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The Epidemiology and Chemoprevention of Canine Vector-Borne Pathogens in the Asia-Pacific Region Using Novel Molecular Diagnostics

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The Epidemiology and Chemoprevention of Canine Vector-Borne Pathogens in the Asia-Pacific Region Using Novel Molecular Diagnostics

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Abstract

Canine vector-borne pathogens (VBPs) are a diverse range of arthropod-transmitted organisms that encompass viruses, bacteria, protozoa and multicellular metazoan parasites. Together they can generate a spectrum of disease in dogs from relatively benign infections to ones that can be fatal, whilst some are also zoonotic and therefore represent a concurrent risk to humans. Although canine VBPs have been extensively studied in Europe and North America there is substantially less relevant information on these pathogens in the tropics, particularly across the Asia-Pacific. The potential for novel and emerging VBPs to surface from such regions is great, whilst international travel and the movement of pets and livestock may facilitate a global spread of such novel diseases, as has been aptly demonstrated by the ongoing COVID-19 pandemic.

Taking this into consideration, my PhD project set out to firstly optimise a novel next-generation sequencing (NGS) based diagnostic for the holistic characterisation of canine bacterial, apicomplexan and kinetoplastid VBPs. Following validation of my method it would be employed to unbiasedly characterise pathogen communities from stray dogs across Cambodia - a country suspected of being hyperendemic for such diseases based on limited prior data. Building upon these findings I then aimed to use our broad-scope NGS data to design a new high-throughput and economical qPCR diagnostic for detection of the common canine VBPs in the region. Such a diagnostic would then be utilisable within the context of a field efficacy trial, to compare the VBP-blocking efficacy of a contemporary ectoparasiticide collar product against a topical ectoparasiticide typically used in the region.

Initial design, testing and optimisation of my NGS metabarcoding method identified it as being more sensitive for the detection of canine VBP than conventional PCR (cPCR) and better able to detect rarer/unexpected pathogens (Chapters 2 and 3). With respect to the detection of bacterial VBPs my method was further augmented in Chapter 4 by the development of a canine DNA blocking primer that reduced the amount of extraneous amplification and sequencing of host DNA and increased the amount of bacterial pathogen DNA detected, thereby increasing my method's sensitivity.

Next, extensive fieldwork and sampling of stray and semi-domesticated dogs from across five Cambodian study sites, followed by use of my novel NGS method identified a diverse range of hyperendemic canine VBPs (Chapter 5). Across all sites 62% of dogs were infected with at least one confirmed VBP with *Anaplasma platys*, the causative agent of infectious cyclical thrombocytopenia, the most common at 32%, followed by *Ehrlichia canis* the agent of the often lethal canine monocytic ehrlichiosis in 20% of dogs. Other key pathogens identified included *Hepatozoon canis* (18%), *Babesia vogeli* (14%), *Mycoplasma haemocanis* (13%), the zoonotic pathogen *Bartonella clarridgeiae* (3%), *Candidatus Mycoplasma haematoparvum* (0.2%) and

Trypanosoma evansi (0.2%) Critical metadata was also collected and analysed which identified the presence of ectoparasites, abnormal mucous membrane condition, anaemia and elevated total protein as significant risk indicators for canine VBP exposure; information that will be key for veterinarians working in the region that may have limited resources to molecularly diagnose dogs.

Subsequently, information gleaned through my NGS-based survey and prior studies permitted the identification of the most prevalent canine VBPs in the Asia-Pacific. Using such data we designed and validated a novel Taq-Man based multiplex qPCR to rapidly and economically test for such pathogens from canine blood samples, in Chapter 6.

Finally, to explore measures for reducing canine VBP transmission within the Asia-Pacific a 12-month Cambodian field efficacy trial was commenced to compare the efficacy of a long-acting ectoparasiticide collar (Seresto®, Bayer) against a monthly application of topical fipronil, a product commonly used throughout the region (Chapter 7). Despite this trial not reaching completion within the duration of my PhD, initial 6-month data suggests that both products are highly effective at blocking the transmission of canine VBPs. Moreover, information accrued at baseline suggests that topical ectoparasiticide products are better than systemic ones, such as the isoxazolines, at preventing VBP infection in dogs; a critical piece of information for veterinarians in the tropics.

Overall, my thesis has garnered data and employed techniques in contexts never previously conducted. For example, I was the first to use NGS metabarcoding to detect pathogens from canine blood and my research has been the first to molecularly identify the canine VBPs *A. platys*, *T. evansi* and *B. clarridgeiae* from Cambodian dogs. Moreover, to the best of my knowledge there has not been such a country-wide epidemiological survey of bacterial and protozoan VBPs using metabarcoding for any host animal to date. I envision that the data and methods presented within this thesis will provide researchers with a critical baseline understanding of common and rare canine VBPs within the Asia-Pacific, whilst also generating valuable tools for future epidemiological surveys that can be employed in a wide range of contexts, not solely restricted to diagnosis in canine populations. Such tools will assist in unpicking the complex interplay between VBPs infecting companion animals, wildlife and humans, allowing researchers to better comprehend transmission cycles, emerging zoonotic threats and the importance of a One Health approach to managing our environment and future public health risks.

Declaration

This is to certify that the work described in this thesis is original work conducted by the author and predominantly performed at the Faculty of Veterinary and Agricultural Sciences in The University of Melbourne between February 2018 and September 2021. Due acknowledgement has been made in the text where other materials have been used or occasional assistance received. The thesis is less than 100,000 words in length, exclusive of tables, figures, references and appendices. No part of this thesis has been submitted for any other degree or diploma.

.....

Lucas Huggins

Date: 10/09/2021

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Publications and Presentations

The publications, conference proceedings and seminars resulting from this thesis are listed:

Peer-reviewed research papers

Huggins L.G., Koehler A.V., Ng-Nguyen D., Wilcox S., Schunack B., Inpankaew T., Traub R.J., 2019. Assessment of a metabarcoding approach for the characterisation of vector-borne bacteria in canines from Bangkok, Thailand. *Parasit. Vectors* 12, 394.

Huggins L.G., Koehler A.V., Ng-Nguyen D., Wilcox S., Schunack B., Inpankaew T., Traub R.J., 2019. A novel metabarcoding diagnostic tool to explore protozoan haemoparasite diversity in mammals: A proof-of-concept study using canines from the tropics. *Sci. Rep.* 9, 12644.

Huggins L.G., Koehler A.V., Schunack B., Inpankaew T., Traub R.J., 2020. Optimal DNA extraction combined with a host-specific blocking primer improves the detection capability of a metabarcoding protocol for canine vector-borne bacteria. *Pathogens* 9, 258.

Huggins L.G., Massetti L., Schunack B., Colella V., Traub R.J., 2021. Novel high-throughput multiplex qPCRs for the detection of canine vector-borne pathogens in the Asia-Pacific. *Microorganisms* 9, 1092.

Huggins L.G., Colella V., Koehler A.V., Schunack B., Traub R.J., 2021. A multipronged next-generation sequencing metabarcoding approach unearths hyper-diverse and abundant dog pathogen communities in Cambodia. *Transbound. Emerg. Dis.* 1-18.

Conference presentations and seminars delivered

Huggins L.G., 2018. Next-generation sequencing based characterisation of canine vector-borne diseases in Cambodian dogs. *Annual Conference of the Australian Society for Parasitology*, Melbourne, Australia. 24.09.18.

Huggins L.G., 2018. Next-generation sequencing based characterisation of canine vector-borne diseases in SE Asian dogs. *Veterinary Science Postgraduate Symposium*, Melbourne, Australia. 29.11.18.

Huggins L.G., 2019. Characterisation and chemoprevention of tropical canine vector-borne diseases in SE Asia. PhD confirmation: Faculty of Veterinary and Agricultural Sciences, University of Melbourne, Australia.14.02.19.

Huggins L.G., 2019. A highly sensitive next-generation sequencing (NGS) approach uncovers unusual haemoparasites from Thai dogs. *Annual Conference of the Australian Society for Parasitology*, Adelaide, Australia. 11.07.19.

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List of Abbreviations

Common abbreviations used throughout this thesis are listed below, they are also introduced in full at their first usage in each chapter.

12S rRNA	Mitochondrially encoded 12S ribosomal RNA
16S rRNA	16S ribosomal RNA; part of the 30S subunit in prokaryotic ribosomes
18S rRNA	18S ribosomal RNA; part of the 40S subunit of eukaryotic ribosomes
3'-spacer C3	Three carbon spacer element attached to 3' end of oligonucleotide
AIC	Akaike information criterion
ASV	Amplicon sequence variant
BB	Battambang; field site location
BC	Buffy coat; DNA extraction kit type by Promega
BI	Bayesian inference
BL	Bioline: DNA extraction kit type by Bioline
BLASTn	Basic local alignment search tool; in nucleotide database
bp	Base pair
BP	Blocking primer
CAPCT	Companion Animal Parasite Council for the Tropics, now TroCCAP
CBC	Complete blood count
CCT	Cyclical canine thrombocytopaenia
CI	Confidence interval
CIRD	Canine infectious respiratory disease
CL	Cutaneous leishmaniasis
CMAC	Cambodian Mine Action Centre; Southeast Asian collaborator
CME	Canine monocytic ehrlichiosis
COVID-19	Coronavirus disease 2019
COX1	Cytochrome c oxidase I; main subunit of the oxidase complex
CP	Control product; in our study topical fipronil (DeTick)
cPCR	Conventional PCR
Cq	Quantitation cycle
CSD	Cat scratch disease
CVBD	Canine vector-borne disease
DNA	Deoxyribonucleic acid

EDTA	Ethylenediaminetetraacetic acid
EHV	Equine herpes virus
FAD	Flea allergy dermatitis
FBSF	Flea-borne spotted fever
ftsZ	Filamenting temperature-sensitive mutant Z gene
GABA	Gamma-aminobutyric acid; inhibitory neurotransmitter
gB	Glycoprotein B
gBlock	Gene blocks; synthetic DNA fragments
gltA	Citrate synthase gene
GPS	Global positioning system
gyrB	DNA gyrase subunit B gene
IgG	Immunoglobulin G; commonest antibody type found in circulation
ITS	Internal transcribed spacer; region between ribosomal RNA subunits
IVP	Investigational product; in our study Seresto [®] collars
KC	Kampong Chhnang; field site location
LNA	Locked nucleic acid
MCMC	Markov chain Monte Carlo
MERS	Middle East respiratory syndrome
MIC	Magnetic induction cyclers
mtDNA	Mitochondrial DNA
NCBI	National Center for Biotechnology Information
NEG	Negative
NGS	Next-generation sequencing
NNTB	Number needed to treat for benefit
NNTH	Number needed to treat for harm
NPA	Norwegian People's Aid; collaborating fieldwork organisation
ompB	Outer membrane protein B gene
ONT	Oxford Nanopore Technologies
OR	Odds ratio
OTU	Operational taxonomic unit
PCR	Polymerase chain reaction
PCR-RFLP	Restriction fragment length polymorphism polymerase chain reaction
PCV	Packed cell volume
POS	Positive

PP	Phnom Penh; field site location
Q30	Quality score of 30; high quality score for NGS read data
QG	Qiagen; DNA extraction kit type by Qiagen
qPCR	Quantitative or real-time PCR
R^2	Correlation coefficient
R1	Read 1 i.e. the forward read in paired end sequencing.
R2	Read 2 i.e. the reverse read in paired end sequencing.
RNA	Ribonucleic acid
Rnase P	Ribonuclease P
ROC	Receiver operating characteristic
SARS	Severe acute respiratory syndrome
SARS-Cov-2	Severe acute respiratory syndrome coronavirus-2
SD	Standard deviation
SE	Standard error
SE Asia	Southeast Asia
secY	Subunit of the bacterial Type II secretory pathway gene
SFG	Spotted fever group
SID	Semil in die, meaning once per day
SOLiD	Sequencing by oligonucleotide ligation and detection
SR	Siem Reap; field site location
SRA	Sequence Read Archive
ssrA	Transfer-messenger RNA gene
TG	Typhus group
TK	Tbong Khmum; field site location
TroCCAP	Tropical Council for Companion Animal Parasites
V4	Hypervariable region 4. V1 and V6 refer to the first and sixth
VBD	Vector-borne disease
VBP	Vector-borne pathogen
VL	Visceral leishmaniasis
VSG	Variable surface glycoprotein
WB	Whole blood; DNA extraction kit type by Promega
χ^2	Chi-squared; statistical test

**Chapter 1 - The epidemiology and chemoprevention of
canine vector-borne pathogens in the Asia-Pacific region
using novel molecular diagnostics**

1.1. Introduction

The modern dog (*Canis familiaris*) was domesticated from the wolf (*Canis lupus*) and has lived in close association with humans for some 12,000 years (Mencke, 2013). Initially employed for guarding and hunting (Driscoll et al., 2009), now dogs predominantly cohabit with people as pets and are well recognised for the benefits they provide to their owners, including important contributions to their physical and mental well-being as well as associations with low blood pressure and reduced stress (McConnell et al., 2011; Ormerod et al., 2005; Robertson et al., 2000).

Today, due to growing popularity, dog ownership is increasing with some of the highest rates seen in nations such as the US where 23.6% of the populace own a dog, some 77 million, (McConnell et al., 2011) followed by Brazil at 17%, or roughly 37 million (Dantas-Torres and Otranto, 2014). Countries in the Asia-Pacific have followed the trend with Australia exhibiting an exceptionally high rate of pet ownership at approximately 53% (Palmer et al., 2008) of which 3.4 million are dogs (Irwin, 2014). At the same time, pet ownership in China has grown by as much as 20% over a 5-year period from the start of the new millennium, starting a trend that has not abated (Chan et al., 2017). Nonetheless, quantification of the number of dogs within a country is not limited to those that are owned alone. Many countries, particularly in developing nations, have large populations of stray dogs; for example, India is predicted to have at least 20 million (Traub et al., 2014), whilst in Thailand an estimated 9.8% of all dogs, roughly 600,000, are strays (Liu et al., 2016).

A plethora of vector-borne pathogens (VBPs) that cause vector-borne diseases (VBDs) exploit these expanding canine populations, using haematophagous arthropods, such as ticks, fleas, mosquitoes and biting flies, amongst others, to be transmitted from host to host (Beugnet and Marié, 2009; Wall and Shearer, 1997). The prevalence of a particular VBP is strongly influenced by the effects climate and geography exert on their respective vectors, however, a number of common pathogenic families and genera can be found worldwide (Beugnet and Marié, 2009; Pfäffle et al., 2013).

Some of the most widespread are alphaproteobacterial VBPs in the families Rickettsiaceae and Anaplasmataceae, including many species in the genus, *Rickettsia* and a few in the genera *Ehrlichia* and *Anaplasma* (Parola et al., 2013; Rar and

Golovljova, 2011). These intracellular bacterial parasites can cause some of the most damaging VBP infections in canine hosts, such as canine monocytic ehrlichiosis (Kaewmongkol et al., 2017) or anaplasmosis (Carrade et al., 2009). Other bacterial genera outside of the alphaproteobacteria can also cause significant disease in dogs, these include an increasingly recognised number of haemotropic *Mycoplasma* species (Compton et al., 2012) as well as members of the genera *Bartonella* and *Borrelia*, the latter being the causative agent of Lyme disease (Chomel et al., 2006; Krupka and Straubinger, 2010).

Protozoan apicomplexan parasites have also long been recognised as the generators of a spectrum of disease in canids, ranging from relatively benign, asymptomatic infections through to rapidly fatal ones (Irwin, 2009; Ivanov and Tsachev, 2008). Piroplasmids such as *Babesia* species infect host erythrocytes and are transmitted by a variety of tick vector species, inducing haemolytic anaemia in their host to varying degrees of severity (Irwin, 2009; Vial and Gorenflot, 2006). In contrast, apicomplexans of the *Hepatozoon* genus are not transmitted via arthropod feeding but instead due to ingestion of the vector. *Hepatozoon* infections are often only associated with severe disease when coinfecting a dog host with other VBPs or if the dog is immunosuppressed due to treatment for other illness, e.g. steroids such as prednisolone have been linked to *H. canis* re-activation and high parasitaemia (Baneth et al., 2003; Ivanov and Tsachev, 2008). All of these apicomplexan parasites have wide global distributions and are found on all continents, excluding Antarctica (Irwin, 2009; Ivanov and Tsachev, 2008; Otranto et al., 2009a).

Unlike the VBPs mentioned thus far, filarial nematode parasites are predominantly transmitted by highly motile volant mosquito vectors, as opposed to ticks or fleas (Megat Abd Rani et al., 2010). The filarial worm *Dirofilaria immitis* is a particularly common parasite of dogs in Asia, The Americas and an expanding range in Europe, exerting a large toll on their canine hosts, causing fatal cardiocirculatory disease or respiratory failure (Baneth et al., 2016; Dantas-Torres and Otranto, 2013; Frangipane di Regalbono et al., 2016; Genchi and Kramer, 2017).

Amongst the spectrum of pathogens that dogs can acquire, some of the most prevalent and damaging can also be transmitted to people, i.e. are zoonotic (Baneth et al., 2016). Ectoparasites that commonly infest dogs or wild animals can feed on

humans to obtain a blood meal, thus providing an opportunity for parasites using sylvatic or peri-domestic hosts to spill over into human populations (Dantas-Torres and Otranto, 2014; Pfäffle et al., 2013). For example, the brown dog tick, *Rhipicephalus sanguineus* sensu lato, can build up large infestations within kennels or homes, increasing the likelihood of human attachment and feeding (Boost et al., 2017; Gray et al., 2013; Jongejan et al., 2016). *R. sanguineus* can transfer the potentially deadly pathogen *Rickettsia rickettsii* the causative agent of Rocky Mountain Spotted Fever in the US or *Rickettsia conorii* an equivalent pathogen, causing Mediterranean Spotted Fever in Southern Europe, the Middle East and India (Parola et al., 2013). Some of these *Rickettsia* species have been shown to have fatality rates as high as 32% depending on strain and local access to healthcare (Mencke, 2013).

In addition, there has been growing recognition of the VBP *Rickettsia felis* as an emergent zoonosis, since its first identification in a human patient in 1990 (Beugnet and Marié, 2009). *R. felis* is transmitted by the cat flea, *Ctenocephalides felis* and alike to *Rickettsia conorii* uses dogs as a reservoir host (Levin et al., 2012; Ng-Nguyen et al., 2020; Reif and Macaluso, 2009). A mounting number of human *R. felis* infections have been observed across the globe due to the ubiquitous prevalence of its vector with this pathogen in humans producing the multi-systemic disease Flea-Borne Spotted Fever (FBSF) responsible for flu-like symptoms, myalgia and malaise (Reif and Macaluso, 2009; Teoh et al., 2016; Williams et al., 2011).

Akin to its severity in dogs, some *Ehrlichia* species can also invoke severe pathogenesis in humans (Rar and Golovljova, 2011). Whilst dogs are predominantly affected by the species *Ehrlichia canis*, they can also become infected with the potently zoonotic species *Ehrlichia chaffeensis* the aetiological agent behind human monocytic ehrlichiosis (Little, 2010). Cases are most common within the US with 4,545 cases reported from 2003 to 2010 although there are increasing reports of infection in other continents too (Rar and Golovljova, 2011). *Anaplasma phagocytophilum* is another important zoonotic agent that can be harboured by dogs which exhibit non-specific clinical signs, such as lethargy, fever and inappetence when infected (Chirek et al., 2018; Fourie et al., 2019b). When transmitted by *Ixodes* ticks to people, human granulocytic anaplasmosis is generated that can manifest as severe symptoms including dyspnoea, vomiting amongst others and can even be fatal (Kolo et al., 2020; Lee et al., 2020).

Finally, dogs are also known to be key reservoirs of potentially zoonotic filarial worm infections (Genchi and Kramer, 2017). The filarial species *Brugia malayi*, *Dirofilaria immitis* (heartworm) and the nodule worms *Dirofilaria repens* and *Dirofilaria hongkongensis* can all produce long-term infection in dogs and be transmitted zoonotically, with human exposure to these parasites correlating with their prevalence in local canine populations (Ambily et al., 2011; Frangipane di Regalbono et al., 2016; Petry et al., 2015; Smout et al., 2017; Thanchomnang et al., 2010; Yilmaz et al., 2016). Whilst, *D. immitis* parasitosis is relatively rare in humans, *D. repens* is regarded as an emergent zoonosis, causing subcutaneous dirofilariasis, ocular complications and eosinophilia in people, whilst *D. hongkongensis* can also be zoonotic (Frangipane di Regalbono et al., 2016; Genchi and Kramer, 2017; Megat Abd Rani et al., 2010; Xing et al., 2020). Areas over which *D. repens* infections are found are now understood to be expanding, moving out of previously confined hotspots such as Southern Europe and into Northern Europe, with recent research now identifying it within Asia as well (Genchi and Kramer, 2017; Petry et al., 2015; Yilmaz et al., 2019).

Given the large breadth of VBPs that can be transmitted from canines to people research focus is needed to elucidate the roles dogs play in maintaining parasite reservoirs and how they facilitate the spill over of VBPs from sylvatic cycles to humans (Colwell et al., 2011; Pfäffle et al., 2013). To combat VBDs a 'One Health' approach is needed, recognising that veterinary treatment, surveillance and communication between medics and public health sectors is key, due to the potential for transmission between animals and people (Dantas-Torres and Otranto, 2016, 2014). This gives dogs an important role as sentinels of zoonotic risk, particularly as VBP prevalence changes in the modern world, signifying the need for veterinarians to keep abreast of updated best practice guidelines and report unusual cases of infection outside of typically endemic zones (Irwin, 2014; Traub et al., 2015).

1.2. Prominent ectoparasites and vector-borne pathogens of canines in the Asia-Pacific

1.2.1. Canine ectoparasites of the Asia-Pacific

Arthropods exhibit staggering levels of diversity, with some estimates attributing 80% of all known species to this phylum (Wall and Shearer, 1997). A considerable portion of the Arthropoda have adopted an ectoparasitic haematophagous existence, obtaining blood meals from vertebrate hosts either obligately for survival and development or facultatively e.g. for egg production (Wall and Shearer, 1997). These traits make them excellent vectors of disease, in particular the Acari (ticks and mites), Siphonaptera (fleas), Phthiraptera (lice), Culicidae (mosquitoes), Phlebotominae (sand flies) and Simuliidae (black flies) (Dantas-Torres and Otranto, 2016; Wall and Shearer, 1997). Together these vectors inflict a huge burden on human morbidity and mortality as well as on that of companion animals and livestock (Conlan et al., 2011; Dantas-Torres and Otranto, 2016).

Hard ticks in the family Ixodidae are vectors of an exceptionally numerous quantity of different bacteria and protozoa and transmit some of the most lethal canine VBPs to dogs (Bonnet et al., 2014; Jongejan et al., 2016). Ixodid ticks show specific morphological and behavioural adaptations to their ectoparasitic lifestyle, such as detection of CO₂, temperature, humidity and chemoreception via their Haller's organ as well as questing behaviour to increase their chances of contact with a host (Pfäffle et al., 2013; Wall and Shearer, 1997). *R. sanguineus* "tropical lineage" which was newly recognised in 2021 as *Rhipicephalus linnaei*, according to mitochondrial genome sequencing, is likely the principal ixodid species infesting dogs in Southeast Asia (Kaewmongkol et al., 2017; Šlapeta et al., 2021). All four life stages of this tick use dogs as a host, meaning ectoparasite populations can build up without seeding events from sylvatic cycles (Gray et al., 2013; Irwin and Jefferies, 2004). This species also vectors many canine VBPs, such as *Babesia* spp., *Hepatozoon canis*, *E. canis* and *Anaplasma platys*, amongst others (Inpankaew et al., 2016; Koh et al., 2016; Megat Abd Rani et al., 2010).

