health sectors across Australia. While the concept has been acknowledged by the Australian government, there are calls to incorporate One Health approaches into the university curriculum in a variety of courses both at undergraduate and postgraduate level (Reid, MacKenzie & Woldeyohannes 2016). The adoption of the One Health policy statement in October 2017 opened a structured collaboration framework to guide multi-institutional networks and enhance collaboration. The One Health policy statement acknowledges One Health principles, the aims of the special interest group, the challenges likely to be encountered and outlines the steps to be undertaken for effective implementation of the One Health concept in Australia (PHAA 2017).

The approaches that have previously been in use are usually siloed or fragmented, national-based and considering only the health and scientific aspects of diseases (Manlove et al. 2016). With a One Health approach being implemented, the focus of zoonotic disease control would be interdisciplinary, multi-sectoral and include social determinants of health that will be anchored on national and international cooperation. Successful application of One Health approaches in Australia would not only improve zoonotic disease surveillance but also strengthen collaboration

and inter-sectoral preparedness, however, there is need to identify challenges being faced in the implementation of a One Health approach. Understanding disease transmission and inter-sectoral collaboration coupled with political goodwill and environmental considerations are some of the key factors that would greatly help in breaking down barriers and building connections for enhanced public health outcomes (Johnson, Hansen & Bi 2017).

4.7. One Health relevance to *Campylobacter* spp. and squamates

Public health risks arising from human campylobacteriosis spread through squamates exposure is a One Health issue. Cognizant of the relevance of the One Health approach to food safety, zoonoses and antimicrobial resistance, the World Health Organization calls for professionals in relevant sectors to share epidemiological and laboratory information across local, national and international fronts: this will enable implementation of joint responses to public health threats arising from zoonoses (WHO 2017). International trade that involves animals such as reptiles, amphibians and squamates among others, is not only a driver of zoonotic risk but also a concern to international health security. Other drivers for increased incidence of zoonotic diseases such as campylobacteriosis include climate change, urbanization and intensification of agriculture which negatively impact on animal habitats therefore causing increased interactions between animals and humans and potential spill-over of diseases to humans (OIE 2020).

Campylobacter contributes a significant disease burden to Australia despite presence of robust food safety standards. The occurrence of *Campylobacter* outbreaks transmitted via food, contaminated water and other environmental sources such as animals calls for integrated surveillance, involving investigation of other potential environmental sources of *Campylobacter* spp.

Antimicrobial resistance in *Campylobacter jejuni* is an emerging global concern not only in Europe, the USA and United Kingdom but also in Australia. A study in Australian retail products, using Whole genome sequencing, showed that the majority of *Campylobacter* isolates possess

fluoroquinolone-resistant genes (Wallace et al. 2020). Further, genomic analysis showed that resistance to fluoroquinolones, macrolides, tetracyclines, aminoglycoside and beta-lactam range from 2.5% to 15.3%. However, multidrug resistance was very low at <5% although it was 10 times more in *C. coli* than in *C. jejuni* (Wallace et al. 2020). Australian legislation regulating the veterinary use of some particular antibiotics on food-producing animals has reduced the development of antibiotic resistant genes (JETACAR 1999; Australian Government, Department of Health 2003). Antimicrobial resistance in *Campylobacter* spp. thus offers a good example of where a One Health approach is applicable. All the drivers of zoonotic risks revolving around *Campylobacter* spp. and environmental reservoirs put human, animal and environmental health at the forefront in tackling the increasing risk of zoonotic diseases. It thus requires a One Health approach that offers multi-sectoral collaboration, communication, coordination and resource sharing.

4.8. The challenges to One Health implementation

One of the challenges being faced in the implementation of the One Health approach is the emerging “trend of professions moving to specialization and expertise within their own realm rather than in collaboration and cross-discipline” (Mackenzie, McKinnon & Jeggo 2014, p. 163). While specialization is necessary, the pursuit of this trend without acknowledging the need for other disciplines may in the long run pose a serious challenge when a community is faced with zoonotic disease outbreaks whose risk factors and effects require inter-sectoral preparedness to contain them.