A few other ixodid species also play an important role as disease vectors in the Asia-Pacific, including *Rhipicephalus haemaphysaloides*, *Ixodes* spp. *Dermacentor auratus* and *Haemaphysalis* spp., although these are not as widespread as *R. linnaei* across

the region (Colella et al., 2020; Inpankaew et al., 2016; Irwin and Jefferies, 2004; Šlapeta et al., 2021). Not only is there a wide diversity of tick vectors in the Asia-Pacific, but also a high abundance, even found infesting pet dogs. A recent study by Colella et al. (2020) found ticks on 22.3% of client owned dogs across eight Southeast Asian nations with prevalence ranging from highs of 67.5% in dogs in the Philippines to lows of 4.4% in Malaysia. Prevalence of fleas was less than that of ticks at 14.8% of dogs sampled across all countries investigated (Colella et al., 2020).

Flea species, such as *C. felis*, are as common throughout the Asia-Pacific as they are globally (Irwin and Jefferies, 2004). Nonetheless, this is not the only flea species found in the region, with others such as *Ctenocephalides canis*, *Ctenocephalides orientis* and *Xenopsylla cheopis* also having been found at differing prevalence (Colella et al., 2020; Hii et al., 2015a; Phoosangwalthong et al., 2018).

Like many tick species, fleas have evolved to live intimately with their hosts. Flea eggs initially laid on the host's body are shed into its environment; flea larvae develop in nesting materials or carpet and a localised population quickly builds up (Wall and Shearer, 1997). Not only can flea infestation cause Flea Allergy Dermatitis (FAD) in dogs but they can also transmit and maintain infections, such as *R. felis*, *Bartonella* spp. and *Dipylidium caninum* (Chomel et al., 2006; Irwin, 2014; Kho et al., 2017; Ng-Nguyen et al., 2020; Reif and Macaluso, 2009).

Mosquitoes are also important vectors of canine disease across the Asia-Pacific and easily transmit pathogens from dog hosts to people (Genchi and Kramer, 2017; Smout et al., 2016). For example, mosquitoes can spread the filarial worm *D. immitis* which is prevalent in wild, stray and domestic canine populations throughout regions of Northern Australia (Smout et al., 2017), Cambodia (Inpankaew et al., 2016) and Malaysia (Koh et al., 2016). In addition, filarial worms of the genus *Brugia* which can cause zoonotic lymphatic filariasis have been found to be transmitted within countries such as Malaysia, Thailand and India (Ambily et al., 2011; Chirayath et al., 2017; Ravindran et al., 2014; Thanchomnang et al., 2010). Mosquito species in the *Aedes*, *Culex* and *Anopheles* genera are some of the principle vectors of filarial VBPs and are dependent on static waterbodies for reproduction, thereby strongly tying the effect of local climate on the prevalence of these filarial diseases (Carrade et al., 2009; Smout et al., 2016).

1.2.2. Bacterial canine vector-borne pathogens of the Asia-Pacific

1.2.2.1. *Anaplasma platys*

Anaplasma spp. are close relatives of *Ehrlichia* spp., that phylogenetically lie alongside them in the Anaplasmataceae family (Carrade et al., 2009). Like *Ehrlichia* spp. they are gram-negative obligately intracytoplasmic bacteria, transmitted by ticks such as *R. sanguineus*, *R. linnaei* or *Dermacentor* spp. in Asia (Carrade et al., 2009; Little, 2010). *A. platys* intracellularly infects host platelets and, in most hosts, can cause a chronic and cyclical thrombocytopaenia that can begin as early as seven days post-infection. Clinical signs of infectious canine cyclic thrombocytopaenia caused by this agent include weight loss, pale mucous membranes (indicative of anaemia), lethargy, fever and bleeding tendencies, whilst it is also commonly found as a coinfection with *E. canis* and *Babesia vogeli* (Gaunt et al., 2010; Hii et al., 2012; Little, 2010; Rar and Golovljova, 2011; Sainz et al., 2015).

Both *A. platys* and the closely related *Anaplasma phagocytophilum* can infect and cause anaplasmosis in dogs, however, it is *A. phagocytophilum* that is considered the principal zoonotic agent of these two species - capable of producing a non-specific febrile illness in people (Carrade et al., 2009; Little, 2010). Human granulocytic anaplasmosis is typically self-resolving within two months, although in rare cases it can cause complications, such as toxic shock and become fatal (Carrade et al., 2009). In addition, there are a few reports of *A. platys* infections in humans; one case of two symptomatic women in Venezuela (Arraga-Alvarado et al., 2014) and others where clinical signs were either absent or coinfections meant they could not be unequivocally confirmed, despite *A. platys* DNA being found in patients' blood samples (Breitschwerdt et al., 2014; Maggi et al., 2013).

Treatment of canine anaplasmosis is relatively simple with clinical improvement visible in as little as 24 to 48 hours using doxycycline, whilst treatment duration must be at least two to four weeks (Gaunt et al., 2010). However, whether complete clearance of the infection can be achieved, without possibility of future chronic relapse in dogs is debated (Carrade et al., 2009; Gaunt et al., 2010).

In Australia *A. platys* has been found in canine hosts over multiple studies including in 27% - 36% of community dogs from Indigenous communities in Queensland and the Northern (Hii et al., 2012; Shapiro et al., 2017). In Southeast Asian studies, PCR

detection has found *A. platys* at a prevalence of 4.4% in Thailand (Liu et al., 2016), whilst there is serological evidence of *A. platys* in canines from Vietnam (13.3%), the Philippines (17%), Indonesia (11.7%), Singapore (2.6%) and Malaysia (11.1%) as well as in Samoan dogs at a prevalence of 8.4% (Carslake et al., 2017; Colella et al., 2020).

1.2.2.2. *Bartonella* spp.

Bartonella spp. are gram-negative alphaproteobacteria of the family Bartonellaceae that infect a wide range of animal hosts, including humans (Boulouis et al., 2005). Both fleas and ticks transmit *Bartonella* spp. although fleas are recognised as the major vector of the key zoonotic *Bartonella* species, *Bartonella henselae* (Chomel et al., 2006). These haemotropic bacteria invade erythrocytes and endothelial cells causing an intracellular bacteraemia and vasoproliferative lesions (Álvarez-Fernández et al., 2018; Boulouis et al., 2005). Cats are the main reservoir of the zoonosis *B. henselae* the aetiological agent of Cat Scratch Disease (CSD), capable of producing fever, malaise and neurological complications in immunocompetent people as well as severe fever and endocarditis in the immunocompromised (Álvarez-Fernández et al., 2018; Boulouis et al., 2005; Breitschwerdt et al., 2010; Chomel et al., 2006). Nonetheless, there is growing recognition that dogs harbour and suffer from a variety of other *Bartonella* spp. that may be of zoonotic significance, such as *Bartonella vinsonii berkhoffii* which was found in febrile dogs in Thailand (Chomel et al., 2004; Irwin and Jefferies, 2004).

Within Australia there has been little research directly demonstrating the existence of *Bartonella* spp. infection in dogs apart from one report of a *B. henselae* infection in a 'Blue Heeler' (Shapiro et al., 2017). Across Southeast Asia there has been more applicable research, for example, a Thai study elucidated prior exposure to *Bartonella* in 38% of canines tested using serological methods (Suksawat et al., 2001). Furthermore, there are numerous researchers that have detected *Bartonella* spp. within canine ectoparasites across the region. *Bartonella clarridgeiae* was detected in 4.7% of *Ctenocephalides orientis* fleas infesting dogs in Lao PDR (Kernif et al., 2012) and 19.1% of fleas infecting dogs and cats in Malaysia (Mokhtar and Tay, 2011). A *Bartonella* genus specific real-time PCR identified *Bartonella* DNA in the ectoparasites

of canines and felines from Indonesia, Malaysia, the Philippines, Thailand and Vietnam (Foongladda et al., 2011; Nguyen et al., 2020). Such data may indicate that *Bartonella* spp. use canines as infection reservoirs across various nations in the Asia-Pacific.

For treatment of *Bartonella* spp. in canines there has been almost no research done, although the use of lipid-crossing antibiotics such as doxycycline, amoxicillin and azithromycin, have demonstrated success in some case reports from dogs and cats (Álvarez-Fernández et al., 2018; Berkowitz et al., 2016; Boulouis et al., 2005; Rolain et al., 2004). In humans, exact treatment depends on the immunocompetency of the patient, however, antibiotics such as azithromycin, ciprofloxacin and rifampicin have been demonstrated to reduce the severity and duration of infection (Chomel et al., 2004; Rolain et al., 2004).

1.2.2.3. *Ehrlichia canis*

E. canis is an alphaproteobacterial VBP in the Anaplasmataceae family, it uses ticks as a vector and as an important infection reservoir as the bacteria can be passed down via transstadial transmission to each instar (Rar and Golovljova, 2011). *E. canis* is an intracellular pathogen targeting canine monocytes and dividing within internal vesicles until host cell rupture occurs (Jongejan et al., 2016; Rar and Golovljova, 2011). This bacterium can produce an acute febrile disease with lethargy, anorexia and bleeding that lasts between 1-4 weeks, after which most dogs initially control the infection and become outwardly asymptomatic (subclinical phase). This is typically followed by a chronic phase some months or years later where the dog relapses into a severe condition with clinical signs reflective of a pancytopenia, hyperglobulinaemia and hypoalbuminemia including pallor, epistaxis, petechiae, haematuria, lameness, ventral oedema, neurological manifestations and secondary infections; this phase of the disease is often fatal (Fourie et al., 2013a; Little, 2010; Mylonakis et al., 2019). *E. canis* has only infrequently been reported as a zoonotic pathogen with reports of people in Venezuela and Costa Rica found to have been symptomatically infected, as verified by molecular methods (Bouza-Mora et al., 2017; Perez et al., 2006). However, the closely related *E. chaffeensis* is a known human pathogen that can be harboured by

dogs and cause a potentially fatal infection in people with hospitalisation rates during *E. chaffeensis* infection as high as 60% (Little, 2010; Rar and Golovljova, 2011).

E. canis is common in many Asia-Pacific countries, for example it was detected via PCR done in the blood of 9.8% of anaemic dogs (Kaewmongkol et al., 2017) and 3.9% of asymptomatic dogs in Thailand (Liu et al., 2016) as well as 21.8% of dogs in Cambodia (Inpankaew et al., 2016). Using serological detection indicating either previous exposure to *E. canis* or current infection 39.5% of dogs across Peninsular Malaysia had seroconverted to produce *E. canis* reactive antibodies (Koh et al., 2016). Prior to 2020, *E. canis* was not detected in PCR-based cross-sectional studies investigating VBPs in Australian dogs (Hii et al., 2015b; Mason et al., 2001; Shapiro et al., 2017). Nonetheless, worryingly since May 2020 a growing number of reports have found *E. canis* in various locations across Western Australia, the Northern Territory and South Australia with high case fatality rates (Australian Government, 2020).

Canine *E. canis* infection is treated via a course of doxycycline for a minimum of 28 days, although incomplete clearance and future relapse has been demonstrated after such therapy (Jongejan et al., 2016; Little, 2010).

1.2.2.4. Haemotropic *Mycoplasma* spp.

Mycoplasma spp. are a genus of bacterial pathogen that lack cell walls in the class, Mollicutes (Chalker, 2005). They are common parasites of mammals, birds and reptiles and are unculturable, resulting in a significant dearth of research into their biology (Compton et al., 2012).

Transmission of haemotropic *Mycoplasma* species remains a heavily debated topic given that conclusive evidence of arthropod transmission has never been demonstrated, although it is suspected given that the DNA of canine pathogenic species is commonly found within fleas and ticks (Millán et al., 2020; Soto et al., 2017). Nonetheless, other routes of transmission have been implicated for some haemotropic *Mycoplasma* species, for example vertical transmission or iatrogenic transmission through blood transfusion (Millán et al., 2020; Nury et al., 2021). Lots of studies have demonstrated the wide host range of many of such mycoplasmas providing insight into

how these pathogens are maintained in nature, whilst also facilitating transmission into wild and domestic dog populations (Cabello et al., 2013; Millán et al., 2019; Soto et al., 2017).

Haemotropic *Mycoplasma* spp. are unusual in that they colonise the surface of canine erythrocytes as opposed to intracellularly infecting cells, causing anaemia and weight loss in immunocompromised dogs, although they are not typically associated with significant disease in healthy hosts (Chalker, 2005; Soto et al., 2017). *Mycoplasma haemocanis* was the first canine infecting species to be identified, nonetheless, since 2004 *Candidatus Mycoplasma haematoparvum* has also been recognised as a globally prevalent and important pathogen of dogs (Chalker, 2005; Compton et al., 2012).

Haemotropic *Mycoplasma* spp. can be effectively treated using doxycycline and are not thought to be a substantial zoonotic risk, although there have been cases where haemotropic *Mycoplasma* DNA has been isolated from people (Chalker, 2005; Maggi et al., 2013).

Both canine infecting haemotropic *Mycoplasma* spp. have been found in Cambodia at a prevalence of 9.9% for *M. haemocanis* and 2.9% for *Candidatus M. haematoparvum* (Inpankaew et al., 2016), whereas a study in Thailand found haemotropic *Mycoplasma* spp. to be the most prevalent VBP of stray dogs at a 19.9% prevalence (Liu et al., 2016). Both haemotropic *Mycoplasma* spp. have also been found in Australia (Hii et al., 2015b; Shapiro et al., 2017).

1.2.2.5. *Rickettsia felis* and other significant *Rickettsia* spp.

The Rickettsiaceae is a large family of obligately parasitic, intracellular gram-negative bacteria of which the genus *Rickettsia* is the most diversified and ubiquitous (Cowan, 2000; Parola et al., 2013). *Rickettsia* are close relatives of other canine VBP genera such as *Ehrlichia* and *Anaplasma*, that together partly comprise the order Rickettsiales (Reif and Macaluso, 2009). *Rickettsia* are broadly categorised into four assemblages; the spotted fever group (SFG), the typhus group (TG), the *Rickettsia bellii* group and the *Rickettsia canadensis* group depending on the type of disease they produce and their comparative genomics (Parola et al., 2013; Piotrowski and Rymaszewska, 2020).

R. felis has typically been considered a member of the SFG group and is a prominent global zoonosis, transmitted by *C. felis* fleas and causing the febrile illness FBSF in humans (Hii et al., 2011a; Inpankaew et al., 2016; Reif and Macaluso, 2009). *R. felis* was experimentally shown to be persistently maintained within its *C. felis* vector for up to twelve generations via transovarial and transstadial transmission, without any need for horizontal transmission from a blood meal (Wedincamp and Foil, 2002).

Despite the early recognition of *C. felis* as the principal invertebrate reservoir of *R. felis* a definitive mammalian host has taken a considerable period of time to be conclusively identified (Hii et al., 2011a, 2013). Recently, a study by Ng-Nguyen et al. (2020) has provided substantial evidence for the role domestic dogs play as the vertebrate reservoir of *R. felis*, demonstrating that dogs may exhibit prolonged periods of rickettsaemia in the blood with few clinical signs, thereby facilitating horizontal transmission to uninfected fleas and maintaining this pathogen's life cycle (Hii et al., 2011a; Ng-Nguyen et al., 2020; Teoh et al., 2016).

In humans, *R. felis* infection mostly manifests as non-specific flu-like symptoms but in some cases may cause severe pathology, including maculopapular rashes, malaise, fever, myalgia and in some cases severe multi-systemic disease (Hii et al., 2011a; Teoh et al., 2016). Fortunately, human rickettsiosis by *R. felis* can be successfully treated using a course of doxycycline (Botelho-Nevers et al., 2012).

R. felis is an important pathogen across the Asia-Pacific due to its zoonotic potential (Irwin and Jefferies, 2004). In Northern Cambodia *R. felis* was detected in 10.9% of dogs as elucidated by PCR (Inpankaew et al., 2016), whilst in Malaysia a serological study found exposure to *R. felis* in 16.1% of farm workers and 22.5% of people in Indigenous communities (Koh et al., 2016). Whilst not explicitly demonstrating canine or human infection a report by Kernif et al. (2011) found *R. felis* within ectoparasites from domestic dogs and other hosts, at levels as high as 76.6% in Laos and 74.4% in Borneo.

In Australia, the prevalence of *R. felis* has been more thoroughly documented, with one study detecting it in 9% of pound dogs in Brisbane (Hii et al., 2011a) and another in 2.3% of dogs in an Indigenous community in the Northern Territory (Hii et al., 2011b). Moreover, Hii et al. (2013) demonstrated *R. felis* seropositivity in 50.7% of dogs sampled over Southeast Queensland and the Northern Territory indicating prior

exposure to this VBP. Cases of human infection and disease by *R. felis* have also been reported in Australia, including a family from Melbourne who acquired flu-like illnesses after adopting flea infested kittens. The aetiological agent responsible was retrospectively identified as *R. felis* (Teoh et al., 2016). Additionally, a serological study by Teoh et al. (2017) found evidence of *R. felis* exposure as high as 16% in Australian veterinarians, highlighting the zoonotic risk of working with animals that are potentially infested with *R. felis* vectors.

Apart from *R. felis* there is some limited research on the role of other *Rickettsia* species as relevant human pathogens in the Asia-Pacific (Irwin and Jefferies, 2004). Species found in the region previously include *Rickettsia australis*, *Rickettsia conorii* subsp. *Indica*, *Rickettsia heilongjiangensis*, *Rickettsia helvetica*, *Rickettsia honei*, *Rickettsia japonica*, *Rickettsia raoultii*, *Rickettsia rickettsii*, *Rickettsia* spp. genotype RF2125, *Rickettsia tamurae*; although only some of these species have been found associated with canine and human infections (Kho et al., 2016; Phoosangwalthong et al., 2018; Piotrowski and Rymaszewska, 2020).

Specific relevance of these other *Rickettsia* species to humans and dogs has been demonstrated in Thailand where canine seroreactivity to *Rickettsia prowazekii* (24%), *Rickettsia rickettsii* (12%) and *Rickettsia canadensis* (4%) antigens was demonstrated, although the ability for serology to accurately identify to species level in this case was debated by the authors (Suksawat et al., 2001). Many other studies have isolated different *Rickettsia* spp. DNA from tick ectoparasites in the region, of which some were infesting dogs, in Lao PDR (Kernif et al., 2012), Bangkok (Foongladda et al., 2011), the Thai-Myanmar border and Vietnam (Parola et al., 2003). There is also evidence of human tick-borne rickettsial infection in Southeast Asia, as Kho et al. (2017) found seropositivity to *R. conorii* at a range of 3.3% to 50% in different communities on the Peninsular Malaysia.

1.2.3. Protozoan and metazoan canine vector-borne pathogens of the Asia-Pacific

1.2.3.1. *Babesia* spp.

Babesia is a genus of protozoan parasite in the order Piroplasmida, that phylogenetically nestle beside the *Theileria*, a closely related group of parasites that

commonly infect a variety of other domestic animals, e.g. cattle (Cassini et al., 2009; Irwin, 2009). They are predominantly transmitted by ticks but can also be spread by blood transfusion, with evidence of the species *Babesia gibsoni* also being transmissible via dog fighting and *in utero* to offspring (Irwin, 2009; Jefferies et al., 2007b; Muhlnickel et al., 2002). Tick species known to vector *B. gibsoni* include *Haemaphysalis bispinosa* and *Haemaphysalis longicornis*, whilst the brown dog tick is the most common vector of *B. vogeli* (Muhlnickel et al., 2002; Penzhorn, 2020).

At the point of vector blood-feeding *Babesia* sporozoites are inoculated into the host, they attach to host erythrocytes, penetrating these cells and dividing until cell membrane integrity is lost causing oxidative injury and haemolysis, particularly in the spleen (Irwin, 2009; Solano-Gallego and Baneth, 2011). Such pathophysiology can lead to poor prognosis depending on the *Babesia* species involved, the host's age, immune status and presence of concurrent VBPs (Rawangchue and Sungpradit, 2020; Schetters et al., 2009). Clinical signs encompass but are not limited to haemolytic anaemia, pyrexia, pallor, splenomegaly and hypoxic injury (Irwin, 2009; Mittal et al., 2019; Vial and Gorenflot, 2006). *B. vogeli* infection can be asymptomatic or cause relatively mild disease except in puppies under the age of 12 weeks and in older immunocompromised dogs or those coinfecting with other VBPs, for which such infections can be fatal (Cardoso et al., 2008; Solano-Gallego and Baneth, 2011). *B. gibsoni* is moderately pathogenic, capable of causing disease and mortality even in immunocompetent adult dogs (Muhlnickel et al., 2002; Solano-Gallego and Baneth, 2011). Some *Babesia* species have zoonotic significance for instance *Babesia microti* and *Babesia divergens* can be fatal if transmitted to humans, however, these do not typically infect canines (Irwin, 2009; Vial and Gorenflot, 2006).

Both *B. vogeli* and *B. gibsoni* are known to infect dogs in regions of the Asia-Pacific, although *B. gibsoni* is more focal in its distribution (Irwin and Jefferies, 2004; Muhlnickel et al., 2002). *B. gibsoni* has been sporadically reported in Australian dogs, for example in American Pit Bull Terriers in the state of Victoria (Jefferies et al., 2007b; Muhlnickel et al., 2002), whilst *B. vogeli* has been identified as being more widely distributed across Australia with between 5 - 44% of dogs infected in the tropics and sub-tropics (Barker et al., 2012; Hii et al., 2015b; Shapiro et al., 2017). Across the Asia-Pacific more generally, *Babesia* has also been found at high prevalence via molecular methods, for example *B. vogeli* in 32.7% of Cambodian dogs (Inpankaew

et al., 2016), but also at lower levels, such as 8% of stray dogs in Thailand (Do et al., 2021). In addition, *B. gibsoni* has been detected molecularly in 6.3% of dogs from Thailand (Do et al., 2021) 2.3% of dogs in China and 0.9% of dogs from Singapore (Colella et al., 2020).

Treatment of infection by *B. vogeli* can be achieved via two doses of imidocarb dipropionate at 5 - 7mg/kg given two weeks apart, whilst *B. gibsoni* infection cannot be cured using the same protocol (Irwin, 2009; Jongejan et al., 2015). Treatment of this infection is more challenging with improvement of clinical signs observed after use of clindamycin, metronidazole and doxycycline in combination as well as buparvaquone or atovaquone and azithromycin used concurrently (Jefferies et al., 2007c; Kirk et al., 2017).

1.2.3.2. *Dirofilaria immitis*

D. immitis is a filarial nematode parasite, commonly known as heartworm, that is transmitted by Culicidae mosquito vectors (Genchi et al., 2001; McCall et al., 2008). L3 larvae are transmitted from mosquito to host during a blood meal, they initially migrate to the host's abdomen and by day 70 post-infection localise in their final infection site; the pulmonary arteries or chambers of the heart (Kotani and Powers, 1982; Kume and Itagaki, 1955; Newton, 1968). Here the *D. immitis* larvae develop into adults after a pre-patency period of between six to nine months, reproducing and releasing microfilariae into the host's bloodstream which can then be picked up by a new mosquito vector, thus perpetuating the lifecycle (Kotani and Powers, 1982; Taylor, 1960). Symptoms of cardiopulmonary dirofilariasis in canines typically progress in severity from asymptomatic infection through to cough and exercise intolerance and then eventually death owing to severe pulmonary disease and congestive right-sided heart failure (Dillon et al., 1995; Frangipane di Regalbono et al., 2016; Strickland, 1998). Caval syndrome and haemolysis may also develop associated with exacerbation of clinical signs to include icterus and haemoglobinuria (Jackson, 1975; Strickland, 1998). *D. immitis* is a rare zoonosis that can lead to the formation of pulmonary granulomas and occasional ocular infections (Avellis et al., 2011; McCall et al., 2008; Mirahmadi et al., 2017).

D. immitis is common throughout the Asia-Pacific and has been best documented in multiple studies in Australia finding it at rates as high as 72.7% in wild dingoes (Smout et al., 2016), and 75% of wild dogs surrounding Townsville (Brown and Copeman, 2003). In Southeast Asia *D. immitis* was detected in the blood of 15.8% of Cambodian dogs via PCR (Inpankaew et al., 2016) and by serology in 46.8% of Samoan dogs (Carslake et al., 2017). Microfilaremia can be effectively treated in dogs using a single dose of a macrocyclic lactones, such as moxidectin, however established adult worms are harder to treat and require adulticidal treatment using melarsomine dihydrochloride or, although not recommended, a 'slow-kill' regime combining a macrocyclic lactone and doxycycline (Bazzocchi et al., 2008; Bowman et al., 2017; Grandi et al., 2010).

1.2.3.3. *Hepatozoon canis*

Hepatozoon species, like *Babesia*, are protozoan apicomplexan parasites that use ticks as vectors for transmission (Ivanov and Tsachev, 2008). They belong to the suborder Adeleorina with as many as 300 species currently recognised, predominantly as blood parasites of reptiles, birds and mammals (Ivanov and Tsachev, 2008). Unlike most VBPs *H. canis* is not transmitted when the arthropod vectors feeds but instead when a host animal ingests the tick vector, whilst transplacental transmission can also occur (Baneth et al., 2003; Little et al., 2009). Once digestion of the tick begins, oocysts in the tick body release sporozoites into the dog's gastrointestinal tract, these then disseminate via the lymph and circulatory systems, targeting hepatocytes or leukocytes to invade and divide in (Baneth et al., 2003; Ivanov and Tsachev, 2008). Symptoms of *H. canis* infection in dogs are generally moderate causing fever, lethargy and anaemia but in worse cases can cause life-threatening emaciation (Ivanov and Tsachev, 2008; Liu et al., 2016). There is as yet no report of any *Hepatozoon* spp. causing infection in people (Dantas-Torres and Otranto, 2016).

H. canis has been reported in dogs across the Asia-Pacific including in Northern Cambodia at a prevalence of 10.9% (Inpankaew et al., 2016) and in Thailand at a prevalence of 18.8% (Liu et al., 2016). Prior to 2018 Australia was considered free of this pathogen possibly due to strict importation laws and screening (Chalada et al., 2018; Hii et al., 2015b; Shapiro et al., 2017). Nonetheless, *H. canis* has now been identified via blood smear and PCR in an Australian dog with no prior history of

international travel, leading to the possibility that this species is already somewhat established within the country (Greay et al., 2018a).

Therapy for *H. canis* involves fortnightly subcutaneous or intramuscular injections of imidocarb dipropionate for as long as 8 weeks to suppress pathology and potentially clear the infection (Ivanov and Tsachev, 2008).

1.2.3.4. *Leishmania* spp.

Leishmania spp. are one of the most important zoonotic vector-borne pathogens globally with approximately 0.2 – 0.4 million human cases of the highly pathogenic visceral leishmaniasis (VL) and 0.7 – 1.2 million cases of cutaneous leishmaniasis (CL) reported annually (Conlan et al., 2011; Okwor and Uzonna, 2016). *Leishmania* parasites are flagellated protozoans that phylogenetically reside within the kinetoplastida class as close relatives of the *Trypanosoma*, another genus with important VBP species of mammals (Dantas-Torres, 2007). *Leishmania infantum* is the most important canine-infecting *Leishmania* species from a zoonotic perspective, as dogs are competent reservoirs of *L. infantum* and where infection levels are high VL in human populations is typically endemic (Dantas-Torres, 2007). Clinical signs in dogs infected with *L. infantum* range from critically ill signalment, including anaemia, lymphopenia and monocytopenia that can be fatal, through to entirely asymptomatic infections (Nicolato et al., 2013). The latter category may explain why this pathogen is so hard to control, particularly when VL is tackled in human populations with a high prevalence of pet dogs (Coura-Vital et al., 2011).

Our understanding of the prevalence of *Leishmania* species across the Asia-Pacific is complex and based on limited research. Canines found serologically positive to *L. infantum* have been identified in Vietnam and the Philippines, whilst in China, molecular techniques have also identified this species (Colella et al., 2020). The zoonotic species *Leishmania siamensis* and *Leishmania martiniquensis* have been detected in Thailand, but no data to date has identified a role for dogs in local transmission of these VBPs (Conlan et al., 2011; Leelayoova et al., 2017; Wiwanitkit and Wiwanitkit, 2015).

Leishmaniasis treatment in dogs can be conducted via various protocols, with the most common being a combination therapy of meglumine antimoniate and allopurinol for one to two months (Oliva et al., 2010). Other effective treatments include amphotericin

B, pentamidine and aminosidine with course durations typically lasting at least one month (Oliva et al., 2010).

1.2.3.5. *Trypanosoma evansi*

Trypanosoma spp. are flagellated protozoan organisms also belonging to the Kinetoplastida class, of which many species are important pathogens of humans and animals (Aregawi et al., 2019; Carnes et al., 2015). *T. evansi* has a particularly large host range for one species, capable of infecting a diverse spectrum of mammals, including dogs, livestock (where it causes the disease ‘surra’) and humans (Aregawi et al., 2019). This pathogen is transmitted mechanically by biting fly vectors, particularly tabanid flies, although oral transmission of infected meat has also been reported (Bui et al., 2021; Panigrahi et al., 2015). Trypomastigote stages of *T. evansi* reside in the host’s bloodstream and extravascular fluids, where they multiply producing waves of parasitaemia that generate a strong immune response that is in part responsible for this VBP’s pathogenicity (Defontis et al., 2012; Habila et al., 2012). Clinical signs of *T. evansi* infection in dogs include pyrexia, anaemia, lethargy, oedema, pancytopenia, corneal opacity and uveitis, with late stage disease almost invariably fatal as the pathogen invades the dog’s central nervous system (Bui et al., 2021; Defontis et al., 2012; Habila et al., 2012; Panigrahi et al., 2015; Rjeibi et al., 2015). *T. evansi* also demonstrates some zoonotic potential with infrequent reports of human infections where it causes moderate pathology, such as fever (Van Vinh Chau et al., 2016; Vanhollebeke et al., 2006).

There have been occasional reports of canine *T. evansi* infections across the Asia-Pacific, including in Vietnam (Bui et al., 2021), Malaysia (Aregawi et al., 2019) and Thailand (Aregawi et al., 2019) as well as in non-canid animals across the region including Cambodia, Indonesia, Laos and the Philippines, with no reports from Australia (Aregawi et al., 2019). The low number of reported cases of *T. evansi* infection in dogs may be partly due to this VBP’s high pathogenicity in this host species, whilst also signifying that dogs are unlikely to play a significant role in maintaining this parasite’s sylvatic and domestic lifecycles (Aregawi et al., 2019; Rjeibi et al., 2015).

T. evansi infection in canines can be treated by intramuscular off-label diminazene aceturate at low dosage over five days (Panigrahi et al., 2015), whilst off-label

suramins have also been shown to lead to resolution of parasitaemia (Defontis et al., 2012). Nonetheless, there is a significant dearth of research into canine treatment options for this VBP and outcomes between dogs after such treatments may be highly variable (Defontis et al., 2012).

1.3. Current and advancing techniques for the diagnosis of vector-borne pathogens

1.3.1. Historical and current techniques for VBD diagnosis

Up until only recently has diagnosis of bacterial diseases been primarily executed via cultivation on different growth media, followed by morphological identification and testing for antibiotic resistance (Lecuit and Eloit, 2015). Such diagnostic techniques can be highly efficacious at identifying common bacterial pathogens, however, they are hampered by the issue that a large portion of bacterial diversity is unculturable, predisposing these methodologies to false negative results (Lecuit and Eloit, 2015; Vayssier-Taussat et al., 2013). Moreover, bacterial culturing techniques are lengthy processes with a long lag time until results are obtained, they also require highly trained personnel to carryout complex processing pipelines to test for the swathes of bacteria that could be causing infection (Lecuit and Eloit, 2015; Pallen, 2014).

For protozoan parasites the challenges of accurate diagnosis have historically been even harder. Most are obligately intracellular parasites that would need a live host to be culturable and maintained (Bonnet et al., 2014). Diagnosis of vector-borne protozoa has therefore commonly relied on blood smears from the infected host, followed by morphological identification using microscopy (Bonnet et al., 2014). However, many species are morphologically almost identical and require great expertise to identify down to species level, for example the large canid *Babesia*; *Babesia canis*, *Babesia rossi* and *B. vogeli* (Sikorski et al., 2010). Furthermore, to detect circulating VBPs in the blood stream, parasitaemia at the point of sampling must be relatively high, otherwise false negatives are obtained and covert infections missed (Otranto et al., 2009b). For the diagnosis of nematode parasitosis the difficulties are similar to those of protozoan ones with many species morphologically almost indistinguishable, regardless of whether an adult or egg life history stage is being observed (Tanaka et al., 2014).

Advancing from microscopy-based diagnosis, serological techniques have also been widely used for VBD diagnosis (Hii et al., 2013; Kho et al., 2017; Koh et al., 2016; Teoh et al., 2017, 2016). Serology typically relies on detection of antibodies produced by the host against a particular VBP, these antibodies indicate the host has been previously exposed to or is currently infected by that pathogen (Abbasi et al., 2003; Pottumarthu et al., 2000). An exception is for serological detection of heartworm (*D. immitis*) that relies on the detection of antigens excreted by the adult female worm (Digangi et al., 2017). Serology alike to morphological identification, is frequently unable to discern similar species apart with cross-reactivity of host antibodies to different pathogen antigens being common (Abd Rani et al., 2011; Kho et al., 2017). There is also a considerable lag time between infection of a host and seroconversion, meaning recent infections are undetectable whilst immunocompromised hosts may be unable to effectively generate antibodies (Hii et al., 2015b, 2012; Koh et al., 2016). Moreover, serology cannot always discern whether the individual sampled is experiencing a current infection or if serological positivity is a sign of prior pathogen exposure that may now have been cleared, due to the continued circulation of antibodies for lengthy periods of time after infection (Wong et al., 2011).

With the advent of molecular technologies, like PCR, the field of pathogen diagnostics has been transformed. Conventional PCR (cPCR) allows for selective amplification of DNA from specific gene(s) targeting a pathogen present in clinical samples, such as bodily fluids, tissue or faeces (Bass et al., 2015; Gasser, 2006). PCR primers can be designed to target a pathogen at a genus, species or sub-species level using a specific sequence, such as the 18S small ribosomal subunit gene (18S rDNA), the internal transcribed spacer regions (ITS) of ribosomal DNA or the cytochrome c oxidase I gene (COX1). These methods provide far greater identification accuracy than morphological or serological techniques (Abbasi et al., 2003; Koehler et al., 2013; Zepeda Mendoza et al., 2015). PCR-based techniques are also typically more sensitive than non-molecular ones, able to detect low burden or asymptomatic infections via detection of minute quantities of DNA even within the femtogram range (Koehler et al., 2013).

Despite this, cPCR technologies do possess some fundamental limitations to their usage. For the design of primers against species-specific DNA sequences *a priori* knowledge of the species of interest's genome or key diagnostic genes is essential (Ondrejicka et al., 2014). This caveat sometimes limits the use of cPCR for identifying

new and undescribed species or those that have not had their DNA sequenced and deposited in open access databases (Lecuit and Eloit, 2015; Ondrejicka et al., 2014). Furthermore, a single species-specific cPCR assay coupled with Sanger sequencing cannot identify coinfections whilst genus or family specific assays can typically only be used to amplify and sequence the DNA from the most predominant pathogen in a sample (Zepeda Mendoza et al., 2015). In the context of VBP diagnosis this may mean that concurrent infections, which are common in VBP generated disease, are missed as only the most prevalent parasite sequences are detected or mixed Sanger sequencing chromatograms generated that cannot be analysed (Dario et al., 2017; Paparini et al., 2015; Schneeberger et al., 2016). Nonetheless, some cPCR formats can overcome these challenges, for example restriction fragment length polymorphism PCR (PCR-RFLP) can produce different amplicon product fragments that can be run on a gel and used to assess for the presence of multiple pathogens coinfecting a host at one time (Jefferies et al., 2007a; Jiménez et al., 2011).

Real-time PCR (qPCR) may obviate some of these challenges, particularly those that employ Taq-Man based fluorescent probe technologies. In this context, different pathogen targeting probes can be designed and multiplexed together so that one assay can detect multiple species' DNA in a sample simultaneously (Espy et al., 2006; Massetti et al., 2020). These qPCR methods are easily scalable into a high-throughput format and generate results from detection of a fluorescent signal as opposed to cPCR which relies on visualisation of bands on a gel that may be faint and difficult to observe or the product of non-specific amplification (Massetti et al., 2020). Nevertheless, qPCR methods alike to cPCR, still rely on prior knowledge of a pathogen's diagnostic genes, with no-to-limited ability to mine for novel species (Takhampunya et al., 2019).

1.3.2. The next-generation sequencing revolution

From the mid-2000s conventional PCR and sequencing technology has taken a major leap forward with the invention of high throughput sequencing platforms or 'next-generation sequencing' (NGS) (Mardis, 2008). Next-generation sequencing can be carried out in two ways; to either generate short sequence reads between 150 to 700 base pairs in length or long sequence reads from 4,000 to as large as 200,000 base pairs (Goodwin et al., 2016).

Short-read methods start by fragmentation of template DNA followed by sequencing that is either carried out via ligation or synthesis. Sequencing by ligation, such as on the SOLiD platform, involves a fluorophore-bound probe sequence, hybridising to a template DNA fragment (Goodwin et al., 2016; Mardis, 2008). The fluorophore then emits a unique fluorescence spectrum indicating the base pairs present and their positions (Goodwin et al., 2016). Alternatively, sequencing by synthesis, like Qiagen's Illumina platform, uses a DNA polymerase in conjunction with fluorophore-labelled nucleotides to detect addition of bases into the DNA strands being synthesised (Goodwin et al., 2016).

Long-read next-generation sequencing has only existed since 2012 and was developed to combat the problems of complex genome assembly, as data from short-read sequencing can be impossible to accurately assemble (Goodwin et al., 2016; van Dijk et al., 2014). Pacific Biosciences was the first to pioneer long-read sequencing technology using a fixed polymerase and labelled nucleotides to visualise the incorporation of bases into one long, continuous DNA strand (Goodwin et al., 2016; van Dijk et al., 2014). Since then, Oxford Nanopore Technologies have developed an entirely new method for DNA sequencing, using a protein pore to measure changes in electrical current as a native DNA molecule is translocated through it (Goodwin et al., 2016). Oxford Nanopore Technologies MinION system can characterise sequences at previously unprecedented lengths on a sequencer the size of a USB stick (Quick et al., 2016, 2015).

Next-generation sequencing mitigates the need to selectively amplify one or a few target DNA sequences from a template as all sequences within a sample can be rapidly characterised (Bass et al., 2015; Goodwin et al., 2016). This can be done in two approaches. Either all the genomes in a sample are sequenced in a process known as shotgun sequencing, producing a metagenome that has extracted as much genetic information from a sample as possible (Pallen, 2014; Zepeda Mendoza et al., 2015). Alternatively, only species diagnostic regions otherwise termed barcodes of a sample may be targeted for sequencing, for example the COX1 gene is a common barcoding gene. This can generate a sequencing output of all the COX1 DNA barcodes in a sample, or a metabarcode, that can be used to elucidate all the species present in a sample (Zepeda Mendoza et al., 2015). Analysis of a sample's metabarcode alone may be sufficient if a sample's biodiversity is all that is required.

However, metagenomic information may be more suitable if in-depth information is needed, such as the antibiotic resistance status of species in a sample (Pallen, 2014; Schneeberger et al., 2016; Zepeda Mendoza et al., 2015). Both approaches typically require comparison of generated sequences with reference databases to correctly identify the species present (Leray and Knowlton, 2016; Zepeda Mendoza et al., 2015).

Even with all the benefits next-generation sequencing brings in terms of quantity and depth of information generated it is also encumbered with various drawbacks. Next-generation sequencing platforms, despite declining prices, are still significantly more expensive than cPCR and qPCR technologies (Goodwin et al., 2016; Ondrejicka et al., 2014). The data obtained can also be large and need costly data storage beyond the financial reach of many laboratories (Pallen, 2014). Furthermore, the bioinformatic processing of complex next-generation sequencing data is much more challenging than that for Sanger sequencing, necessitating complex work pipelines to extract the meaningful information from a complete dataset (Nascimento et al., 2016; Zepeda Mendoza et al., 2015). Gonzalez et al. (2016) highlighted the risks of obtaining false positives, using next-generation sequencing workflows. The authors found that when poor reference databases were used, alongside unchecked data processing pipelines, that unusual taxonomical matches could be found and reported. These included platypus DNA in environmental samples from countries as wide-ranging as China, Kyrgyzstan, Brazil and the USA, as well as *Yersinia pestis* (plague) and *Bacillus anthracis* (anthrax) DNA in the New York subway (Gonzalez et al., 2016).

1.3.3. Next-generation sequencing technologies for diagnostic parasitology

Ever since its inception, next-generation sequencing has been recognised and used as a powerful diagnostic tool to identify and thus help treat infection (Lecuit and Eloit, 2015; Ondrejicka et al., 2014). For example, metagenomic sequencing has been used to elucidate disease pathogens in situations where conventional microscopy and PCR techniques had failed. In one study, Schneeberger et al. (2016) found that metagenomic sequencing of faecal samples from patients presenting with chronic gastrointestinal disease could elucidate the responsible agents and interactions between them, that previous techniques had missed. Similarly, Jerome et al. (2019)

found metagenomics to be more sensitive than conventional molecular diagnostics in identifying previously undetected viral infections in febrile returning travellers whilst Rozo et al. (2019) used NGS to increase the number of successfully diagnosed patients suffering from sepsis in Cambodia. Chen et al. (2020) also found metagenomic sequencing to be superior than conventional diagnosis of visceral leishmaniasis, an important finding given that this zoonosis accounts for roughly 20,000 – 40,000 deaths annually. All of these cases powerfully demonstrate NGS-methodologies' role as an important addition to the global armoury of utilisable diagnostics (Chen et al., 2020; Jerome et al., 2019; Rozo et al., 2020).

Recent advances in next-generation sequencing, such as Oxford Nanopores MinION platform, have allowed whole genome data to be accrued from samples in as little as 24 hours using highly portable equipment (Quick et al., 2016, 2015). For instance, Quick et al. (2016) used the USB sized MinION sequencer along with other small-scale laboratory equipment that could collectively fit into a 50kg suitcase for their study. This level of portability allowed their team to transport a crude genetics laboratory to remote regions of West Africa, to monitor the 2014 Ebola Outbreak. Despite intermittent access to internet and electricity their team could carry out real-time genome surveillance to monitor the evolution of different Ebola strains and infer where transmission events were occurring over country borders (Quick et al., 2016). Recent advances in MinION technology have improved base-calling up to an accuracy of 99%, permitting the development of bioinformatic pipelines that can identify bacteria down to strain-level classification as well as assisting in the construction of genomes previously too structurally complex for short-read technology alone (Amarasinghe et al., 2020; Hall et al., 2020; Petersen et al., 2020).

Protozoan pathogens can show vast diversification and speciation within taxonomical groups that can be difficult to categorise, nonetheless, NGS-based diagnostics can mitigate such challenges and have been extensively used to investigate protozoan parasite communities (Bass et al., 2015). NGS can provide sufficiently in-depth genomic data to greatly assist in the construction of accurate phylogenetic placement, assisting in the demarcation between new species or just new strains of parasite (Bass et al., 2015; Vermeulen et al., 2016). Such a method was used to investigate the genomic relationships of *Eimeria* parasites a genus known to contain over a thousand species (Vermeulen et al., 2016).

Next-generation sequencing has been directly applied to the field of vector-borne diseases. Collection of ticks from the field followed by whole DNA extraction and deep sequencing can generate detailed information on circulating VBPs in the wild and therefore highlight current risks to livestock, pets and humans (Bonnet et al., 2014; Egan et al., 2020; Gofton et al., 2015a; Vayssier-Taussat et al., 2013). A study by Vayssier-Taussat et al. (2013) carried out this method to uncover a range of bacterial VBPs infecting *Ixodes ricinus* ticks in Europe, identifying a dominance of rickettsial pathogens, including the first example of zoonotic *R. felis* isolated from a tick (Vayssier-Taussat et al., 2013). In Australia, similar methods have been used to identify potential pathogens in wildlife and human-biting ticks (Egan et al., 2020; Gofton et al., 2015b), whilst in Thailand a targeted metabarcoding approach was used to characterise pathogenic *Rickettsia* species in ectoparasites from wildlife, domestic animals and rodents, demonstrating potential transmission pathways as well as zoonotic threats in the region (Chaorattanakawee et al., 2021). Studies have used the same methods to detect protozoan pathogens in ectoparasites, for example the discovery that zoonotic *Babesia* species, such as *B. divergens* and *B. microti*, use *I. ricinus* ticks as sylvatic reservoirs (Bonnet et al., 2014).

Recent large expansions in the number of NGS-based tick microbiome studies being conducted has led to a much greater understanding of the roles of bacterial endosymbionts in ticks, the effects of coinfection with different vector-borne pathogens as well as differences in microbiome based on environmental factors, tick age, sex and the tick organ sampled (Chauhan et al., 2020; Gofton et al., 2015b; Greay et al., 2018b; Guizzo et al., 2020; Landesman et al., 2019; Narasimhan and Fikrig, 2015; Thapa et al., 2018). As this work continues it highlights the possibility of NGS-led control strategies for tick-borne diseases such as detailed xenosurveillance or tick microbiome manipulation (Greay et al., 2018b).

Alongside the growing exploration of the tick microbiome, there is a mounting number of investigations into the bacterial microbiomes of mammalian blood, particularly in populations prone to bacterial VBP infection. Whilst the blood compartment contains low amounts of bacterial DNA even in severe VBP infections, 16S rRNA-based metabarcoding has still proven itself to be a valuable tool in identifying bacterial VBP infections, particularly coinfections that may have been missed by traditional molecular techniques (Eisenhofer et al., 2019; Rodino et al., 2021). These methods have been

used to characterise zoonotic VBP from human blood (Rodino et al., 2021), a range of VBPs from Chinese rodents (Ge et al., 2018) and *Rickettsia* in Thai rodents (Chaorattanakawee et al., 2021), *Anaplasma* spp. from Sudanese camels (Mohamed et al., 2021) as well as from dogs and humans in South Africa (Kolo et al., 2020). As these methods are further optimised, especially with regard to maximising bacterial DNA liberation whilst reducing that of the host, we can only expect to see a widening scope of mammals that have their blood microbiomes sequenced (Oney et al., 2021).

Alike to the success of microbiome studies exploring bacterial microbiomes, more and more research is now being published on protozoan parasitic assemblages or the 'haemoprotobiome', using the eukaryotic 18S rRNA gene for barcoding (Chaudhry et al., 2019). Recent studies have explored this in wildlife and livestock in Zambia (Squarre et al., 2020), African buffalo in South Africa (Glidden et al., 2020), cattle and water buffalo in Pakistan (Ghafar et al., 2021), Asian buffalo and sheep from Pakistan (Chaudhry et al., 2019) camels in Egypt (Mohamed et al., 2021) and wild marsupials, including koalas, bandicoots and wallabies from Australia (Barbosa et al., 2017; Ortiz-Baez et al., 2020). Many of these studies found novel sequences or pathogen species not previously identified in the relevant host before.

More experimental approaches have even used NGS metabarcoding to attempt to characterise all the eukaryotic parasites from a host simultaneously, using a pan-eukaryotic primer pair (Flaherty et al., 2021, 2018). Such approaches typically have low sensitivity given the preponderance for PCR amplification to amplify host 18S rRNA sequences which are in abundance compared to parasite sequences. Nonetheless, Flaherty et al. (2018) used restriction endonucleases to specifically target and digest host 18S rRNA sequences, meaning most of the resultant sequencing data was from parasites, thus creating a protocol for universal eukaryotic parasite detection (Flaherty et al., 2021, 2018).