Secondly, negative perceptions, invisibility and underrating of some professions, that make significant impacts on the health status of populations may contribute negatively to advancement of the approach. The environmental health profession has often been overlooked and underrated yet it plays a key role in disease prevention (Whiley et al. 2018). It is estimated that emerging and re-emerging diseases account for nearly 73% of zoonoses (Woolhouse & Gowtage-Sequeria 2005). One of the ways to successfully contain these zoonotic diseases is through the application of a One

Health approach which has been shown to provide mechanisms for multisectoral communication, collaboration and coordination to tackle diseases that have a huge impact on public health, the economy, social stability and security of the society (PHAA 2017).

Identifying barriers to collaboration between the sectors most relevant to the One Health approach will enable seamless implementation of an integrated system to manage zoonotic diseases. For example, in the Australian National Notifiable Disease Surveillance System, notification of animal and human diseases differs significantly as they are monitored and managed by different sectors where data sharing and timely communication may be limited or inherently compromised and priorities divergent (Australian Institute of Health and Welfare 2016). While notifications in human communicable diseases directly prioritize disease, infection control and risk of zoonotic disease to humans, animal disease notifications prioritize first and foremost, minimization of adverse impacts on trade (Australian Government Department of Health 2015; 2016). The need for understanding disease transmission, political good will (Johnson, Hansen & Bi 2017), communication and collaboration in addressing these multifaceted and interlinked challenges cannot be over emphasized. Since no single sector can adequately address the intricacies of food safety, zoonoses and antimicrobial resistance that relate to campylobacteriosis, a One Health approach is indispensable and thus recommended.

4.9. Adoption and operationalization of the One Health approach

The One Health policy statement as adopted in 2017 by the Public Health Association of Australia (PHAA) recommends awareness creation as one of the sustainable ways to enhance operationalization of One Health approaches among practitioners and policy makers in the human, animal and environmental sectors in Australia. There are concerted efforts to ensure curriculum development for training and education incorporate One Health concepts so as to improve management of existing and emerging disease threats (PHAA 2017). In addition, the PHAA suggests creation of support frameworks to forge a way forward in networking and collaboration,

leadership, capacity-building and resource mobilization to sustainably operationalize the One Health approach across the diverse disciplines and sectors that are relevant to public and environmental health management.

The development of the One Health paradigm that recognizes human, animal and ecosystem health is key to tackling public health challenges that are inter-sectoral and disease threats that are newly emergent or zoonotic in nature. To better understand and effectively respond to public health needs and enhance networking among diverse disciplines that are relevant to One Health, there is a need to identify cross-sectoral approaches and initiatives that enhance leadership, collaboration, capacity building and resource sharing between human, animal and environmental health sectors.

5.0. CONCLUSION

The presence of *C. jejuni* in lizard faeces has zoonotic potential and public health implications. While the disease is typically foodborne-transmitted, research shows zoonotic transmission through squamates has the potential to pose a public health risk to humans through pet handling and cross-contamination. The increased interest in keeping lizards and snakes as pets in developing countries calls for implementation of approaches that reduce public health risks to humans. Climate change, intensification of agriculture, demand for alternative sources of proteins, urbanization and trends in international trade are some of the drivers of zoonotic risks that may exacerbate shedding of *Campylobacter* spp. and spill-over from squamates to humans as a result of increased interactions. The proximity of human habitation and interactions with animals at the human-wildlife-ecosystem interface, influences potential transmission of pathogens between animals and humans.

Squamates can also play a role in cross-contamination of other environmental sources of human campylobacteriosis. This is particularly a concern with captive squamates which have increased interaction with the built environment. Previous studies show that there is a higher pathogen carriage rate and shedding in captive lizards compared with free-living wild lizards. This may explain the absence of *C. jejuni* in lizard faeces samples collected at Bunday Bore station where there is minimal human interaction with lizards due to low human habitation. A recent systematic literature review by Masila et al. (2020) found only one environmental surveillance study in the Netherlands that reported *Campylobacter* spp. (*C. fetus* subsp. *testudinum*) in sleepy lizards, thus implying that *C. jejuni* may be rare in sleepy lizard (*Tiliqua rugosa*) species.