The utility of next-generation sequencing to shed light on currently undescribed parasite diversity that may pose a threat to public health has been elegantly demonstrated in a study by Dario et al. (2017). These authors looked at trypanosomatids in Brazilian bats a protozoan parasite group that contains the human infecting species *Trypanosoma cruzi* that causes Chagas disease. A metabarcoding approach identified trypanosomatid coinfections with as many as eight different

species per host, including five different *T. cruzi*-like species (Dario et al., 2017). The use of deep-sequencing methods exposed pronounced *Trypanosoma* diversity harboured by predominantly asymptomatic bats and was of particular pertinence as a recent human coinfection of *Trypanosoma dionisi* and *T. cruzi* in the region had been fatal (Dario et al., 2017).

The interplay between pathogens infecting wildlife, domestic animals, vectors and humans has also been more explicitly investigated through a study by Takhampunya et al. (2019). Here, the researchers used bacterial metabarcoding on samples from people with undifferentiated febrile illness as well as from domestic mammals, wild rodents and the ectoparasite vectors found on them. The results identified significant overlap between the pathogenic bacteria identified within all of these animal cohorts, whilst also identifying putative novel pathogens implicated in human disease (Takhampunya et al., 2019).

1.4. Chemoprevention of ectoparasites and vector-borne pathogens

1.4.1. Prevention is better than cure

Upon diagnosis, due to the potential morbidity and mortality VBPs can inflict upon a canine host, a course of antibiotics is typically given to attempt cure (Baneth et al., 2012). Nonetheless, achieving complete elimination of a VBP once it is already established in a host's body can be extremely difficult, with some showing regular recrudescence after a treatment course and disease relapse (Baneth et al., 2012; Otranto et al., 2009b). For example, canine monocytic ehrlichiosis is typically treated with a tetracycline derivative, such as doxycycline, however after initial elimination of parasitaemia and acute disease, chronic relapses are commonly observed months later (Little, 2010; Otranto et al., 2009b). Furthermore, because sustained drug courses are often needed, owner compliance may become a problem as owners decide to finish antibiotic courses prematurely or fail to administer them at appropriate time points (Mencke, 2013). In developing countries, the cost of therapeutic drugs can also pose a challenge, exacerbated by the lengthy duration over which they must be provided (Otranto et al., 2009b). Some countries may also have limited access to the latest and safest anti-parasitic drugs. For example, reliance on older treatments for VBP infection may use antimonials or similar heavy metal based compounds that can

themselves cause serious harm to the host (Khaw and Panosian, 1995; Oliva et al., 2010).

Taking all this into consideration, it is clear that to fundamentally restrict transmission and alleviate the health risks posed to canines by VBPs, it is deterrence of ectoparasites and prevention of feeding that warrants research effort (Mencke, 2013; Otranto et al., 2009b).

1.4.2. Chemopreventive agents for ectoparasites

Given the risks posed by an infected ectoparasite transmitting a VBP during feeding, effective chemoprevention and killing of ectoparasites is essential (Wall and Shearer, 1997). Mites and lice are permanent parasites, remaining with their host for life, thus application of an effective chemopreventive drug to the animal host and its bedding may eliminate the entire local population. However, non-permanent parasites such as fleas, ticks and mosquitoes are harder to control as only a small portion of the local population are killed or deterred at any one time (Taylor, 2001; Wall and Shearer, 1997). Therefore, chemoprevention for such ectoparasites must be sustained as re-infestation events may be more likely (Witchey-Lakshmanan, 1999).

Since the first ectoparasiticides and preventives were developed in the 1920s, our available armoury has grown considerably, although, in the last few decades development of novel chemical classes has reduced (Taylor, 2001; Wall and Shearer, 1997). Ectoparasiticides and chemopreventives work in two ways; either by systemic uptake into the host's tissues, which is encountered by the ectoparasite during feeding or via topical application and dissemination over the hosts skin and hair, which deters parasites on contact (Taylor, 2001).

Most ectoparasiticides and preventives are neurotoxins of arthropods, allowing them to act quickly on synapses and neuromuscular junctions to inhibit neurotransmission and cause paralysis (Wall and Shearer, 1997). One of the first ectoparasiticide chemical classes to be developed and applied to canines was the formamidines, including the widely used acaricidal product Amitraz, a dipping chemical that could deter ticks and treat infection caused by sarcoptic and demodectic mites (Taylor, 2001; Wall and Shearer, 1997). After the formamidines, ectoparasiticide development diversified significantly to include key new groups, such as the pyrethrins and synthetic

pyrethroids, phenylpyrazoles, nitroguanidines, macrocyclic lactones and isoxazolines (Taylor, 2001; Zhou et al., 2021).

Pyrethrins are the most widely used insecticides and acaricides globally, derived from the naturally occurring chemical pyrethrum, from the chrysanthemum plant (Taylor, 2001). They have a rapid knock-down effect on insects but very low toxicity to mammals and have been synthetically augmented to produce pyrethroids that use the same base structure with minor chemical changes to increase their stability (Pfister and Armstrong, 2016). The phenylpyrazoles, such as fipronil, and nitroguanidines, e.g. imidacloprid, are two highly arthropod-specific neurotoxins, the former affecting gamma-aminobutyric acid (GABA) transmission generating a state of hyperexcitation and the latter irreversibly binding to nicotinic acetylcholine receptors generating paralysis (Taylor, 2001; Wall and Shearer, 1997). Another important ectoparasiticide class is the macrocyclic lactones, that include the chemicals selamectin and moxidectin. Macrocyclic lactones were developed from fermentation chemicals found in different *Streptomyces* bacteria species and are broad-spectrum antibiotics against arthropods and nematodes (Taylor, 2001; Wall and Shearer, 1997). They are typically highly lipophilic allowing them to build up in the fat stores of the host they are administered to and then be slowly released around the body and its tissues (Taylor, 2001). The final and most contemporary class of ectoparasiticides developed are the isoxazolines that include afoxolaner and fluralaner amongst others (Zhou et al., 2021). These target GABA-gated and glutamate-gated ion channels in many ectoparasite species, disrupting neurotransmission in the invertebrate central nervous system thereby causing paralysis and death (Shoop et al., 2014; Zhou et al., 2021).

Whilst the particular chemopreventive or ectoparasiticide given to host animals is important, so too, is the manner in which it is delivered. The first of such products were typically powders or shampoos which could easily slough off the host, meaning they had short windows of efficacy and could contaminate the environment (Witchey-Lakshmanan, 1999). Subsequently, more advanced delivery systems were invented, such as spot-on treatments or collars. Spot-on treatment is the application of a highly concentrated formula to one point on the animal which then spreads over its skin, covering the body and providing sustained protection, typically for one month (Taylor, 2001; Witchey-Lakshmanan, 1999). Collar technologies use a matrix collar which is impregnated with the ectoparasiticide and acts as a reservoir to constantly deliver

chemicals from its matrix to the animal's skin and body, with some products conferring protection for as long as eight months (Stanneck et al., 2012c; Witchey-Lakshmanan, 1999). Benefits of these longer acting products is that owner compliance becomes less of an issue, as reapplication is less frequent and thus there are fewer windows of infestation risk if owners forget to reapply a product (Brianti et al., 2013; Stanneck et al., 2012a).

1.4.3. Chemoprevention to block vector-borne pathogen transmission

Chemopreventives and ectoparasiticides have typically had their efficacy tested by how well and how quickly they kill the arthropod vector, nonetheless, there is now growing scientific understanding that product efficacy is better defined by how well they block VBP transmission (Fourie et al., 2013b, 2013a; Jongejan et al., 2016). How well a vector has been killed is of little relevance if it has had time to transmit a VBP to a new host. Therefore, more research is now focussed on feeding prevention as well as investigation of how long it takes different VBPs to be transmitted during a blood meal (Jongejan et al., 2016).

One product that has recently become available in some countries in the Asia-Pacific and shown great promise at blocking VBP transmission is a 10% imidacloprid and 4.5% flumethrin chemopreventive collar (Seresto®) by Bayer (Brianti et al., 2013; Dantas-Torres et al., 2013). Imidacloprid is a nitroguanidine insecticide and acaricide that has been widely used since the early 2000s, whilst flumethrin is an alpha-cyano pyrethroid registered as an acaricide since 1986 (Stanneck et al., 2012b, 2012a). Together the two chemicals have been demonstrated to generate a potent synergistic parasitocidal effect (Stanneck et al., 2012a). Imidacloprid acts as a nicotinic acetylcholine receptor agonist causing neuron depolarisation and opening sodium channels. Flumethrin can only bind to these neuronal sodium channels when they are in their open configuration and hence imidacloprid opens more of these channels to allow flumethrin to bind (Stanneck et al., 2012a). Once bound, subsequent closing of these channels is prevented, meaning a difference in intra and extracellular Na⁺ concentration cannot be regenerated and neuron function is destroyed (Stanneck et al., 2012a). Overall the two chemicals act together, with imidacloprid increasing

sensitivity to flumethrin and thereby producing parasitocidal results that are greater than the sum of either alone (Stanneck et al., 2012a, 2012b).

Seresto® collars have been substantially tested in both the lab and the field to elucidate their efficacy as a repellent, ectoparasiticide and ability to block VBP transmission (Brianti et al., 2013; Dantas-Torres et al., 2013; Fourie et al., 2013b; Stanneck et al., 2012b; Stanneck and Fourie, 2013). Laboratory experiments have found Seresto®'s therapeutic efficacy against a pre-existing *C. felis* flea infestation to be 99.8-100% by the second day of collar application whilst its effectiveness at preventing re-infestation by a number of different tick species was 96% seven days post-collaring. However, the collar's ability to repel ticks that had already attached was not as efficacious, varying between 32.2% to 96.4% depending on the tick species involved (Stanneck et al., 2012b). Seresto® has also demonstrated high efficacy (> 95%) to repel *Ixodes holocyclus*, the Australian paralysis tick, a species of great veterinary importance given that a single tick bite can be fatal for dogs due to envenomation with holocyclotoxin (de Burgh et al., 2017).

Moreover, Seresto® collars have also been demonstrated to have great chemopreventive capacity in the field. A study by Brianti et al. (2013) used the product on dogs in a Sicilian shelter of which 96.3% were infested with ticks at levels as high as 434 ticks per dog at the study's commencement, due to great infestation pressure from the shelter and local environment. Flea infestation was also rampant with 68.8% of dogs infested and 16.8% of these showing symptoms of FAD. Seven days after collaring, tick infestation was reduced to 15% and flea infestation down to 3.9%, with no ectoparasites found at the 14-day sampling point. This high level of deterrence was maintained for the duration of the 90-day study, whilst all symptoms of FAD were cleared after just 30 days (Brianti et al., 2013). These results are mirrored by other studies into Seresto®'s long-term effectiveness, including one that used a cohort of 400 dogs to find sustained reduction in flea counts at 96.7% and ticks at 94.7% compared to controls over the eight months the product is claimed to work (Stanneck et al., 2012c).

The VBP transmission blocking capacity of Seresto® has also been investigated in detail. Dantas-Torres et al. (2013) carried out a study using dogs from an Italian shelter where transmission of tick-borne VBPs was common. They compared the

efficacy of Seresto® collaring on acquisition of new VBPs with an uncollared control group, finding that it controlled new *A. platys* infection by 91.1%, *B. vogeli* by 100% and *H. canis* by 43.4% (Dantas-Torres et al., 2013). Prevalence of fleas in the collared dog group was also observed to reduce by 100%. The authors postulated that the collars lower efficiency at reducing *H. canis* infection was due to this protozoan being transmitted via tick ingestion, highlighting the complexities and differences between effective VBD control between pathogen species (Dantas-Torres et al., 2013). Krämer et al. (2020) found Seresto® collars 100% efficacious at blocking the transmission of *A. phagocytophilum* and *Borrelia burgdorferi* sensu lato, whilst Fourie et al. (2013) found similar results regarding blocking *B. canis* infection with frequent challenges. This finding was further supported by a second study that demonstrated this efficacy persisted across the collar's entire eight-month duration of action (Fourie et al., 2019a; Fourie et al., 2013b; Krämer et al., 2020). An even longer study demonstrated Seresto® to be 94% effective at preventing *E. canis* transmission during regular challenges over a 420-day period (Stanneck and Fourie, 2013).

Given the long eight-month duration over which Seresto® is purported to act and the large amount of environmental exposure dogs are likely to have over such time periods, there have also been investigations into whether the parasite repellent effects of Seresto® remain even with regular water exposure. Two studies have demonstrated that repellence by Seresto® against *C. felis* fleas is reduced when regular exposure to rain is simulated, to an efficacy as low as 68.3% in some cases, although no concurrent reductions in efficacy were seen against *R. sanguineus* ticks (Beugnet et al., 2018; Halos et al., 2014).

Regardless of whether Seresto® or another chemopreventive is used, it is the growing recognition that ectoparasites should be immediately deterred and prevented from feeding, that is crucial to a products benefit (Jongejan et al., 2016). This was elegantly demonstrated by Jongejan et al. (2016) that investigated the VBP transmission blocking efficacy of a topical chemopreventive that acts from the skin of the host, against systemic ectoparasitocides which act once the parasite has begun feeding. They tested three different products using sustained challenges of *R. sanguineus* ticks infected with *E. canis* and found that blocking efficacies were between 33.3% to 66.6% in the systemic products but 100% in the spot-on chemopreventive product (Jongejan et al., 2016). Comparable results have been obtained in other field trials, finding that

chemopreventive agents such as permethrin, fipronil and amitraz can together significantly block VBP transmission to dogs in a manner that cannot be replicated in products that rely on vector feeding (Fourie et al., 2013a; Jongejan et al., 2015; Witche-Lakshmanan, 1999).

Research into the transmission times of different VBPs after tick attachment has shown that the speed at which transmission occurs is highly dependent on the pathogen in question and the species of vector (Jongejan et al., 2016). *B. canis* is only transmitted 24-48 hours after tick attachment (Schein et al., 1979), whilst rickettsial pathogens are thought to be transmitted within 4-48 hours (Nicholson et al., 2010). However, some studies have recorded *E. canis* as being inoculated into hosts after just three hours post-attachment of a tick (Fourie et al., 2013). Recently, *A. phagocytophilum* was shown to be transmitted by feeding ticks after just a few hours of attachment, although duration of feeding had to be over 48 hours to produce an inoculation of pathogen substantive enough to infect a naive dog host (Fourie et al., 2019b).

The picture is further complicated by an understanding that other factors, such as whether the tick vector has recently fed, influence transmission time (Saraiva et al., 2014). For example, the tick vector *Amblyomma aureolatum* was observed to decrease the transmission time of *R. rickettsii* from ten hours to ten minutes, depending on whether it had recently fed or not (Saraiva et al., 2014). Such potentially short periods over which VBPs can be transmitted highlight the risks posed to the host by ectoparasiticides that rely on attachment and feeding to take effect, demonstrating why fast-acting chemopreventives are superior at interrupting VBP transmission (Fourie et al., 2013b; Jongejan et al., 2016).

Ticks and fleas are not the only important disease vectors of canines, thus for ectoparasite chemoprevention in canines to be fully effective it must also protect against mosquito and other biting fly-borne pathogens, like *D. immitis* and *L. infantum* (Brianti et al., 2013; Frangipane di Regalbono et al., 2016; Genchi and Kramer, 2017). A recent metanalysis by Yimam & Mohebbi (2020) identified Seresto® collars as having an average efficacy of 90% at reducing canine visceral leishmaniasis, via decreasing the amount of phlebotomine sand fly vector biting — an important finding

given that canines act as the principal reservoir of zoonotic *Leishmania* in endemic areas (Brianti et al., 2013; Yimam and Mohebbi, 2020).

For endoparasites, macrocyclic lactones, are another crucially important parasiticide class that are used to prevent nematode infections in canines. Chemicals such as ivermectin and moxidectin have demonstrated great parasitocidal activity against numerous nematodes taxa, including hookworms, roundworms, heartworms and skinworms (Bowman et al., 2016; Frangipane di Regalbono et al., 2016; Hellmann et al., 2003). However, differences between the macrocyclic lactone used, the delivery method and the nematode life cycle stage targeted can all greatly affect the nematocidal efficacy (Fok et al., 2010; Petry et al., 2015; Traversa et al., 2011). For example, one spot-on application of 2.5% moxidectin has been demonstrated to eliminate 88.9% of microfilaremia in dogs caused by *D. repens* infection, however, the same treatment was needed over a six-month duration to achieve an adulticidal efficacy of 96.2% of dogs (Petry et al., 2015; Traversa et al., 2011). Such results, demonstrate how nematode life history stage, site of infection, drug type and duration form a complex interplay to define treatment efficacy (Bowman et al., 2017; Petry et al., 2015). Our understanding of nematode chemopreventive value is further convoluted by the rising development of macrocyclic lactone drug resistance (Bowman, 2012). For instance, in the US some strains of *D. immitis* have begun to emerge in the Mississippi Delta region that demonstrate resistance to the commonly used macrocyclic lactone preventive, milbemycin oxime (Bowman, 2012; Genchi and Kramer, 2017).

In the Asia-Pacific *D. immitis* is one of the most important mosquito-borne VBPs of canines and fortunately there is a substantial body of research into its chemoprevention. Advantage Multi® (Bayer) a spot-on combination of 10% imidacloprid and 2.5% moxidectin has been shown to be a highly effective treatment and prophylactic against *D. immitis* infection, showing 100% efficacy against a subcutaneously delivered L3 challenge (Bowman et al., 2017, 2016). The same product was also tested in field conditions in areas highly endemic for *D. immitis* and *D. repens* in Italy, finding a single dose to be 99.97% effective at blocking transmission of the former species and 100% effective of the latter (Frangipane di Regalbono et al., 2016). The topical application of moxidectin in Advantage Multi®, is believed to be particularly effective as a chemopreventive because moxidectin is observed to

bioaccumulate in host tissues, maintaining protective levels for longer (Bowman et al., 2016). In comparison other macrocyclic lactones such as ivermectin and selamectin are metabolised faster, producing shorter half-lives and duration of efficacy, within the host (Bowman et al., 2016).

The effective treatment and chemoprevention of VBPs in canines is not a trivial matter given the preponderance of species that can also infect and cause disease in humans (Conlan et al., 2011; Irwin and Jefferies, 2004). VBPs, such as *Bartonella* spp., *Rickettsia* spp., *A. phagocytophilum*, *L. infantum*, *D. immitis* and *D. repens* are all zoonotic, causing pathogenesis in humans, however, they can also be treated and prevented in canine hosts, interrupting cycles of transmission (Dantas-Torres and Otranto, 2014; Day, 2011; Otranto et al., 2009b; Parola et al., 2013; Smout et al., 2017). Scientific understanding of the overlap between disease in people and animals has led to the principle of One Health i.e. that the reduction of risk of infection in pets and livestock also reduces the risk of disease in human populations (Dantas-Torres and Otranto, 2014; Smout et al., 2016). This guiding framework to direct research promotes collaboration between scientists, medics and veterinarians, comprehending that these different scientific and clinical areas benefit when working together seamlessly to foresee new zoonotic threats (Conlan et al., 2011; Day, 2011; Traub et al., 2015). Exchange of information between these different parties also improves the services doctors and veterinarians can provide. For example, such dialogue may make medical clinicians more aware of potential zoonotic risks in an area and therefore be better able to diagnose patients presenting with unusual symptoms (Conlan et al., 2011; Traub et al., 2015). At the same time, veterinarians cognizant of up-to-date VBP risks in their local area can encourage appropriate chemoprevention to clients of their practice (Baneth et al., 2012; Conlan et al., 2011).

Never before in recent history has the importance of One Health principles and understanding been more clearly demonstrated than through the ongoing COVID-19 pandemic (McNamara et al., 2020; Watsa and Wildlife Disease Surveillance Focus Group, 2020). As yet, a definitive animal host for SARS-CoV-2 has not been discovered nonetheless there is little dispute within the scientific community that the virus originated from a wildlife source that then made the jump into human populations. This is supported by phylogenetic analysis which shows a very high similarity between SARS-CoV-2 and previously sequenced bat coronaviruses, whilst similar epidemics

caused by SARS and MERS both had their animals origins eventually traced back to civets and camels, respectively (Bonilla-Aldana et al., 2020; Ferri and Lloyd-Evans, 2021). Moreover, outbreaks of disease originating from animals appear to be accelerating with COVID-19 only the latest in a steady stream of emergent zoonotic viral epidemics including Zika, Ebola and SARS, amongst others (Gardy and Loman, 2018; Overgaauw et al., 2020; Watsa and Wildlife Disease Surveillance Focus Group, 2020). Given such a deteriorating situation, the ability to detect pathogens of zoonotic potential and predict which pose a substantial threat is critical, prior to them moving into human populations (Hu et al., 2017).

1.4.4. Vector-borne pathogen protection in the tropics: Parasite infection pressure at its highest

Whilst risk of canine VBD is global, nowhere is this threat more pronounced than in developing countries in the tropics (Traub et al., 2015). High year-round temperatures coupled with elevated humidity provide ideal conditions for burgeoning ectoparasite diversity and thus VBP species (Irwin and Jefferies, 2004). Many countries in the tropics also have large stray dog populations with limited access to veterinary care or the latest chemopreventive drugs (Inpankaew et al., 2007; Koh et al., 2016; Megat Abd Rani et al., 2010; Ng-Nguyen et al., 2015; Traub et al., 2015, 2014). Sanitation infrastructure and hygiene may also be under resourced, further increasing the likelihood of zoonotic transmission e.g. through unclean drinking water (Inpankaew et al., 2007; Traub et al., 2015).

Itinerant dogs are a particularly significant risk factor that increase canine VBP infection pressure and intensity (Abd Rani et al., 2011; Koh et al., 2016). These dogs are at a higher risk of acquiring VBPs, due to increased contact with natural, non-urban environments or through having more contact with other strays, allowing easy transmission of ectoparasites from the wild or from dog-to-dog (Abd Rani et al., 2011; Irwin and Jefferies, 2004; Koh et al., 2016). Consequently, ectoparasite pressure and VBD risk increases for domestic dogs as well, due to intermittent contact with stray dogs or the environments they frequent (Irwin and Jefferies, 2004; Pumidonming et al., 2016). Here, non-domesticated dogs act as a constant VBP reservoir that remains untreated, maintaining the threat of infection. Several studies have shown that when

people live in close proximity to animals infected with zoonotic VBPs there is an increased chance of infection in people (Dantas-Torres and Otranto, 2014; Irwin and Jefferies, 2004; Traub, 2013). For instance, *R. sanguineus* and *R. linnaei* rely on just a single dog host to feed and complete its life cycle and therefore local populations can build up in kennels or human homes, capable of transmitting VBPs, such as *R. rickettsii*, *R. conorii* or zoonotic *Ehrlichia* spp. to humans (Boost et al., 2017; Gray et al., 2013; Levin et al., 2012; Moraes-Filho et al., 2009).

Many of the aforementioned chemopreventives and ectoparasiticides have been substantially tested in European countries or North America, however, the outcomes of such experiments are not necessarily indicative of how the same products will perform in the tropics (Brianti et al., 2013; Dantas-Torres et al., 2013; Stanneck et al., 2012c). Due to typically higher VBP prevalence in some Asia-Pacific countries as well as more ectoparasite infestation risk from stray animals, there is a necessity to trial the same products in these more extreme conditions (Irwin and Jefferies, 2004). For example, Seresto® was demonstrated as being 100% effective at blocking *B. vogeli* transmission to canines in field conditions in Southern Italy (Dantas-Torres et al., 2013). Initial prevalence of infection was demonstrated as being 4.9% for *Babesia* spp. with this data corroborating with a similar Italian study finding *Babesia* spp. at a 6% prevalence (Cassini et al., 2009; Dantas-Torres et al., 2013). Nevertheless, *B. vogeli* has been found in 32.7% of stray dogs in Northern Cambodia (Inpankaew et al., 2016), a prevalence approximately six times greater than that in Italy, whilst Thailand has also exhibited greater *Babesia* spp. prevalence at a level of 9.4% (Liu et al., 2016). Whether the same product could afford a comparable level of protection under these conditions of elevated disease intensity is therefore not something that can be presumed.

Recognising that tropical settings present unique challenges regarding animal VBP prevention and treatment the Companion Animal Parasites Council for the Tropics (CAPCT) was developed in 2015, today renamed as TroCCAP (Traub et al., 2015). This council, mirroring counterparts supporting European countries (European Scientific Council of Companion Animal Parasites) and North America (Companion Animal Parasite Council) aims to instead focus on creating best practice guidelines for treatment and control of VBPs in poorly researched countries in the tropics (Traub et al., 2015). Working as independent, non-profit organisations that draw together

expertise from parasitologists, medics, veterinarians and the public health sector these councils provide up-to-date advice and surveillance on VBPs and zoonotic risks to the countries they provision (Traub et al., 2015). TroCCAP is no exception and since its inception has a growing body of best practice guidelines and original articles to inform veterinary practitioners in the Asia-Pacific, Latin America, Africa and the Caribbean (Traub et al., 2015). TroCCAP's information and resources are specific, recognising that many parasites are unique or highly endemic to regions of the tropics and thus pose distinct threats to the pets and people that live there (Traub et al., 2015).

The founding of such advisory research councils has come at a time of great need, to assist with finding solutions to problems generated by a rapidly changing globe with transforming zoonotic threats (Beugnet and Marié, 2009). The last 20 years have seen abrupt changes in VBP epidemiology, such as shifting endemicity, prevalence and pathogenicity, best documented in Europe and North America (Beugnet and Marié, 2009; Dantas-Torres and Otranto, 2016; Otranto et al., 2009a). Causative factors are believed to be of a predominantly anthropogenic nature, including climate change, habitat alteration, biodiversity loss, overpopulation and urban expansion which all affect local ecosystems and thus arthropod vectors (Beugnet and Marié, 2009; Conlan et al., 2011; Zinsstag et al., 2018). One of these factors; warmer climates, is an ideal change for many endothermic ectoparasites allowing them to increase the geographic range over which they can survive, permitting movement into new regions and thus spreading vector-borne diseases to naïve populations (Rocklöv and Dubrow, 2020).

A growing body of evidence has now found a changing climate to be responsible for the increase in frequency and distribution of tick-borne diseases across Europe as well as mosquito-borne *D. repens* infection, which has seen range expansion out of Southern Europe to the north and east (Beugnet and Marié, 2009; Genchi and Kramer, 2017). Swift urban expansion is also problematic as it can bring people and their pets into greater contact with wild animals, increasing the chances of disease spill-over, whilst also changing the local environment to potentially make it more favourable to disease vectors, like mosquitoes (Ramasamy and Surendran, 2011; Smout et al., 2016). Furthermore, increased travel of humans and their pets can rapidly translocate disease within and between countries, a fact well exemplified by the Pet Travel Scheme in the UK (Hii et al., 2012; Shapiro et al., 2017). This scheme reduced barriers and quarantine measures for pets travelling into the UK from the EU but at the same

time led to the importation of *Babesia* spp., *Ehrlichia* spp. and *Leishmania* spp. parasites, which had been rarely or never observed in the country before (Beugnet and Marié, 2009; Hii et al., 2012). Similar outcomes can emerge due to natural disasters as well. For example, after Hurricane Katrina, massive relocation of dogs in affected areas led to *D. immitis* infection becoming established in areas of the US where it was previously absent (Otranto et al., 2009a). Overall, reported cases of VBP infections in humans have increased over the last 30 years, with lots of research focused on an elevation in the incidence of mosquito and tick-borne infections in Europe and North America (Booth, 2018; Rizzoli et al., 2019).

The Asia-Pacific is not exempt from such patterns of change, identified in other parts of the world. Many countries in these regions are observing climactic alteration whilst also undergoing ecological degradation, deforestation, industrialisation with urban expansion as well as growing livestock production and agriculture (Conlan et al., 2011; Zinsstag et al., 2018). In addition, countries in the tropics typically bear a larger burden of disease outbreaks, especially regarding mosquito-borne disease, when compared to their counterparts in temperate regions (Rizzoli et al., 2019). The interaction between human environmental modification and changing patterns of disease will undoubtedly continue to evolve with the effects of these changes on the distribution and emergence of VBPs multifaceted and intertwined (Rocklöv and Dubrow, 2020). Altogether, this puts great onus on the research community to pull apart such interplay and keep abreast of this rapidly developing field of study in the region.

1.4.5. Cambodia – a humanitarian effort in jeopardy

Cambodia has one of the highest amounts of antipersonnel landmines of any country today (Norwegian People's Aid, 2014). Numerous conflicts and periods of political upheaval, including the Vietnam war, the reign of the Khmer Rouge and civil war have cumulatively built up heavy mine contamination across the country (Somasundaram and Renol, 1998). Estimates from the late 1990s put the number of landmines as being anywhere from four to ten million, the latter figure implying a ratio of almost one mine per person at the time of its estimation (Harris, 2000; Somasundaram and Renol, 1998). The human cost of such extensive mining is great, with Cambodia suffering from the highest proportion of physically disabled people worldwide (Harris, 2000;

Somasundaram and Renol, 1998). At its worst point roughly 100 to 300 people were involved in landmine detonation accidents a month, with 50% of these losing a limb and 31% being killed outright (Somasundaram and Renol, 1998). Not only have landmines in Cambodia caused marked cost to human life but also had vast socioeconomic impacts for the country, making swathes of suitable agricultural land unusable due to landmine risk (Somasundaram and Renol, 1998).

Thankfully, this dire situation has improved substantially since the 1990s with extensive humanitarian efforts to demine the most heavily affected areas. In 2013, improving upon trends set out in previous years, 45.9 km² of mined land was cleared and 21,618 mines destroyed bringing estimates of remaining contaminated land down to 1,915 km² across the whole country (Norwegian People's Aid, 2014). Moreover, accidents caused by landmines have also decreased significantly, with the number of deaths and injuries per year dropping from figures in the hundreds to just over twenty fatalities and 89 injuries in 2013 (Norwegian People's Aid, 2014). Such pronounced improvements in Cambodia's situation are the results of extensive strategic planning, global and local financial support (\$25.86 million USD in 2013) and the remarkable actions of demining groups such as the Cambodian Mine Action Centre (CMAC) and Norwegian People's Aid (NPA).

Both CMAC and NPA rely considerably on the use of mine detection dogs to detect minute levels of explosive chemicals released by landmines and remnants of war, to assist in technical survey of mined areas (Norwegian People's Aid, 2016). These specially trained Belgian Shepherd dogs (Malinois) can be released and GPS tracked through dense vegetation to identify contaminated land that needs demining resources, thus speeding up mine clearance operations overall (Norwegian People's Aid, 2016). Nonetheless, training just one mine detection dog is expensive at approximately \$30,000 USD per dog, excluding the costs of attempting to train the 50% of dogs that are unable to make it through the rigorous two-year training program (Cambodian Mine Action Centre, 2014). Dogs that do make it through training are extremely valuable, some being used for up to a decade in different countries for demining activities (South China Morning Post, 2018).

Given the value of such mine detection dogs for humanitarian demining efforts in Cambodia it is of particular concern that many become clinically affected by VBPs

through their work. One NPA veterinarian has emphasised that as many as a third of NPA's mine detection dogs are afflicted by VBP infection by adulthood, with potentially higher levels present in CMAC's dogs (Dr Charlotte Søyland, pers. comm., July 2016 and March 2018). This is maybe unsurprising given that the ratio of dogs to humans in Cambodia is estimated at 1:3, giving a dog population of at least 5 million, many of which are stray with little access to veterinary care (Ly et al., 2009; Sor et al., 2018). These dogs provide a large reservoir from which VBPs can be harboured and transmitted to working mine detection dogs. Taking this into account, it is of paramount importance that research efforts be directed at exploring the causative agents of such infections, particularly given the high diversity of VBPs known to be present in the region (Inpankaew et al., 2016). Furthermore, this situation provides an ideal tropical context within which the latest ectoparasite chemopreventives can be tested to elucidate their efficacy in a high VBP intensity setting.

In light of the significant dearth of research into canine VBPs in the Asia-Pacific and the impact these VBPs have on dog populations, in particular those that conduct valuable landmine detection and decontamination work, I set out to accomplish the following aims:

- 1) Develop a novel next-generation sequencing-based method capable of holistically characterising the bacterial, apicomplexan and kinetoplastid pathogen communities from canine blood.
- 2) Estimate the prevalence, diversity and risk factors associated with established, emerging and vector-borne pathogens infecting pet, community and mine detection dogs in Cambodia using the aforementioned next-generation sequencing diagnostic techniques.
- 4) Using vector-borne pathogen prevalence data gleaned through next-generation sequencing to design, develop and optimise a novel multiplex qPCR capable of economically detecting the common canine VBPs for the Asia-Pacific.
- 5) Evaluate the field efficacy of a long-acting 10.0% imidacloprid/4.5% flumethrin collar (Seresto®, Bayer) to monthly applied fipronil (DeTick, Thailand), for the chemoprevention of vector borne pathogens in working mine detector and pet dogs in Cambodia using our new qPCR diagnostic.

1.5. Hypotheses

I hypothesise that (i) canine ectoparasites and the vector-borne pathogens they transmit are highly endemic in pet, community and free-roaming dogs in Cambodia and reflect typical infection-pressures faced in similar tropical settings in the Asia-Pacific; (ii) domestic dogs in Cambodia harbour novel vector-borne pathogens not previously identified in the Asia-Pacific, made readily detectable by NGS diagnostic methodologies (iii) Seresto[®] collars (Bayer) replaced at eight-monthly intervals have superior efficacy to monthly fipronil (DeTick) application, for the prevention of ectoparasite infestation and vector-borne pathogen transmission in dogs.

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Chapter 2 - Assessment of a metabarcoding approach for the characterisation of vector-borne bacteria in canines from Bangkok, Thailand

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2.1. Abstract

Globally, bacterial vector-borne disease (VBD) exerts a large toll on dogs in terms of morbidity and mortality but nowhere is this more pronounced than in the tropics. Tropical environments permit a burgeoning diversity and abundance of ectoparasites some of which can transmit an extensive range of infectious agents, including bacteria, amongst others. Although some of these vector-borne bacteria are responsible for both animal and human diseases in the tropics, there is a scarcity of epidemiological investigation into these pathogens' prevalence. The situation is further exacerbated by frequent canine co-infection, complicating symptomatology that regular diagnostic techniques may miss or be unable to fully characterise. Such limitations draw attention to the need to develop screening tools capable of detecting a wide range of pathogens from a host simultaneously. Here, we detail the employment of a next-generation sequencing (NGS) metabarcoding methodology to screen for the spectrum of bacterial VBD that are infecting semi-domesticated dogs across temple communities in Bangkok, Thailand. Our NGS detection protocol was able to find high levels of *Ehrlichia canis*, *Mycoplasma haemocanis* and *Anaplasma platys* infection rates as well as less common pathogens, such as "*Candidatus* *Mycoplasma haematoparvum*", *Mycoplasma turicensis* and *Bartonella* spp. We also compared our high-throughput approach to conventional endpoint PCR methods, demonstrating an improved detection ability for some bacterial infections, such as *A. platys* but a reduced ability to detect *Rickettsia*. Our methodology demonstrated great strength at detecting coinfections of vector-borne bacteria and rare pathogens that are seldom screened for in canines in the tropics, highlighting its advantages over traditional diagnostics to better characterise bacterial pathogens in environments where there is a dearth of research.

2.2. Introduction

Bacteria transmitted by blood-feeding arthropods, such as ticks and fleas, generate some of the most prevalent and life-threatening illnesses of canines in the tropics (Kaewkong et al., 2014; Nguyen et al., 2019a). The brown dog tick *Rhipicephalus sanguineus* is of particular importance in such regions, being able to transmit a wide range of different pathogens that cause vector-borne diseases (VBDs), including *Ehrlichia canis*, *Anaplasma platys*, and putatively some haemotropic *Mycoplasma* spp., whilst *Ctenocephalides* fleas transmit *Rickettsia felis* and *Bartonella* species (Corales et al., 2014; Dahmani et al., 2018; Hii et al., 2015; Nguyen et al., 2019; Phoosangwalthong et al., 2018). One of the most prevalent tick transmitted bacteria contracted by canines in the tropics is *E. canis* the causative agent of canine monocytic ehrlichiosis, which produces a chronic pancytopenia in its later stages that is often fatal (Gal et al., 2008; Kaewkong et al., 2014). *Anaplasma platys* is another common, tick-borne pathogen of canines that specifically targets platelets, creating a relapsing thrombocytopenia. This pathogen has been observed to reach a prevalence as high as 27% in some regions of tropical northern Australia and when found co-infecting the same host may greatly exacerbate the pathogenesis of other diseases (Kaewkong et al., 2014; Little, 2010; Shapiro et al., 2017). In fact, the co-infection of multiple vector-borne pathogens in the same canine host is common in the tropics, due to the high diversity of both infectious agents and vectors in such regions, in conjunction with frequently poor access to veterinary care (Corales et al., 2014; Irwin and Jefferies, 2004).

Assessment and monitoring of canine VBDs is important, not only due to the mortality and morbidity they generate in dogs but also due to the risk they potentially pose to people (Dantas-Torres and Otranto, 2016; Nguyen et al., 2019). Both *Ehrlichia chaffeensis* and *R. felis* can infect canines and are also zoonotic, the former of which is potentially lethal to man and the latter being the aetiological agent of flea-borne spotted fever (FBSF) an emerging zoonosis (Hii et al., 2011a; Little, 2010; Rar and Golovljova, 2011; Teoh et al., 2016). Furthermore, it is now recognised that the incidence of tick-borne infections in humans, pets and domestic animals is increasing, due to a range of factors, including habitat alteration, greater contact with wildlife and changing population dynamics of the relevant vector (André, 2018; Dantas-Torres and

Otranto, 2016). To tackle this, adoption of a One Health approach is of paramount importance, whereby thorough surveillance of VBDs in domestic and wild animal populations is carried out and the reporting of findings disseminated between veterinarians and clinicians to better broadcast and predict the risk of emerging zoonotic threats to humans (Dantas-Torres and Otranto, 2016; Skotarczak, 2018).

Detection and diagnosis of bacterial disease has for a long time principally been carried out via cultivation on specific growth media, followed by morphological identification with the aid of biochemical and antibiotic testing (Lecuit and Eloit, 2015). Nonetheless, many vector-borne bacteria are unculturable, making serological or molecular techniques preferable (Vayssier-Taussat et al., 2013). PCR-based diagnosis is particularly useful given that it can provide information on current infection status and can be much more specific than serology which relies on antibodies that often demonstrate interspecific cross-reactivity (Abd Rani et al., 2011; Kho et al., 2017; Pottumarthy et al., 2000). Such methods do, however, have some limitations including a reliance on the presence of bloodstream circulating pathogens at the time of sampling (Peters et al., 2004).

With the arrival of next-generation sequencing (NGS) technologies vector-borne bacteria detection and diagnosis has been taken further than what was previously possible, as massive parallelisation of sequencing reactions allows for thorough characterisation of all variants of a sequence of interest (Decuypere et al., 2016; Vayssier-Taussat et al., 2013; Zepeda Mendoza et al., 2015). When targeting a barcoding gene, such as the bacterial 16S small ribosomal RNA subunit locus (16S rRNA), a 'metabarcoding' is created, detailing information on all the bacterial genera and sometimes species present in a sample, depending on the particular 16S rRNA region targeted and sequence reference library used (Zepeda Mendoza et al., 2015). NGS-based 'metabarcoding' has led to a wealth of research investigating the microbiome of different internal and external environments; however, there is a distinct paucity of research investigating the bacterial blood microbiome in species other than humans (Decuypere et al., 2016; Faria et al., 2015; Fliegerova et al., 2014; Gyarmati et al., 2015; Païssé et al., 2016; Waldor et al., 2015). Moreover, 'metabarcoding' is better able to detect novel and rare bacterial species, that family, genus or species-targeting conventional PCR (cPCR) would normally miss (Zepeda Mendoza et al.,

2015). This is of great relevance in regions where there has been little research into vector-borne bacteria of canines, such as in the tropics.

In the present study, we endeavoured to assess for the first time, whether pan-bacterial primers targeting 16S rRNA could be used to detect vector-borne bacteria of canines with focus on the assay's ability to detect mixed infections. In addition, we compared this NGS-based method to endpoint cPCR protocols to evaluate differences in both technique's relative sensitivity and range of species detected. Populations of semi-domesticated Thai dogs were chosen as study subjects, given that canine VBD is known to be highly endemic in these cohorts (Foongladda et al., 2011; Kaewmongkol et al., 2017; Liu et al., 2016; Suksawat et al., 2001).

2.3. Methods

2.3.1. Sampling and DNA extraction

This study utilised a subset of 100 whole blood samples collected from temple community dogs that make up part of an ongoing project at Kasetsart University, exploring canine and feline VBDs across Thailand. After obtaining informed consent from the relevant monk or caregiver, canine blood samples were collected from 35 Buddhist temple communities. A qualified veterinarian carried out blood sample collection through a cephalic or jugular puncture. This was held in anti-coagulation EDTA tubes and stored at -20 °C until required. DNA extraction was performed using the E.Z.N.A.[®] Blood DNA Mini Kit (Omega Biotek Inc., Norcross, GA, USA) from a starting quantity of 250 µl of blood according to the manufacturer's instructions. The only modification to protocol made was a reduced final DNA elution volume of 100 µl.

2.3.2. Bacterial 16S rRNA metabarcoding

The pan-bacterial 16S rRNA targeting primers 515f Modified (5'-GTG YCA GCM GCC GCG GTA A-3') from Parada et al., 2016 and 806r Modified (5'-GGA CTA CNV GGG TWT CTA AT-3') from Apprill et al., 2015 were chosen, which amplify an approximately 250–300 bp region spanning the 16S hypervariable 4 (V4) region. This primer pair was selected over others tested, based on its accuracy at identifying VBD present in mock communities that combined between one and five different vector-borne bacterial DNA

positive controls. Initially, these primers when tested on canine blood DNA returned many reads identified as canine mitochondrial rRNA sequences, demonstrating cross-reactivity with host DNA (data not shown). To limit this, a degenerate base was removed from both the forward and reverse primers to reduce cross-reactivity, after primer alignment with canine mitochondrial rRNA sequences. The final primers used were Wehi_Adp_515F (5'-GTG YCA GCAGCC GCG GTA A-3') and Wehi_Adp_806R (5'-GGA CTA CNV GGG TAT CTA AT-3') with the modified base underlined.

Three separate physical containment areas were utilised for DNA extraction, pre-PCR and post-PCR experiments. All PCRs were prepared in a PCR hood under aseptic conditions following UV sterilisation. Optimal reaction mixtures for amplification were found to be 20 µl comprising 10 µl of OneTaq® 2× Master Mix with Standard Buffer (New England Biolabs, Ipswich, MA, USA) 0.2 µM of both forward and reverse primers, 1 µl of template DNA and 8.2 µl of Ambion Nuclease-Free Water (Life Technologies, Carlsbad, CA, USA). All PCRs were run with positive and no-template negative controls to check for cross-contamination. Field-based DNA extraction negative controls were also run, utilising blood from canines known to be vector-borne bacteria free, to ascertain the normal bacterial microbiota of healthy canines from the sampling region and to detect contaminant bacteria from the DNA extraction kits used.

Optimal thermocycling conditions for the selected 16S rRNA primers were found to be an initial denaturation of 95 °C for 3 min, followed by 35 cycles of 95 °C for 45 s, 56 °C for 60 s and 72 °C for 90 s with a final elongation at 72 °C for 10 min. During PCR optimisation experiments amplicons were run and visualised on a 1.5% agarose gel using a ChemiDoc™ System (Bio-Rad, Hercules, CA, USA).

Deep sequencing of 16S rRNA amplicon metabarcodes was carried out according to Aubrey et al., 2015. Briefly, the aforementioned first-step PCR was completed with the addition of overhang sequences at the 5' end of the Wehi_Adp primers. The overhang sequence added to the 5' end of the forward primer was 5'-GTG ACC TAT GAA CTC AGG AGT C-3' and to the 5' end of the reverse primer was 5'-CTG AGA CTT GCA CAT CGC AGC-3'. PCR product was then cleaned using 1× Ampure Beads (Beckman Coulter, Brea, CA, USA). A second PCR step was then carried out introducing eight-base forward and reverse indexing sequences, permitting multiplexing of amplicons

onto a single run. Eight forward indexes and 13 reverse indexes were used allowing for multiplexing of 104 bacterial 16S amplicons, including two no template negative controls and two positive controls (a uniquely identifiable *Rickettsia felis* strain URRWXCal2 from cell culture).

Thermocycling conditions for this second PCR were an initial denaturation of 95 °C for 2 min, followed by 24 cycles of 95 °C for 15 s, 60 °C for 15 s and 72 °C for 30 s with a final elongation at 72 °C for 7 min. Amplicon size distribution was analysed using an Agilent 2200 TapeStation (Agilent, Santa Clara, CA, USA), pooled and then purified using 0.7X Ampure Beads to exclude primer-dimer products (Aubrey et al., 2015). The purified amplicon pool was then quantified using a Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA) and run on an Illumina MiSeq (Illumina, San Diego, CA, USA) using 600-cycle v3 chemistry (2 × 300 bp paired-end reads) at the Walter & Eliza Hall Institute Proteomics Facility, Parkville, Australia.

2.3.3. Bioinformatics analysis

Raw data was demultiplexed using in-house software at the Walter & Eliza Hall Institute and then imported into the QIIME 2 (version 2018.8) environment for bioinformatic processing (Caporaso et al., 2010; McDonald et al., 2012; McKinney, 2010; Rideout et al., 2018). The cutadapt plugin (Marcel, 2011) was used to trim primer, adapter and index sequences from raw reads, followed by inspection for quality, using QIIME2 View. Removal of low quality reads, denoising, dereplicating, filtering of chimeras and merging of forward and reverse reads was then performed using DADA2 (Callahan et al., 2016). Truncation parameters for DADA2 were decided upon using visual assessment of read quality plots, so that truncation was performed where read quality dropped off (quality score of approximately 35). Next, VSEARCH (Rognes et al., 2016) was used to cluster reads into 97% similarity *de novo* operational taxonomic units (OTUs). The scikit-learn classifier (Pedregosa et al., 2011) was then used to taxonomically assign these OTU consensus sequences, against the SILVA version 132 reference database, downloaded from docs.qiime2.org. SILVA was preferable to the Greengenes database as it was able to taxonomically assign more OTUs. SILVA-based scikit-learn assignment was corroborated using the BLASTn

program in GenBank (NCBI) to taxonomically identify the same OTUs, in some cases this permitted identification to a lower taxonomic level. Unassigned sequences or those only assigned to kingdom and phylum were excluded from the final dataset. Sequences reported from negative control samples were subtracted from the overall dataset and only the results of known, or suspected, vector-borne bacteria were reported. Alpha rarefaction plots were generated, using MAFFT (Kato and Standley, 2013) and FastTree 2 (Price et al., 2010), to ensure that OTU diversity plateaued and hence a sufficient sequencing depth had been achieved. All NGS data produced in the present study are available from the BioProject database, BioProjectID: PRJNA528154 and SRA data accession numbers SRR8894273 to SRR8894371.

Infections were considered true by NGS, if a sample had a vector-borne bacterial read count of 113 or over. This threshold was determined as the mean reads of four canine DNA samples that were identified as having sequences from the positive controls used within the library preparation, due to occasional index misreading or hybridisation errors during Illumina sequencing (Kim et al., 2017). This was supported by assessment of where on the 96-well plate the samples with positive control sequences appeared, which showed no relationship with proximity to positive control location. The average Phred quality score over the adapter and indexing regions for the raw data was 33 which indicates an error rate of between one in 1,000 to 10,000, highlighting how occasional sequencing artefacts may have led to index misreading.