From the systematic literature review, *C. fetus* subsp. *testudinum* and *C. fetus* subsp. *fetus* were identified as the most frequently isolated species in squamates and the predominate cause of human campylobacteriosis from a squamate host. *C. jejuni* and *C. iguaniorum* were also isolated from

lizard faecal samples and reported to pose potential health risks to humans. The common squamate hosts identified included bearded dragons (*Pogona vitticeps*), green iguana (*Iguana iguana*), western beaked gecko (*Rhynchoedura ornate*) and botched blue-tongued skink (*Tiliqua nigrolutea*). One environmental surveillance study (Whiley, McLean & Ross 2017) reported presence of *Campylobacter jejuni* in lizard faeces collected from Central Australia. This informed the need to carry out surveillance of *C. jejuni* in sleepy lizards in South Australia using quantitative PCR.

People with underlying chronic illnesses, young children below the age of five years, the immunocompromised and the elderly are the most vulnerable populations hence they should observe personal hand hygiene and ensure appropriate but minimal human-animal contact practices. Exposure to pet squamates, wild animals, consumption of reptilian cuisines and cross contamination with untreated water were risk factors associated with *Campylobacter* infections. The findings from this environmental surveillance study as well as the systematic literature review provide insights into the mechanisms and approaches to apply to reduce the spread of *Campylobacter* infections to humans via squamates. Proper hand hygiene practices, responsible pet ownership, ‘One Health’ education and awareness on zoonotic diseases will help reduce the public health risks arising from *Campylobacter* exposure through squamates. Continued surveillance using molecular diagnostic methods will also enhance detection and response to squamate-linked campylobacteriosis.

5.1. Limitations of the study

There are a number of limitations associated with this study. These limitations need to be considered in interpreting the results.

- i. Small sample size: More lizard faecal samples would increase the confidence level and statistical power of the study. It was not possible to collect more lizard faecal samples from the field in time due to COVID-19 restrictions, hence previously collected and preserved lizard faecal samples were used.

- ii. A limitation of the study was that other common lizard species that are found in the wild and also kept as pets could not be investigated, as the only lizard faecal samples that were available to work on were from sleepy lizards.
- iii. The long duration of preservation of the lizard faecal samples (two to three years) may have contributed to possible degradation of the faecal samples. While presence of PCR inhibitory substances was tested and confirmed to be eliminated by the standard DNA extraction protocol, all the sixty samples whose DNA was extracted were found to be negative for *C. jejuni* through qPCR. Due to time constraints, it was not possible to design another control to measure the effect of lizard faecal DNA extraction on template amount of DNA.
- iv. The assumption was that all precautions were taken during lizard faecal sample collection, transportation to the laboratory, appropriate preservation and storage. Some studies recommend that for long storage of samples over several years, they should be kept at -80°C. The 60 samples that were used had been preserved and stored for up to three years at -20°C. It was not determined whether the long storage could have led to DNA degradation by comparing the preserved samples with recently collected faecal samples, as this was hampered by the COVID-19 conditions.
- v. As the scope of this study was to determine only the presence or absence of *C. jejuni* in the faecal samples from sleepy lizards, and not to quantify the *Campylobacter* spp. DNA, serial dilutions were not performed to determine the limit of detection.

5.2. Future research

Due to paucity of data on epidemiology of *Campylobacter* spp. in lizards and snakes, and limited prior research studies on human campylobacteriosis cases associated with pet and wild squamates, there is need for further research on this topic to inform better strategies to reduce potential public health risks to humans. The following study areas are therefore suggested for further research in order to fill the knowledge gaps that have been identified.