2.3.4. Conventional PCR and Sanger sequencing

To compare the detection ability of our NGS method with traditional molecular techniques all 100 samples were tested for *E. canis*, *A. platys*, *Mycoplasma* spp. and *Rickettsia* Spotted Fever Group (SFG) species by specific endpoint conventional PCR screens from the literature (**Table 2.1**).

To confirm vector-borne bacteria identification by NGS, a subset of samples from each taxon were corroborated by Sanger sequencing. This subset of PCR amplicons was purified using the ExoSAP-IT™ PCR Product Cleanup Reagent kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. Cleaned amplicons were sent to Macrogen (Seoul, South Korea) for Sanger sequencing.

2.3.5. Statistical analysis

Analysis of results was conducted in Excel 2016 version 1803 (Microsoft), whilst Kappa statistics to compare concordance of NGS vs endpoint cPCR results were calculated in SPSS Statistics 24 (IBM).

Table 2.1. Primers used for conventional PCR, real-time PCR and taxonomic cross-validation of NGS results.

Taxon targeted	PCR type	Primer pair (5'-3')	Gene targeted	Product size (bp)	Reference
<i>Ehrlichia canis</i> -specific	cPCR	ECA: AACACATGCAA GTCGAACGGA HE3: TATAGGTACCG TCATTATCTTCCCTAT	16S rRNA	400	(Gal et al., 2008)
<i>Anaplasma platys</i> -specific	cPCR	PLATYS-F: GATTTTGT TCGTAGCTTGCTATG EHR16S-R: TAGCACT CATCGTTTACAGC	16S rRNA	678	(Inokuma et al., 2002)
<i>Mycoplasma</i> spp.-specific	cPCR	HBT-F: ATACGGCCCA TATTCCTACG HBT-R: TGCTCCACCA CTTGTTCA	16S rRNA	600	(Criado-Fornelio et al., 2003)
<i>Rickettsia</i> Spotted Fever Group	cPCR	ompB-F: CGACGTTAAC GGTTTCTCATTCT ompB-R – ACCGGTTTCT TTGTAGTTTTCGTC	Outer membrane protein B (<i>ompB</i>)	252	(Paris et al., 2008)
<i>Bartonella</i> spp.-specific	cPCR	prAPT0257: GCCTTCAAGGAGTTGAT TTTGTTGTTGCCAAT prAPT0258: ACGACCCATTTCAT GCATAACAGAAC	Filamenting temperature-sensitive mutant Z (<i>ftsZ</i>)	500	(Belkhiria et al., 2017)
Filarial worm-specific	cPCR	DIDR-F1: AGTGCGAATTGCA GACGCATTGAG DIDR-R1 – AGCGGGTAATCACG ACTGAGTTGA	5.8S-ITS2-28S	430– 660	(Rishniw et al., 2006)
<i>Rickettsia</i> Spotted Fever and Typhus Groups	qPCR	CS-F: TCGCAAATGTTCA CGGTACTTT CS-R: TCGTGCATTCTT TCCATTGTG CS-P (Probe): 6-FAM-TGCAATAGAAGA ACCGTAGG CTGGATG-BHQ-1	Citrate synthase (<i>gltA</i>)	74	(Stenos et al., 2005)

2.4. Results

2.4.1. NGS bioinformatic analysis and characterisation

In total 15,162,431 (median 148,045) raw paired-end reads were obtained for the 104 multiplexed bacterial 16S amplicons, including two positive and two negative controls. After the DADA2 quality filtering, dereplication, chimera removal and pair-joining step, a total of 7,570,278 (median 73,088) joined sequences (49.9%) were carried forward to the next bioinformatic step. At the OTU clustering stage 723 OTUs were formed of which 94 could not be given any taxonomical assignment and 42 could only be identified to the level of kingdom (39 bacteria; 3 eukaryota). Unassigned OTUs represented 47% of the total filtered reads and were removed from the dataset, many of these sequences were identified as canine mitochondrial rRNA sequences using BLASTn in GenBank. Of the remaining 587 OTUs, 386 were identified down to at least genus level, whilst 42 received a species level classification, using the scikit-learn plug-in. All other OTUs were either assigned to taxonomic levels between kingdom and genus or had top matches with records in the SILVA database that had not originally been classified to the level of species e.g. '*Pasteurellaceae* bacterium canine oral taxon'. Positive control DNA sequences were detected at the end of bioinformatic processing. Bacterial sequences found in negative controls were subtracted across all samples in our dataset and only species known to, or suspected to be, vector-borne bacteria were reported. After taxonomic assignment of OTUs eight were from relevant, or suspected, vector-borne bacteria including, *Ehrlichia canis*, *Mycoplasma haemocanis*, "*Candidatus* *Mycoplasma haematoparvum*", *Mycoplasma turicensis*, *Anaplasma platys*, *Bartonella* spp., *Rickettsia* spp. and *Wolbachia* spp. A diverse range of other bacterial sequences were also detected via our NGS-methodology, but their detection was not the focus of this study, some information regarding these can be found in **Table 2.2**.

From the 100 blood samples tested, our NGS-based detection method found 40 to be positive for *E. canis*, 39 for *Mycoplasma* spp. (34 identified as *M. haemocanis*, three as "*Ca. M. haematoparvum*" and two as *M. turicensis*), 25 for *A. platys* and one for *Bartonella* spp., whilst no samples were found to be positive for *Rickettsia* spp. DNA (**Table 2.3**). Other noteworthy taxonomic hits were two samples found positive for *Wolbachia* spp. DNA known to be an endosymbiont of nematodes and arthropods and

one sample found positive for *Brucella* spp. DNA. Many commensal or contaminant bacterial species of the skin and environment were also identified from samples (**Table 2.2**).

NGS detected a total of 27 bacterial species co-infections, of which 22 comprised two and 5 comprised three canine vector-borne bacteria species. **Table 2.3** shows the number and composition of all bacterial co-infections found. Infections were considered true, if a sample had a vector-borne bacterial read count of 113 or over (see Methods for determination of this cut-off).

2.4.2. Comparison and confirmation of metabarcoding results

Conventional and real-time PCR assays were carried out with which to compare the results of our NGS methodology. Of the 100 canine DNA samples tested, 38 were found positive by an *E. canis*-specific cPCR (Gal et al., 2008), 40 by a *Mycoplasma* genus-specific cPCR (Criado-Fornelio et al., 2003), 15 for a *Rickettsia* spp. Spotted Fever Group (SFG) and Transitional Group-specific cPCR (Paris et al., 2008) and 12 for an *A. platys*-specific cPCR (Inokuma et al., 2002). Combining the results of the separate cPCR screens, 30 dogs were found to be co-infected with vector-borne bacteria; 26 with two bacterial species and 4 with three species (**Table 2.3**). In addition, a separate *Rickettsia* genus-specific citrate synthase gene (*gltA*) targeting real-time PCR (Stenos et al., 2005) was conducted to explore differences in PCR detection ability depending on bacterial gene targeted. This real-time PCR assay found all samples to be negative for *Rickettsia* spp., providing 100% agreement with the NGS results for *Rickettsia*.

Table 2.4 displays the agreement statistics between the NGS and cPCR methodologies. Detection of *Mycoplasma* spp. proved to be the most concordant between the two screening methods with a good level of agreement as defined by the Kappa statistic. Agreement between the two methods when detecting *A. platys* and *E. canis* was not as strong, with both pathogens achieving concordance at a moderate level of agreement, indicating a significant amount of disparity between the results of the two tests for these bacteria. Overall, the two tests demonstrated similar detection capabilities for identifying *E. canis* and *Mycoplasma* spp. as determined by the number

of infections found by both tests. However, the NGS method was better at detecting *A. platys* infection, and more varied bacteria such as *Bartonella* spp. or *Wolbachia* spp., in contrast to the cPCR screen which outperformed the NGS method in its ability to detect *Rickettsia* spp. infection.

Table 2.2. Most abundant bacteria not suspected to be vector-borne pathogens. Bacterial species, genera and families detected via our NGS methodology that are not suspected pathogens across all samples as a percentage of total reads that passed filtering. *Mycoplasma* spp., *E. canis* and *A. platys* were all more abundant than these commensal or contaminant bacterial groups.

Taxonomic Assignment	Reads (% of Total)
<i>Stenotrophomonas</i> spp.	2.49
Burkholderiaceae	1.97
<i>Sediminibacterium</i> spp.	0.63
<i>Staphylococcus</i> spp.	0.36
Caulobacteraceae	0.38
<i>Wolbachia</i> spp.	0.37
<i>Acinetobacter</i> spp.	0.27
Enterobacteriaceae	0.23
Geminicoccaceae	0.20
<i>Pseudomonas</i> spp.	0.19
<i>Enhydrobacter</i> spp.	0.19
<i>Corynebacterium</i> spp.	0.17
<i>Bradyrhizobium</i> spp.	0.17
<i>Sphingomonas</i> spp.	0.15
<i>Pasteurellaceae</i> spp.	0.14
<i>Anaerobacillus</i> spp.	0.11
<i>Streptococcus</i> spp.	0.10

Table 2.3. Percentage of canine blood samples found positive for a vector-borne bacteria using NGS and conventional PCR screening ($n = 100$ dogs).

Pathogen	NGS % Positive	cPCR % Positive
<i>Ehrlichia canis</i>	40	38
<i>Mycoplasma</i> spp.	39	40
<i>Anaplasma platys</i>	25	12
<i>Bartonella</i> spp.	1	1
<i>Rickettsia</i> spp.	0	15
<i>E. canis</i> & <i>Mycoplasma</i> spp.	9	12
<i>E. canis</i> & <i>A. platys</i>	7	5
<i>Mycoplasma</i> spp. & <i>A. platys</i>	5	1
<i>Mycoplasma</i> spp. & <i>Bartonella</i> spp.	1	1
<i>E. canis</i> & <i>Rickettsia</i> spp.	0	2
<i>A. platys</i> & <i>Rickettsia</i> spp.	0	1
<i>Rickettsia</i> spp. & <i>Mycoplasma</i> spp.	0	4
<i>E. canis</i> & <i>A. platys</i> & <i>Mycoplasma</i> spp.	5	2
<i>E. canis</i> & <i>Rickettsia</i> spp. & <i>Mycoplasma</i> spp.	0	2
Total infected dogs	75	74
Total co-infected dogs	27	30

Table 2.4. Bacterial NGS and cPCR agreement statistics.

VBD	cPCR	Bacterial NGS		Total agreement (%)	Kappa ^a (agreement statistic)	Kappa SE
		POS	NEG			
<i>E. canis</i>	POS	26	12	74	0.454	0.091
	NEG	14	48		(Moderate)	
<i>A. platys</i>	POS	12	0	87	0.581	0.098
	NEG	13	75		(Moderate)	
<i>Mycoplasma</i> spp.	POS	35	5	91	0.812	0.06
	NEG	4	56		(Very good)	

^aKappa agreement level: $K < 0.2$, poor; 0.21–0.40, fair; 0.41–0.60, moderate; 0.61–0.80, good; 0.81–1.00, very good

Cross-validation of NGS results was carried out using endpoint cPCR to amplify longer 16S rRNA sequences or sequences from other barcoding genes to assess the accuracy of, or improve upon, the level of identification achieved using NGS. In some cases, these were additional to the cPCR assays used to compare detection ability with our NGS method (**Table 2.1**). The majority of amplicons produced using an *E. canis*-specific PCR (Gal et al., 2008) achieved a 100% query cover and identity match with *E. canis* isolate b2-15 (GenBank: KY594915.1) using the GenBank BLASTn tool. A subset of samples identified as *A. platys* by NGS achieved a 100% query cover and identity match with *A. platys* isolate D35 (GenBank: KX792089.2) using an *A. platys*-specific PCR (Inokuma et al., 2002). In addition, the three different *Mycoplasma* species elucidated using NGS were supported by Sanger sequencing, which found a 100% query cover and identity match with *M. haemocanis* isolate F17 (GenBank: KY117659.1) and “*Ca. M. haematoparvum*” (GenBank: KF366443.1) as well as a 99% query cover and 100% identity match with *M. turicensis* isolate F21 (GenBank: KY117663.1).

The sample identified by NGS as having *Bartonella* spp. DNA was successfully cross-validated using a *Bartonella* ftsZ targeting PCR (Belkhiria et al., 2017) that upon sequencing obtained a 100% query cover and 97% identity match with *Bartonella clarridgeiae* strain 73 (GenBank: FN645454.1).

The two samples that were identified as having *Wolbachia* spp. endosymbiont DNA by NGS were reanalysed using a filarial worm specific PCR (Rishniw et al., 2006). This was done to attempt to elucidate whether the presence of blood-borne *Wolbachia* spp. might represent microfilaremia at the time of sampling, as filarial worms harbour these bacterial endosymbionts (Taylor et al., 2005). One of the two *Wolbachia* spp.-positive samples amplified a filarial worm specific PCR product using endpoint cPCR and was identified as *Brugia* spp. via a BLASTn search with query cover 100% and identity 98–99% to both *Brugia pahangi* (GenBank: EU373655.1) and *Brugia malayi* (GenBank: EU373619.1). One of four randomly tested samples that was *Wolbachia* spp. negative by NGS was amplified using the same filarial worm specific PCR and returned a BLASTn match with *Dirofilaria immitis* clone D2 5.8S (GenBank: JX866681.1; query cover 100%; identity 98%), demonstrating that the presence of *Wolbachia* spp. DNA may highlight filarial infections by some species but not others.

Sanger sequencing of amplicons produced by the *Rickettsia* SFG specific PCR consistently returned BLASTn hits with *Rickettsia asembonensis* (GenBank: LC431491.1; query cover 100%; identity 99%), followed by *Rickettsia felis* clone Ar3 (GenBank: GQ385243.1; query cover 100%; identity 99%), making exact discrimination of the *Rickettsia* spp. involved inconclusive.

2.5. Discussion

To the best of our knowledge this study represents the first use of an Illumina-based NGS detection screen to identify vector-borne bacteria in canine blood. Our method found 40 samples to be positive for *E. canis*, 39 for *Mycoplasma* spp., 25 for *A. platys* and one for *Bartonella* spp. finding an equivalent number of vector-borne bacteria positive dogs when compared to targeted cPCR analysis. Moreover, our method was able to accurately identify bacterial pathogens to species level taxonomic assignment whilst at the same time also identifying rare or unusual pathogens that would not typically be screened for using cPCR assays. Both techniques demonstrated substantial disparity in which bacteria they were able to detect and to what degree. For example, endpoint cPCR screening missed 13 *A. platys* NGS-positive results, whilst our NGS method was unable to detect any of the 15 *Rickettsia* spp. infections detected by endpoint PCR. The two methodologies showed good concordance when detecting *Mycoplasma* spp. DNA from blood. On the other hand, detection of *E. canis* varied greatly between the two techniques, despite both methods finding a similar number of total individuals with *E. canis* infection.

A. platys is an important intracytoplasmic platelet infection of dogs capable of generating thrombocytopenia, fever and lethargy with symptomology being exacerbated during mixed infections with other vector-borne pathogens (Little, 2010; Rar and Golovljova, 2011). Detection of this bacteria was much more sensitive when using our high-throughput approach, compared to the cPCR method by Inokuma et al. (2002) as demonstrated by the 13 infections missed by this screen. In the context of *A. platys* nested conventional PCR screens that first use bacterial generic primers followed by a species specific internal pair have been shown to be more sensitive for the detection of this pathogen in canines (Martin et al., 2005) potentially explaining the discrepancy in the current study. The 25 *A. platys* infections found in our subset of

Thai dogs was higher than those found in a canine VBD study in the same country which found a prevalence of 4.4% from 181 individuals (Liu et al., 2016). Nonetheless, a cPCR as opposed to nested PCR was utilised in this study whilst different sampling locations were also investigated, potentially explaining this difference.

Haemotropic *Mycoplasma* species are ubiquitous pathogens of dogs and other mammals across the globe, able to produce haemolytic anaemia, particularly in immunocompromised hosts (de Sousa et al., 2017; Willi et al., 2007a). The two most prevalent canine infecting *Mycoplasma* species are *M. haemocanis* and “*Ca. M. haematoparvum*” which were also the most common species identified by our NGS-based protocol in the present study (Soto et al., 2017; Wengi et al., 2008). In the case of this bacterial genus, the results of both detection methods corroborated well, with a high Kappa statistic of 0.812 and a similar number of individuals found infected using both methods. Furthermore, our results are supported by other studies completed in the region which found 19.9% of stray dogs in South Thailand (Liu et al., 2016) and 12.8% of dogs in northern Cambodia (Inpankaew et al., 2016) to be infected by a *Mycoplasma* species. The identification of two canines infected with *M. turicensis* was unexpected given that this is typically associated as a pathogen of felines (Sykes, 2010). Nonetheless, this species has been identified in a domestic dog in Brazil (González et al., 2010) and Chile (Soto et al., 2017), as well as wild animals including Darwin’s foxes (Cabello et al., 2013), lions and ocelots (Willi et al., 2007b), amongst others. Therefore, whether the presence of *M. turicensis* within the Thai dogs sampled in the present study represents sustained infection and transmission in these populations or occasional spill over from wild animals is not possible to ascertain. Nonetheless, it must be acknowledged that molecular-based diagnosis detects the presence of pathogen DNA but does not necessarily provide an indication of current and/or viable infection, despite this often being the case (Peters et al., 2004).

Although *B. clarridgeiae* has been identified in fleas and cats in Thailand before, to the best of the authors’ knowledge, this is the first report of this species from a dog in the country (Maruyama et al., 2001; Parola et al., 2003). *Bartonella clarridgeiae* has been detected in canines previously and shown to cause severe host pathology, such as aortic endocarditis and hepatic disease (Chomel et al., 2001; Gillespie et al., 2003; Gundi et al., 2004; Kim et al., 2009). Furthermore, this species is now a suspected zoonotic pathogen, due to a veterinarian reporting the development of cat-scratch

disease (CSD), following the bite of a *B. clarridgeiae* infected cat (Kim et al., 2009; Maruyama et al., 2001; Parola et al., 2003). Taking this into consideration, the detection of *Bartonella* spp. via our NGS method highlights the main benefit of NGS-based techniques to permit the detection of rare and/or unexpected pathogens not typically screened for but potentially able to cause animal and human disease.

The detection of *Wolbachia* spp. endosymbiont sequences via NGS in samples from two canines was further explored to assess whether the presence of these sequences could be utilised as a proxy for filarial worm infection (Casiraghi et al., 2001; Taylor et al., 2005). This was supported by a study that demonstrated that *Wolbachia* species phylogeny is largely congruent with that of the filarial worm host (Casiraghi et al., 2001). However, a filarial worm-specific cPCR screen (Rishniw et al., 2006) only achieved amplification from one of these *Wolbachia*-positive samples, identified as either *B. pahangi* or *B. malayi*, the latter of which is a causative agent of lymphatic filariasis in humans (Nuchprayoon et al., 2005). Another sample that was *Wolbachia* sequence negative but was also screened using the filarial PCR assay returned positive amplification for *D. immitis* demonstrating that the presence of *Wolbachia* DNA was an unreliable proxy for infection with filaria. Furthermore, many arthropod vectors also harbour *Wolbachia* endosymbionts and therefore the appearance of this bacteria DNA may represent the incidental presence of *Wolbachia* on the host dog's skin at the time of sampling, deposited by a dead or passing arthropod (Werren et al., 2008).

When detecting the important canine pathogen *E. canis*, which generates severe disease in infected individuals (Alexandre et al., 2009), the two detection methods assessed differed substantially in which samples they found positive for this bacteria. Twenty-six samples had discordant results; with the NGS methodology finding 14 positive results that were missed by the conventional screen, in comparison to 12 that were missed by NGS. Despite this, both methodologies reported rates of *E. canis* infection higher than the 3.9% rate found previously in Thailand (Liu et al., 2016). *Ehrlichia canis* detection can be substantially improved via fractionation of blood and targeting of the Buffy Coat layer that acts to concentrate circulating monocytes; the principal cell type infected by this pathogen (Alexandre et al., 2009; Jongejan et al., 2016; Mylonakis et al., 2003; Rar and Golovljova, 2011). Fractionation to test Buffy Coat extracted DNA has been demonstrated to provide good molecular detection of

E. canis and therefore the absence of this concentration method within our DNA extraction protocol may explain some of the putative missed infections (Alexandre et al., 2009). Furthermore, the 16S rRNA gene our NGS method targets could be partially responsible for the lower ability to detect infections in some samples. For example, the *E. canis* p30 outer membrane protein genes which are present in very high copy numbers per bacterial cell can improve detection ability by as much as 100 times compared to 16S rRNA based screens (Jittapalapong et al., 2002). On the other hand, the accuracy of the conventional PCR screen must also be assessed. With this pathogen a nested PCR was not used, therefore, future rectification to use a nested screen (Alexandre et al., 2009), alongside replicates (Harrus and Waner, 2011), would assist in ascertaining the true infection status of such discordant results.

The lack of the NGS-assay's ability to detect natural *Rickettsia* spp. infection is problematic given that the *R. felis*-complex of rickettsiae are being increasingly detected in dog blood, making the canine host a potential reservoir for this flea-borne zoonosis (Hii et al., 2011a, 2011b; Inpankaew et al., 2016). In addition, a highly sensitive rickettsial *gltA*-targeting real-time PCR (Stenos et al., 2005) also found no *Rickettsia* spp. infection. This discrepancy between the cPCR's ability to detect these infections compared to real-time PCR and NGS is likely due to the target gene used by each technique. To maintain a pan-bacterial range of detection, our NGS method had to use the highly conserved 16S rRNA gene, whilst the cPCR screen used the rickettsial genus-specific *ompB* gene (Stenos and Walker, 2000). It has been established that amplification of outer membrane protein genes is more sensitive than 16S rRNA targeting, because these genes exist in higher copy numbers per bacterial cell and are therefore easier to detect (Paris et al., 2008; Santib   ez et al., 2013). PCR-based detection of *Rickettsia* is further exacerbated by typically low quantities of circulating bacteria, especially during chronic, relapsing infections which may have further hindered the ability of our NGS method to detect this genus (Portillo et al., 2017). Our NGS methodology used positive control DNA from a highly concentrated, cell culture grown *R. felis* strain URRWXC  2, which was detected by NGS. This indicates that the 16S rRNA primers used by our NGS methodology are capable of amplifying *R. felis* DNA, although potentially not at the concentrations found in natural infections. Future development of our technique may need to consider a

supplementary PCR screen using an alternative rickettsial gene target that would improve detection ability and provision species level assignment.

After initial pilot experiments, modifications were made to the 515f (Apprill et al., 2015) and 806r (Parada et al., 2016) bacterial 16S rRNA primers to reduce base pair degeneracy and therefore lessen the cross-reactivity potential on host mitochondrial 12S rRNA sequences. Despite this, as many as 47% of total filtered paired end reads were unable to be taxonomically assigned by the scikit-learn classifier against the SILVA database of which a large proportion represented continued primer cross-reactivity to canine sequences. This is likely due to the sheer abundance of host mitochondrial DNA compared to the relatively small proportion of circulating bacterial DNA, signifying that even with poor primer complementarity to host sequences, the overwhelming amount of these sequences meant bacterial DNA was outcompeted for primer binding. A similar problem was tackled by Gofton et al. (2015) within the context of the tick microbiome which is dominated by the endosymbiotic bacterium "*Ca. Midichloria mitochondri*". These authors used blocking primers to inhibit the amplification of "*Ca. M. mitochondri*" 16S rRNA sequences during the first round of PCR amplification, allowing better characterisation of the tick microbiome and uncovering of new species that had previously been masked by dominating endosymbiont sequences (Gofton et al., 2015). Further development of our NGS-based methodology could explore the possibility of a similar approach by preventing mitochondrial sequence amplification and thus improving the detection of low abundance pathogens to augment the assay's ability to detect vector-borne bacteria in general.

Our deep sequencing method also elucidated many non-pathogenic bacterial OTUs from our canine blood samples with 380 being identified down to genus level (**Table 2.2**). This is likely due to contamination of blood samples during insertion of the collection needle through the skin, hence the prevalence of common skin commensal species such as *Staphylococcus* spp., *Corynebacterium* spp. and *Streptococcus* spp. (Chiller et al., 2001). Similar findings have been achieved by other researchers working on blood 16S rRNA metabarcoding, unearthing environmental contaminant species, despite the supposed sterile nature of the blood compartment (Decuypere et al., 2016; Gyarmati et al., 2015). The situation is further compounded by the frequent contamination of DNA extraction kits and PCR reagents with *Bradyrhizobium* spp.

(Laurence et al., 2014). This genus, amongst others, was also detected in the present study from negative controls, with such identifications then subtracted from the overall dataset, permitting identification of bacteria arising from the host from those that were commensal or contaminant.

Finally, when comparing our metabarcoding approach with traditional cPCR methods and Sanger sequencing both financial, time and workload considerations must be factored in. For each canine sample to be screened via NGS the associated cost was $\text{AU\$}2415 \div 104 = \text{AU\$}23.2$ per sample, whilst Sanger sequencing of each positive band typically costs AU\$19.9. Given the present results, the total cost of Sanger sequencing of all cPCR positive results would have been $105 \times \text{AU\$}19.9 = \text{AU\$}2089.5$ for detection of the four-principle bacterial groups, i.e. *E. canis*, *A. platys*, *Rickettsia* spp. and *Mycoplasma* spp. This represents a relatively modest price difference, with conventional methods being AU\$325.5 cheaper. NGS methods do accrue additional time costs via the need for lengthy bioinformatic processing that must be conducted to handle the large datasets they generate. However, the employment of automatic bioinformatic pipelines can make NGS quicker than cPCR when factoring in the additional labour and time required to conduct the multiple cPCR reactions needed to thoroughly characterise pathogen diversity using this technique. Taking all this into consideration, our metabarcoding methodology could be more economical in terms of cost and time spent, particularly if used in areas where canine vector-borne bacteria is even more prevalent than found here, as higher infection levels would require more Sanger sequencing and thus greater per sample expenditure.

2.6. Conclusions

For the first time, we have developed and assessed the use of a 16S metabarcoding methodology for the simultaneous detection of vector-borne bacteria from canine blood. This assay has proven to be more sensitive than endpoint cPCR and Sanger sequencing for the detection of vector-borne bacteria, such as *A. platys*, better able to characterise rare pathogens and with greater potential to characterise bacterial pathogen species diversity. Despite limitations regarding the detection of *Rickettsia*, this study lays down a crucial framework from which our method can be refined i.e. via use of blocking primers to achieve a greater depth of bacterial sequences returned or

the use of auxiliary screens for pathogen groups that are difficult to characterise. Our methodology demonstrates great potential as a tool in the armoury of veterinary screens that can be used for surveillance of canine vector-borne bacteria due to its ability to detect rare and novel organisms. This tenet is especially important in the tropics where vector-borne pathogen diversity reaches its peak, but data is limited, whilst also equipping us with a tool that can be used to elucidate and monitor emerging zoonotic threats from these regions (Irwin and Jefferies, 2004; Traub et al., 2015).

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Chapter 3 - A novel metabarcoding diagnostic tool to explore protozoan haemoparasite diversity in mammals: A proof-of-concept study using canines from the tropics

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3.1. Abstract

Haemoparasites are responsible for some of the most prevalent and debilitating canine illnesses across the globe, whilst also posing a significant zoonotic risk to humankind. Nowhere are the effects of such parasites more pronounced than in developing countries in the tropics where the abundance and diversity of ectoparasites that transmit these pathogens reaches its zenith. Here we describe the use of a novel next-generation sequencing (NGS) metabarcoding based approach to screen for a range of blood-borne apicomplexan and kinetoplastid parasites from populations of temple dogs in Bangkok, Thailand. Our methodology elucidated high rates of *Hepatozoon canis* and *Babesia vogeli* infection, whilst also being able to characterise co-infections. In addition, our approach was confirmed to be more sensitive than conventional endpoint PCR diagnostic methods. Two kinetoplastid infections were also detected, including one by *Trypanosoma evansi*, a pathogen that is rarely screened for in dogs and another by *Parabodo caudatus*, a poorly documented organism that has been previously reported inhabiting the urinary tract of a dog with haematuria. Such results demonstrate the power of NGS methodologies to unearth rare and unusual pathogens, especially in regions of the world where limited information on canine vector-borne haemoparasites exist.

3.2. Introduction

Protozoan haemoparasites generate some of the highest rates of morbidity and mortality in canines worldwide, whilst some are also zoonotic, capable of producing significant infections in humans as well (Cassini et al., 2009; Irwin and Jefferies, 2004; Megat Abd Rani et al., 2010; Otranto et al., 2009). The principal taxonomic groups responsible are the bloodborne piroplasmids and kinetoplastids which are transmitted by haematophagous arthropods, such as ticks, fleas, sand-flies and mosquitoes, as vector-borne diseases (VBDs) (Beugnet and Marié, 2009; Otranto et al., 2009). Examples of haemoparasite zoonoses include leishmaniasis which has long been identified as an important canine VBD with a widespread, and in some regions expanding distribution (Baneth et al., 2016; Colella et al., 2019), whilst non-zoonotic diseases such as canine, equine or bovine babesiosis are nevertheless critically important diseases from a veterinary standpoint, with some species now recognised as key emerging pathogens (Fourie et al., 2013; Prakash et al., 2018).

Apicomplexan *Babesia* spp. parasites are transmitted by tick vectors which invade erythrocytes and cause a spectrum of anaemia-related pathology depending on the species, from the relatively benign *Babesia vogeli* to the more virulent *Babesia canis* and *Babesia rossi* species (Irwin, 2009; Megat Abd Rani et al., 2010). Whilst it has not been confirmed that canine-infecting *Babesia* spp. can infect people, other members of the genus, including *Babesia microti* present a severe zoonotic threat (Prakash et al., 2018; Vial and Gorenflot, 2006). In the tropics, kinetoplastid parasites such as *Trypanosoma evansi*, that are important livestock pathogens, can also frequently produce fatal infections in dogs (Irwin and Jefferies, 2004). Furthermore, canines are the primary zoonotic reservoir for *Leishmania infantum*, a kinetoplastid capable of causing a visceral, multi-organ disease in dogs and immunocompromised humans and children, particularly in regions of South America, the Middle East and the Mediterranean (Colwell et al., 2011; Johnson and Fooks, 2014; Mencke, 2013).

Many of these haemoparasites are united by their ability to create enduring infections, that can last years, with periods of immunological control followed by remission (Dantas-Torres, 2007; Irwin, 2009; Ivanov and Tsachev, 2008). This tenet facilitates the formation of a haemoparasite microbiome as a single host accumulates more infections, including those from bacteria, viruses and metazoans, some of which can

be chronic and others short lived. Within the context of the canine blood microbiome a single haemoparasite may not be lethal, but still exert a toll on the dog in which it resides that may make the host more susceptible to other VBDs or make the pathogenesis of another parasite worse (Baneth et al., 2003; Rojas et al., 2014). For instance, *Hepatozoon canis*, typically generates a subclinical infection (Ivanov and Tsachev, 2008), however, when found to be coinfecting with *Babesia* spp. or bacterial VBDs a much more severe anaemia and overall disease outcome is generated (Rojas et al., 2014). Taking this into consideration, canine VBD diagnostic methods must be able to characterise the entire haemoparasite microbiome and not just a dominant pathogen within a host.

With the advent of next-generation sequencing (NGS) technologies the field of parasite diagnostic tests is in the process of being transformed. Conventional PCR (cPCR) methodologies such as endpoint or quantitative PCR, which themselves superseded laborious microscopy and culture-based methods, have always been limited in their assessment of microbiomes by the need for *a priori* data on a target species taxonomic barcoding sequence (Lecuit and Eloit, 2015; Ondrejicka et al., 2014). This restrains such methods to only detecting known and genetically characterised species, whilst ignoring rare or undiscovered species (Ondrejicka et al., 2014). Additionally, endpoint PCR coupled with Sanger sequencing is typically unable to detect more than the dominant sequence in an amplicon of potentially many, thereby making the technique of limited utility for recognising mixed infections (Zepeda Mendoza et al., 2015). NGS-based diagnosis mitigates such limitations as class, phylum or even kingdom specific primers can be used to amplify around barcode regions that are unique to each species. Amplified DNA from all the different species barcodes in a sample can be sequenced in massive parallelisation, generating a sample metabarcode of every species present from a taxonomic group of interest, thereby elucidating an entire microbiome from within a specific host environment (Barbosa et al., 2017; Vermeulen et al., 2016).

The aim of this study was to develop an NGS-based diagnostic tool to fully characterise the apicomplexan and kinetoplastid haemoparasite microbiome from blood DNA samples, thereby including the ability to detect novel, rare or poorly documented species. Moreover, we aimed to compare this novel NGS-based method to the sensitivity and detection range of conventional PCR methods. Semi-

domesticated and temple community canine populations from Thailand were chosen as there is relatively limited information regarding haemoparasite infection in Southeast Asian dogs, whilst the few studies that have been conducted have found parasite prevalence to be high (Inpankaew et al., 2016; Liu et al., 2016; Traub et al., 2015).

3.3. Methods

3.3.1. Ethical approval

Ethical approval was obtained from the Animal Ethics Committee of Kasetsart University, Bangkok, Thailand with work conducted under Ethics Permit: OACKU-00758. All experiments were performed in accordance with relevant guidelines and regulations as defined by the University of Melbourne and Kasetsart University, Bangkok.

3.3.2. Sampling and DNA extraction

This study was part of a larger umbrella project investigating canine and feline VBDs across Bangkok conducted by Kasetsart University, Faculty of Veterinary Medicine. A subset of 100 samples from a total of 1100 collected were used to conduct our NGS diagnostic comparison. Canine blood samples were collected from 35 Buddhist temple communities, after acquisition of informed consent from monks and caregivers. Sampling was done through cephalic or jugular puncture by a qualified veterinarian, collected into EDTA tubes and stored at -20°C until ready for use. DNA extraction was done using the E.Z.N.A.[®] Blood DNA Mini Kit (Omega Biotek Inc.) from a starting quantity of 250 µl whole blood according to the manufacturer's instructions, apart from a reduced final elution volume of 100 µl.

3.3.3. Apicomplexa and Kinetoplastida 18S rRNA metabarcoding

Sequence alignments for primer design were conducted using Geneious version 11.1.5 (Biomatters Ltd.), whilst Primer3 version 0.4.0 was used to assist in selection of primer sequences. Primer3 parameters were set to amplify regions of approximately 100 - 200 bps so that they could be successfully amplified by paired-end Illumina

sequencing. A large breadth of blood-borne apicomplexan and kinetoplastid genera were chosen for primer design so that our designed primers had the potential to amplify from as large a range of putative pathogenic organisms as possible. This was particularly important given the exploratory nature of this study and dearth of research into canine haemoparasites in SE Asia. The complete list of sequences used in our alignments are observable in **Table 3.1**.

The designed primers for Apicomplexa were ApicomplexF: (5'-CRAGGAAGTTTRAGGCAATAACAG -3') and ApicomplexR: (5'-CTAGGCATTCCTCGTTHAHGATT -3') which amplify an approximately 130 bp region towards the end of the 18S rRNA gene. The designed Kinetoplastida primers were Kinetof: (5'-CAAACGATGACACCCATGAA -3') and Kinetor: (5'-CCCCCTGAGACTGTAACCTC -3') which amplify an approximately 170 bp region in the middle of the 18S rRNA gene. Confirmed positive controls for *B. vogeli*, *B. gibsoni* and *H. canis* were used to optimise and find optimal reaction conditions for the Apicomplexa specific primers. DNA Positive controls for *L. infantum* and *T. theileri* clade from an Indonesian hill rat, *Bunomys penitus*, were used to find ideal PCR conditions for the Kinetoplastida primers (**Table 3.2**). All PCRs were prepared in a PCR hood under aseptic conditions following UV sterilisation. Optimal reaction mixtures for amplification were found to be 20 µl comprising 10 µl of OneTaq® 2X Master Mix with Standard Buffer (New England Biolabs), 0.5 µM of both forward and reverse primers, 1 µl of template DNA and 7 µl of Ambion Nuclease-Free Water (Life Technologies). All PCRs were run with no-template negative controls to check for cross-contamination. Primers were also tested for cross-reactivity against a range of different blood-infecting bacteria, protozoa and metazoa from outside of their target group (**Table 3.2**), from which they did not amplify. Optimal thermocycling conditions for the Apicomplexa primers were found to be an initial denaturation of 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 52 °C for 30 s and 72 °C for 30 s with a final elongation at 72 °C for 5 min. Thermocycling conditions for Kinetoplastida primers were initial denaturation of 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 56 °C for 30 s and 72 °C for 30 s with a final elongation at 72 °C for 5 min. During PCR optimisation experiments amplicons were run and visualised on a 1.5% agarose gel using a ChemiDoc™ System (Bio-Rad).

Deep sequencing of 18S rRNA amplicon metabarcodes was carried out according to Aubrey et al. (2015). Briefly, the aforementioned first-step PCR was completed with the addition of overhang sequences at the 5' end of the Apicomplexa and Kinetoplastida primers. The overhang sequence added to the 5' end of the forward primer was 5'- GTGACCTATGAACTCAGGAGTC -3' and to the 5' end of the reverse primer was 5'-CTGAGACTTGACATCGCAGC -3'. PCR product was then cleaned using 1X Ampure Beads (Beckman Coulter). A second PCR step was then carried out introducing 8-base forward and reverse indexing sequences, permitting multiplexing of amplicons onto a single run. Sixteen forward indexes and 26 reverse indexes were used allowing multiplexing of 104 Apicomplexa amplicons and 104 Kinetoplastida amplicons. For each target group 100 canine blood DNA samples were run alongside two no-template negative controls and two DNA positive controls that consisted of a previously sequenced unique *B. gibsoni* strain for the Apicomplexa PCR and two unique *T. theileri* strains for the Kinetoplastida PCR. These unique strains allowed for identification of the appearance of positive control sequences in samples other than controls during NGS data analysis. Thermocycling conditions for this second PCR were an initial denaturation of 95 °C for 2 min, followed by 24 cycles of 95 °C for 15 s, 60 °C for 15 s and 72 °C for 30 s with a final elongation at 72 °C for 7 min. Amplicon size distribution was analysed using an Agilent 2200 Tapestation (Agilent), pooled and then purified using 0.7X Ampure Beads to exclude primer-dimer products (Aubrey et al., 2015). The purified amplicon pool was then diluted using a Qubit 2.0 Fluorometer (Life Technologies) and run on an Illumina MiSeq (Illumina) using 300-cycle v2 chemistry (2 x 150 bp paired-end reads) at the Walter & Eliza Hall Institute Proteomics Facility.

Table 3.1. Parasite and host 18S rRNA sequences used in primer design. Asterisks denotes sequences used to check for primer to host cross-reactivity potential.

Apicomplexa Primer Design		Kinetoplastida Primer Design	
Species	NCBI Accession Number	Species	NCBI Accession Number
<i>Babesia gibsoni</i>	FJ769388	<i>Leishmania amazonensis</i>	GQ332354
<i>Babesia gibsoni</i>	KC461261	<i>Leishmania amazonensis</i>	JX030052
<i>Babesia vogeli</i>	KT333456	<i>Leishmania donovani</i>	GQ332356
<i>Babesia sp. EU1</i>	AY046575.1	<i>Leishmania chagasi</i>	GQ332357
<i>Babesia microti</i>	AB241631.1	<i>Leishmania infantum</i>	GQ332359
<i>Hepatozoon americanum</i>	AF176836	<i>Leishmania mexicana</i>	GQ332360
<i>Hepatozoon canis</i>	AY150067	<i>Leishmania major</i>	GQ332361
<i>Hepatozoon sipedon</i>	JN181157	<i>Leishmania tropica</i>	GQ332363
<i>Hepatozoon felis</i>	KM435071	<i>Leishmania aethiopica</i>	GQ920678
<i>Theileria velifera</i>	AF097993	<i>Trypanosoma cruzi</i>	CP015675
<i>Theileria ovis</i>	AY260172	<i>Trypanosoma simiae</i>	AJ404608
<i>Theileria buffeli</i>	DQ104611	<i>Trypanosoma rotatorium</i>	AJ009161
<i>Theileria annulata</i>	EU083801	<i>Trypanosoma avium</i>	AF416559
<i>Theileria sergenti</i>	EU083802	<i>Trypanosoma ranarum</i>	AF119810
<i>Theileria sinensis</i>	KF559355	<i>Trypanosoma neveulemairei</i>	AF119809
<i>Plasmodium berghei</i>	AJ243513	<i>Trypanosoma mega</i>	AF119808
<i>Plasmodium cathemerium</i>	AY625607	<i>Trypanosoma chattoni</i>	AF119807
<i>Plasmodium ovale</i>	KF018656	<i>Trypanosoma fallis</i>	AF119806
<i>Plasmodium fragile</i>	XR001111607	<i>Trypanosoma evansi</i>	AY904050.1
<i>Plasmodium vinckei</i>	XR552294	<i>Trypanosoma wauwau</i>	KT030835
<i>Toxoplasma gondii</i>	L24381.1	<i>Trypanosoma brucei</i>	XR002989632.1
<i>Canis lupus familiaris</i> *	AAEX03025866	<i>Canis lupus familiaris</i> *	AAEX03025866

Table 3.2. VBD species positive controls used to test designed primer specificity. Asterisks denote a VBD outside of the primer's target group and therefore a test for cross-reactivity.

Apicomplexa Primer Pair	Species Positive Control	PCR Result	Primer Pair Kinetoplastida	Species Positive Control	PCR Result
	<i>Babesia gibsoni</i>	+		<i>Leishmania infantum</i>	+
	<i>Babesia vogeli</i>	+		<i>Trypanosoma theileri</i> type 1	+
	<i>Hepatozoon canis</i>	+		<i>Trypanosoma theileri</i> type 2	+
	<i>Toxoplasma gondii</i>	+		<i>Babesia gibsoni</i> *	-
	<i>Leishmania infantum</i> *	-		<i>Babesia vogeli</i> *	-
	<i>Trypanosoma theileri</i> type 1*	-		<i>Dirofilaria immitis</i> *	-
	<i>Dirofilaria immitis</i> *	-		<i>Rickettsia typhi</i> *	-
	<i>Rickettsia typhi</i> *	-		<i>Anaplasma platys</i> *	-
	<i>Rickettsia felis</i> *	-		<i>Coxiella burnetti</i> *	-
	<i>Anaplasma platys</i> *	-		<i>Mycoplasma haemocanis</i> *	-
	<i>Coxiella burnetti</i> *	-		<i>Bartonella spp.</i> *	-
	<i>Mycoplasma haemocanis</i> *	-			

3.3.4. Bioinformatics

Raw data was demultiplexed using in-house software at the Walter & Eliza Hall Institute. All subsequent bioinformatic analysis was conducted in the QIIME 2 (version 2018.8) environment (Caporaso et al., 2010; McDonald et al., 2012; McKinney, 2010; Rideout et al., 2018). Primer, adapter and index sequences were trimmed from raw reads using cutadapt (Marcel, 2011) and then imported into the QIIME 2 environment and inspected for quality. DADA2 was then used to remove low quality reads, denoise, dereplicate, filter chimeras and merge forward and reverse reads (Callahan et al., 2016). Prior observation of read quality plots informed the selection of truncation parameter values when executing the DADA2 program. DADA2 was used to generate Amplicon Sequence Variants (ASVs) instead of Operational Taxonomic Units (OTUs), that provide finer scale resolution of reads (Callahan et al., 2017). ASVs were then taxonomically assigned using the scikit-learn classifier (Pedregosa et al., 2011) against the SILVA version 132 reference database, downloaded from docs.qiime2.org. ASVs were also taxonomically identified using the BLASTn program in GenBank (NCBI) to corroborate scikit-learn assignment and in some cases identify to a lower taxonomic level. Sequences that were unassigned or only assigned to kingdom and phylum or bacterially assigned were excluded from the final dataset. Sequencing depth was validated by generation of alpha rarefaction plots, using MAFFT (Kato and Standley, 2013) and FastTree 2 (Price et al., 2010) plugins, to ensure that ASV diversity plateaued and thus a sufficient sequencing depth had been achieved. All NGS data produced in the present study is available from the BioProject database (<https://www.ncbi.nlm.nih.gov/bioproject>), with BioProjectID: PRJNA528154 and SRA data accession numbers SRR8872668 to SRR8872867.

3.3.5. Conventional PCR and Sanger sequencing

To compare the sensitivity of our NGS method with traditional molecular techniques all 100 samples were tested for *Babesia* spp. and *H. canis* by specific endpoint cPCR screens from the literature (**Table 3.3**).

To confirm VBD identification in blood DNA samples by NGS, a subset of samples from different taxon were corroborated by Sanger sequencing. This subset of PCR amplicons was purified using the ExoSAP-IT™ PCR Product Cleanup Reagent kit

(Thermo Fisher Scientific) according to the manufacturer's protocol. Cleaned amplicons were sent to Macrogen (Seoul, South Korea) for Sanger sequencing.

3.3.6. *Statistical analysis*

Analysis of results was conducted in Excel 2016 version 1803 (Microsoft), whilst Kappa statistics to compare concordance of NGS vs cPCR results were calculated in SPSS Statistics 24 (IBM).

3.3.7. *Phylogenetic analysis of NGS sequence data*

To assess the accuracy of the scikit-learn classifier's taxonomic assignment, classified ASVs were incorporated into phylogenetic trees to ensure the classification clustered with relevant reference sequences. 18S rRNA sequences were taken from GenBank, aligned with our ASVs in Mega X (Kumar et al., 2018) and the appropriate primer targeted region extracted. Phylogenetic analyses was conducted using the Bayesian inference (BI) and Monte Carlo Markov Chain (MCMC) method in MrBayes version 3.2.3 (Huelsenbeck and Ronquist, 2001). The necessary likelihood parameters required for BI analysis were obtained using the Akaike Information Criteria (AIC) test in jModelTest 2 version 2.1.10 (Darriba et al., 2012). To calculate BI posterior probability values, four simultaneous tree-building chains running 2,000,000 iterations were conducted with trees saved every hundred iterations. Burn in was conducted with the first 25% discarded. A 50% majority rule consensus tree for each analysis was constructed based on the final 75% of trees generated by BI. Trees were viewed in FigTree version 1.4.4 (<http://tree.bio.ed.ac.uk/software/figtree/>).

Table 3.3. Apicomplexa and Kinetoplastida primers used for cross-validation of NGS results. Asterisks denote nested PCR primers designed in the present study, thermocycling reagents and conditions for these primers are as detailed in the Kinetoplastida metabarcoding methods section with a lower annealing temperature of 52°C.