- i. Characterization of the epidemiology and ecology of zoonotic *Campylobacter* spp. through investigating prevalence and genotype diversity in diverse lizard populations.
- ii. Investigation of eco-epidemiology of *C. jejuni* and *C. coli*, prevalence, genotype diversity and potential animal contamination of various surface water types such as wastewater for agricultural use, recreational water, surface water at discharge points of wastewater treatment plants, in different seasons so as to inform better management strategies for prevention, surveillance and response to *Campylobacter* spp. in human-animal-ecosystem interfaces.
- iii. Analysis for isolate diversity and phenotypic characteristics of *Campylobacter* spp. by obtaining the isolates through culturing.
- iv. Assess the effect of environmental factors such as temperature, humidity and rainfall on survival of *Campylobacter jejuni* in lizard faeces.
- v. Establish the appropriate environmental conditions, especially temperature, that limits degradation of *Campylobacter* DNA during long periods of storage, through preservation and storage of lizard faecal samples or the extracted DNA in different environmental conditions.
- vi. Identifying the significance of *C. jejuni*-contaminated lizard faeces and the implications for horizontal transmission in food production farms via lizards and other reptiles.
- vii. Examine how lizard captivity affects the odds of pathogen/parasite infection in pet lizards in comparison with the free-living/wild lizard species in Australia so as to establish whether or not, and how, host conservation threatens parasites.

- viii. A scoping review on successful One Health case studies involving collaborations of public health, environmental health, veterinary and agricultural research in managing increasing risk of foodborne zoonoses such as campylobacteriosis and salmonellosis in Australia.

5.3. Community engagement

Community engagement is vital in research, for informing policy and practice, as well as engaging and getting feedback from beneficiaries, stakeholders and potential consumers of the information that has been generated by research in order to address issues that affect the well-being of the people.

The author of this thesis is an invited poster presenter at the “Developing Northern Australia Conference” on 23-25 November 2020 at Pilbeam theatre, Rockhampton. The presentation will summarize key findings of the published review paper, <https://doi.org/10.3390/pathogens9100799> on zoonotic and public health implications of *Campylobacter* spp. associated with both pet and wild squamates, and the relevance of One Health approaches in prevention, surveillance and response to squamate-linked campylobacteriosis. The submitted abstract entitled “Repurposing One Health Approach for indigenous-led development: Zoonotic and public health implications of *Campylobacter* spp. associated with pet and wild squamates” was categorized under indigenous-led development. The need for community engagement and One Health education in tackling increasing risk of emerging zoonotic diseases will thus be highlighted in the presentation.

The author also participated in the National Public Health think-tank competition 2020, an initiative of the Students and Young Professionals in Public Health Committee (SYPPH) of the Public Health Association of Australia. The submission titled “One Health approach key in tackling increasing risks of zoonotic diseases” was selected for publication in the Croakey Health Media – a social journal for health initiatives. The link to the publication is: <https://www.croakey.org/one-health-approach-key-in-tackling-increasing-risk-of-zoonotic-diseases/>.

These submissions reaffirm the need to disseminate research findings through conferences and the media to raise awareness on emerging public and environmental health threats to the community so as to aid in informing policy and/or practice.

5.4. Concluding observations

The experiments conducted for this study were to identify *Campylobacter jejuni* in lizard faeces collected from one of the common lizard species in Bunday Bore station, South Australia. None of the lizard faecal samples was positive for *C. jejuni*. A systematic literature review was done to determine and understand the current state of knowledge about squamate-linked campylobacteriosis globally. It was important to collate literature evidence on trends in *Campylobacter* spp. spread via squamates, and the exposure routes because campylobacteriosis cases are increasing globally and there is more interest by people in keeping lizards and snakes as pets. It was confirmed that squamates are reservoirs of *Campylobacter* spp. and this has zoonotic and public health implications for humans. Handling of pets, consumption of contaminated water, eating reptilian cuisines and poor hand hygiene were identified as risk factors for human campylobacteriosis. Although the percentage of squamate-linked campylobacteriosis was low, people with low immunity, the elderly, those with underlying illnesses and young children were identified as the most vulnerable populations. It was noted that people who are at greatest risk of contracting the disease were unaware of the risk factors associated with human-animal contact practices and poor hand hygiene after handling pets. Effective approaches for educating the general public and coordinated interventions by the human, animal and environmental health experts through a One Health approach should be implemented to reduce public health risks that may be acquired from pet and wild squamate hosts.

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