Apicomplexa Taxon-target	Primer Pair	Gene Target	Product Size	Reference
Canine piroplasms nested PCR	BTF1 & BTR1 BTF2 & BTR2	18S rRNA gene	930 bp 800 bp	(Jefferies et al., 2007)
<i>Hepatozoon canis</i>	HepF & HepR	18S rRNA gene	666 bp	(Inokuma et al., 2002b)
Coccidia	COC-1 & COC-2	18S rRNA gene	280 - 350 bp	(Ho et al., 1996)
<i>Toxoplasma gondii</i>	Unnamed (see reference)	<i>T. gondii</i> 529 bp repeat element	529 bp	(Lélu et al., 2011)
Tissue coccidia	COX10F & COX500R	Cytochrome c oxidase I (COX1)	470 - 510 bp	(Ogedengbe et al., 2018)
Coccidia specific nested PCR	Sarc-int-2F* (5'-AGCTCGTAGTTGGATA TCTGCTG-3') & Sarc-int-2R* (5'-CCTATCTTGTTATTCCAT GCTGCA-3')	Cytochrome c oxidase I (COX1) Uses product from COX10F & COX500R primers	150 bp	This study*
Kinetoplastida Taxon-target	Primer Pair	Gene Target	Product Size	Reference
Kinetoplastida specific	Kin24SF & Kin24SR	24S alpha-subunit rRNA	440 - 520 bp	(Rasmussen et al., 2001)
<i>Trypanosoma evansi</i>	RoTat1.2F & RoTat1.2R	<i>T. evansi</i> 1.2 Variable Surface Glycoprotein (VSG)	205 bp	(Claes et al., 2004)
Kinetoplastida specific	KinSSUF1 & KinSSUseqR2	18S rRNA	650 bp	(Vandersea et al., 2015)
Kinetoplastida specific nested PCR	PCaud1F* (5'-CTACCACTTCTACGGAGGG C-3') & PCaud1R* (5'-GCAC CAGACTTGTCTCCAA-3')	18S rRNA. Uses product from KinSSUF1 & KinSSUseqR2	130 bp	This study*

3.4. Results

3.4.1. Design of metabarcoding primers for Apicomplexa and Kinetoplastida

Two primer pairs were designed to exclusively amplify a partial stretch of the 18S rRNA gene from the phylum Apicomplexa and the class Kinetoplastida using a diverse range of sequences from GenBank. As the metabarcoding was to be carried out on canine blood samples, Apicomplexa and Kinetoplastida sequences from common blood-infecting species were chosen (for the complete list see the “Methods” section). Primers were designed to bind to highly conserved 18S rRNA sequences but flanking areas of high sequence diversity (barcode regions) to provide species-level discrimination. Host, canine 18S rRNA sequences were also included in the alignment to ensure that the designed primers did not co-amplify canine host DNA.

3.4.2. Metabarcoding assay validation

Confirmed haemoparasite positive controls were used to find optimal PCR conditions, whilst primer cross-reactivity was tested for, using positive controls from species outside of the taxa targeted by each primer pair (see the “Methods” section). Mock communities were also generated by mixing haemoparasite positive controls. After completion of our developed metabarcoding pipeline these mock communities were consistently and accurately reflected in the final results.

3.4.3. Bioinformatic analysis of Apicomplexa data

In total 6,649,169 (median 62,120) raw paired-end reads were obtained for the 104 multiplexed Apicomplexa amplicons, including two positive and two negative controls. After the DADA2 quality filtering, dereplication, chimera removal and pair-joining step, a total of 564,332 joined sequences were retained, representing a retention of 16.97% of original reads ($564,332 \times 2 \div 6,649,169$).

3.4.4. NGS characterisation of Apicomplexa

Of the 100 canine blood samples tested 13 were found to be positive for *B. vogeli* (mean reads 9,846; range 84 - 43,347) and 38 for *H. canis* (mean reads 8,774; range 90 - 58,207); six of these dogs were infected with both haemoparasite species. Two canine DNA samples returned sequences that could only be identified to the level of phylum Apicomplexa (mean reads 120; range 119 - 121). When this sequence was run through a BLASTn search it returned a 100% identity result with a diverse range of sarcocystidae family pathogens, making species level assignment impossible with this sequence alone. In total 47% of dogs were found infected with at least one apicomplexan VBD via deep sequencing (**Figure 3.1**).

Classification of NGS Amplicon Sequence Variants (ASVs) by the scikit-learn classifier was supported by phylogenetic analysis (**Figures 3.2 and 3.3**) which demonstrated ASVs classified as *H. canis*, and *B. vogeli* clustered with high posterior probability between the relevant reference sequences taken from GenBank.

ASVs classified to species had higher nucleotide homology to reference sequences and less sequence variation than sequences assigned to higher taxonomic levels. For example, across all apicomplexan species used in our primer design alignment the mean nucleotide pairwise identities in the 130 bp region amplified by our primers was $71\% \pm 16.4\%$ (mean $\pm$ standard deviation). In comparison, the 10 ASVs classified as *H. canis* had a mean nucleotide pairwise identity of $98.8\% \pm 0.4\%$ when compared to six *H. canis* 18S rRNA reference strains (GenBank accession numbers in **Figure 3.2**). The four ASVs classified as *B. vogeli* had a mean nucleotide pairwise identity of $99.0 \pm 0.5\%$ when compared to four *B. vogeli* reference strains (GenBank accession numbers in **Figure 3.3**). This demonstrates the range of nucleotide diversity within our apicomplexan barcode region, and the observed inter- and intraspecies nucleotide diversity that was used to accurately taxonomically classify each ASV.

Infections were considered true by NGS if a sample had a VBD read count of over 44. This threshold was calculated as the mean reads of two canine DNA samples that were identified as having sequences from the positive controls used, potentially due to index misreading or hybridisation errors during Illumina sequencing (Kim et al., 2017). This was supported by observation of where on the 96-well plate the samples with positive control sequences appeared, which showed substantial distance from the

positive control location. The mean Phred quality score over the adapter and indexing regions for the raw data was 31, highlighting a base call error rate of between one in 1,000 to 10,000, that may have potentially led to occasional index misreading. This conclusion was further corroborated by separate cPCR reactions to target canine piroplasms (Jefferies et al., 2007) that found the two samples that were thought to contain positive control sequences, as negative for piroplasm DNA.

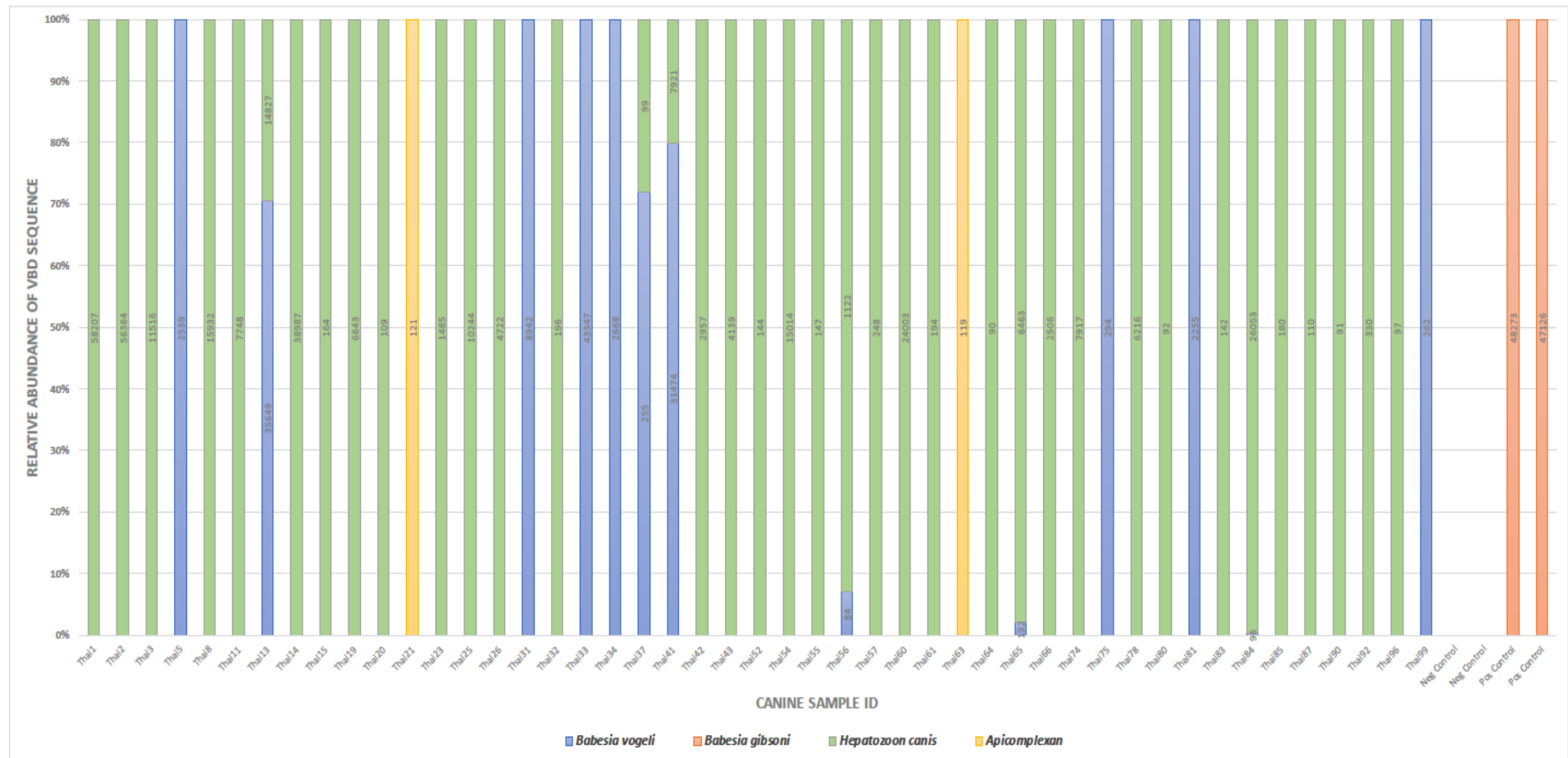


Figure 3.1. Relative sequence composition of canine blood samples found to be positive for an apicomplexan infection. Numbers in columns represent actual read counts.

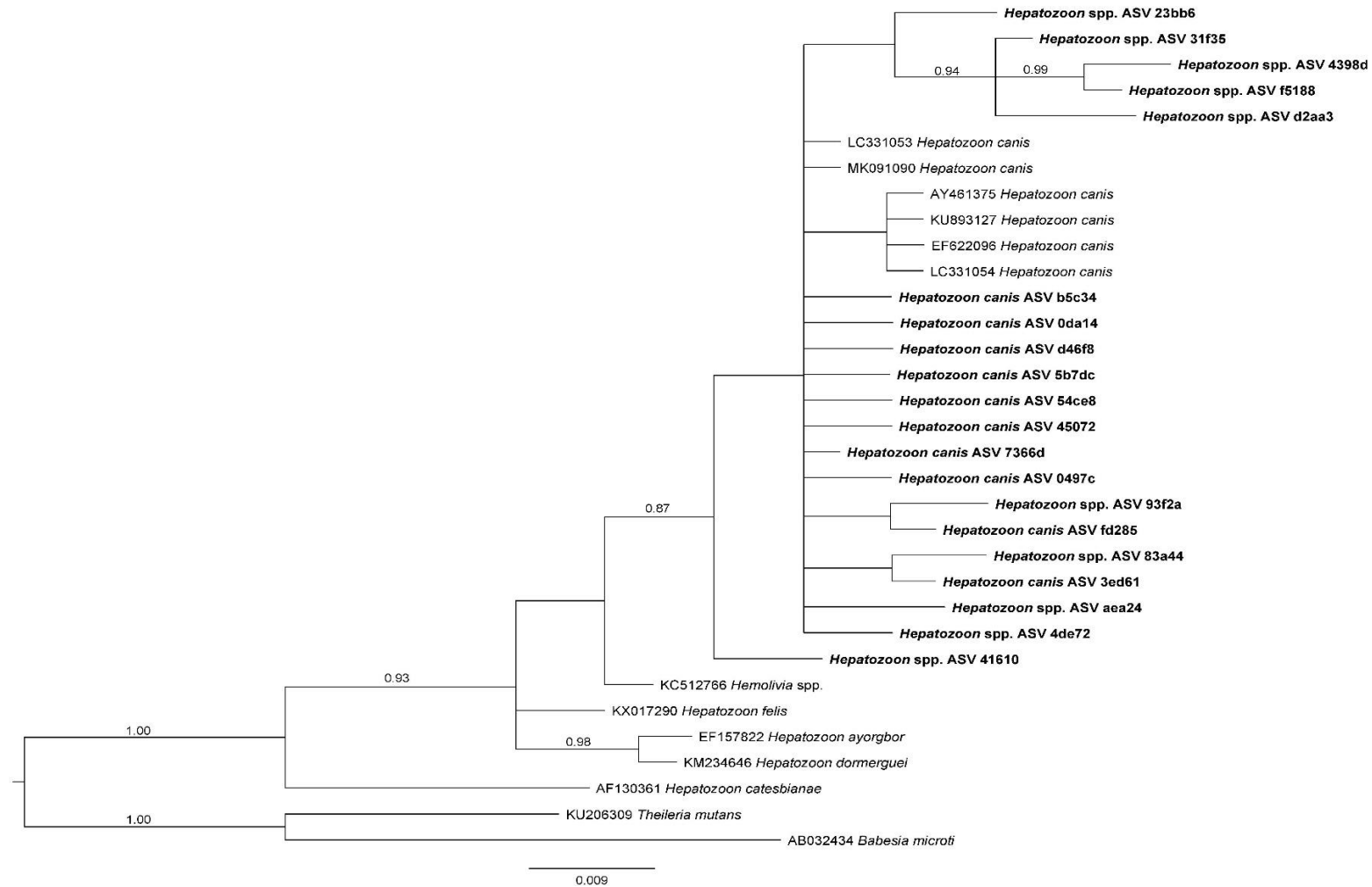


Figure 3.2. *Hepatozoon* spp. phylogenetic analysis by Bayesian inference (BI) of a 130 bp region of the 18S ribosomal RNA (rRNA) gene targeted by our apicomplexan primers. ASVs assigned as *Hepatozoon* spp. or *Hepatozoon canis* generated from our NGS data are in bold and identified by a 5-character string code. Other sequences are references taken from GenBank, including a variety of *H. canis* sequences from different geographical regions, four closely related *Hepatozoon* species and two piroplasmid species as an outgroup. Posterior probabilities (> 0.85) are indicated on branches. The scale-bar represents the number of substitutions per site. Our *H. canis* classified ASVs cluster within *H. canis* reference sequences.

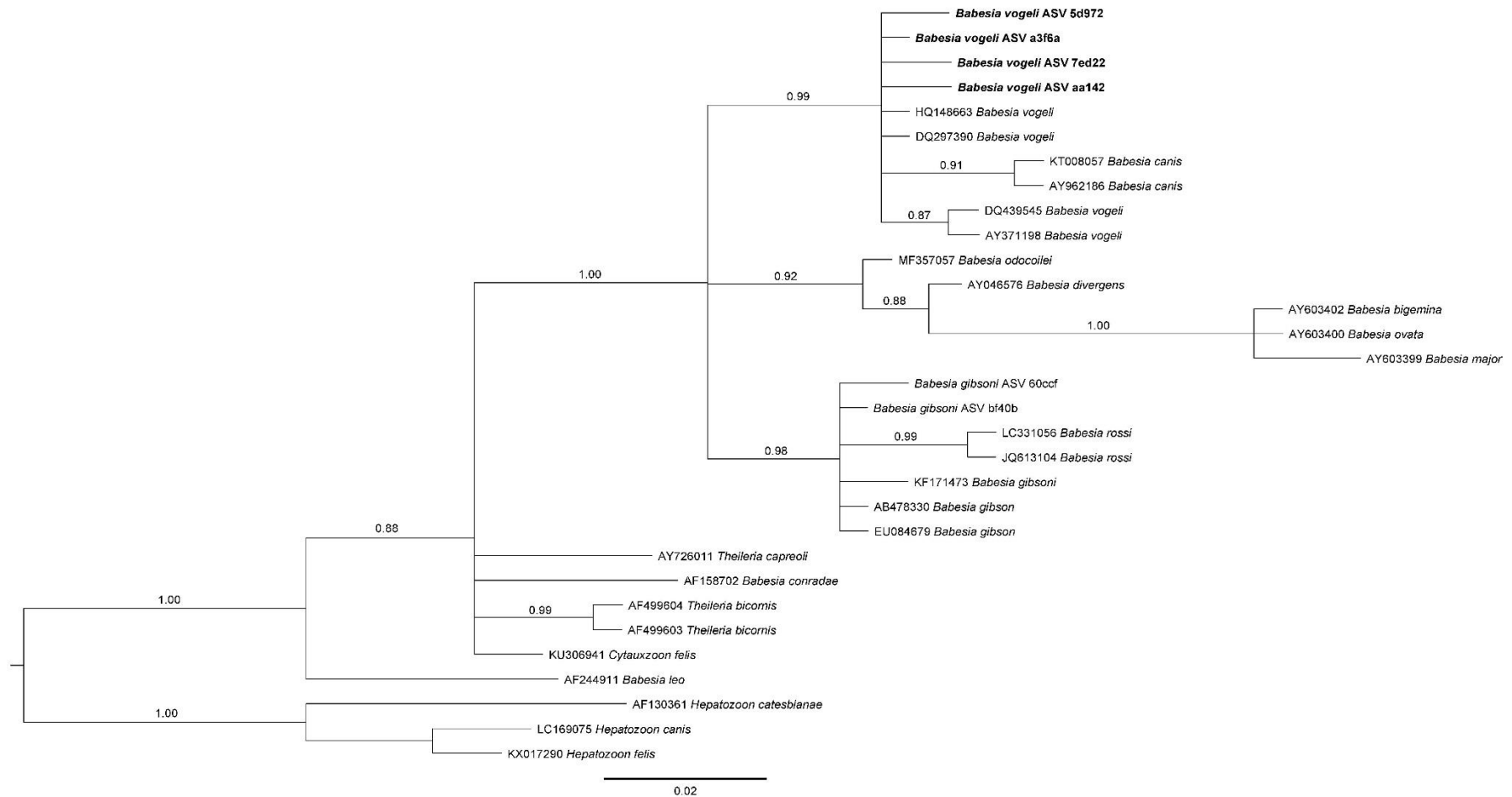


Figure 3.3. *Babesia* spp. phylogenetic analysis by Bayesian inference (BI) of a 130 bp region of the 18S ribosomal RNA (rRNA) gene targeted by our apicomplexan primers. ASVs assigned as *Babesia vogeli* or *Babesia gibsoni* generated from our NGS data are in bold and identified by a 5-character string code. Other sequences are references taken from GenBank, including *B. vogeli* and *B. gibsoni* sequences from different geographical regions. Three *Hepatozoon* species are used as an outgroup, whilst *Theileria* spp. and *Cytauxzoon* spp. are used for comparison. Posterior probabilities (> 0.85) are indicated on branches. The scale-bar represents the number of substitutions per site. Our *B. vogeli* and *B. gibsoni* classified ASVs both cluster next to *B. vogeli* and *B. gibsoni* reference sequences, whilst closely related *Babesia canis* and *Babesia rossi* show a significant number of nucleotide substitutions from their relevant clusters.

3.4.5. Comparison of NGS diagnostic methods with conventional PCR for *Apicomplexa*

A highly specific canine piroplasm nested PCR (Jefferies et al., 2007) capable of amplifying all canine *Babesia* species found 13 blood samples to be infected with this pathogen, whilst a *H. canis* specific cPCR (Inokuma et al., 2002) found 17 samples to be infected with this VBD. The results of both cPCR results taken together identified four dogs as being infected with both *Babesia* spp. and *H. canis*.

Table 3.4 shows the relevant agreement statistics between the two diagnostic tests. The Kappa statistic for the piroplasm specific cPCR showed very good agreement with the NGS results, whilst the Kappa value when comparing the *H. canis* PCR was fair. The NGS method demonstrated superior sensitivity regarding its ability to detect *H. canis* infections, identifying 21 more positives than the cPCR method.

Table 3.4. Apicomplexa NGS and conventional PCR (cPCR) agreement statistics.

VBD	cPCR	Apicomplexa NGS		Total agreement (%)	Kappa ^a (agreement statistic)	Kappa SE
		POS	NEG			
<i>Babesia</i> spp.	POS	12	1	98	0.912	0.062
	NEG	1	86		(Very good)	
<i>Hepatozoon canis</i>	POS	15	2	75	0.406	0.088
	NEG	23	60		(Fair)	

^aKappa agreement level: K < 0.2, poor; 0.21–0.40, fair; 0.41–0.60, moderate; 0.61–0.80, good; 0.81–1.00, very good

3.4.6. Apicomplexa NGS cross-validation

To cross-validate the taxonomic assignment provided by scikit-learn within the bioinformatics pipeline, endpoint PCR experiments were conducted on larger taxonomic barcode regions followed by Sanger sequencing. Amplicons produced by the piroplasmid specific PCR (Jefferies et al., 2007) achieved a 100% query cover and identity match with *B. vogeli* isolate 68SR (GenBank accession no. MH100721.1)

using the GenBank BLASTn tool. *H. canis* taxonomic classification was confirmed by a nested PCR designed in the present study, using Sarc-int-2F and Sarc-int-2R primers (see “Methods” section, **Table 3.3**), returning a 100% query cover and 99.26% identity match with *H. canis* voucher Junagadh 590 (GenBank accession no. MH922768.1).

For the two canine samples that had sequences that could not be classified below the taxonomic level of Apicomplexa, two different tissue coccidia-specific PCRs were conducted (Ho et al., 1996; Ogedengbe et al., 2018). These were chosen as the apicomplexan sequence provided by NGS was found to be highly conserved across the sarcocystidae family by a BLASTn search. Unfortunately, no amplification could be achieved with these endpoint PCRs nor with a *T. gondii* specific real time PCR (Lélu et al., 2011), which was a suspected pathogen.

3.4.7. Bioinformatic analysis of Kinetoplastida data

In total 4,457,913 (median 43,188) raw paired-end reads were obtained for the 104 multiplexed Kinetoplastida amplicons, including two positive and two negative controls. After the DADA2 quality filtering, dereplication, chimera removal and pair-joining step a total of 117,262 joined sequences were retained, representing a retention of 5.26% of original reads ($117,262 \times 2 \div 4,457,913$).

3.4.8. NGS characterisation of Kinetoplastida

Out of the 100 canine blood samples only two demonstrated amplification of sequences from potential haemoparasites. One sample had reads taxonomically assigned to *T. evansi* (18 reads), whilst the other was identified as *Parabodo caudatus* (126 reads). Both *Trypanosoma theileri* clade positive controls were successfully amplified and detected by the NGS diagnostic test.

Classification of NGS ASVs by the scikit-learn classifier was supported by phylogenetic analysis which demonstrated that the ASV classified as *T. evansi* clustered with high posterior probability between *T. evansi*, *Trypanosoma equiperdum* and *Trypanosoma brucei* reference sequences (**Figure 3.4**), the latter two are known to be indistinguishable at the 18S rRNA gene (Carnes et al., 2015; Lai et al., 2008).

Nonetheless, *T. evansi* is the most likely infective agent in this case, as *T. equiperdum* is a horse-specific venereal pathogen, whilst *T. brucei* is geographically only found in Africa (Carnes et al., 2015; Lai et al., 2008). The ASV classified as *P. caudatus* clustered with *P. caudatus* and *Bodo caudatus* reference sequences (**Figure 3.5**), *Bodo* being the former genus name for this species (Moreira et al., 2004).

3.4.9. Kinetoplastida NGS cross-validation

Two Kinetoplastida specific PCRs (Rasmussen et al., 2001; Vandersea et al., 2015) and one *T. evansi* specific PCR (Claes et al., 2004) all failed to amplify the sample that was found to be *T. evansi* positive by NGS, without any product generated for sequencing. However, a nested PCR designed in the present study using PCaud1F and PCaud1R primers (**Table 3.3**) amplified a region from which 41 nucleotides achieved a 95% identity hit with two *Trypanosoma* spp. entries (GenBank accession numbers: JN315385.1 and AF359482.1). The same endpoint PCRs all failed to amplify from the sample found to be *P. caudatus* positive by NGS (Claes et al., 2004; Rasmussen et al., 2001; Vandersea et al., 2015). Blood smears from individuals identified as having Kinetoplastida DNA were also assessed microscopically for the presence of relevant organisms but returned negative results.

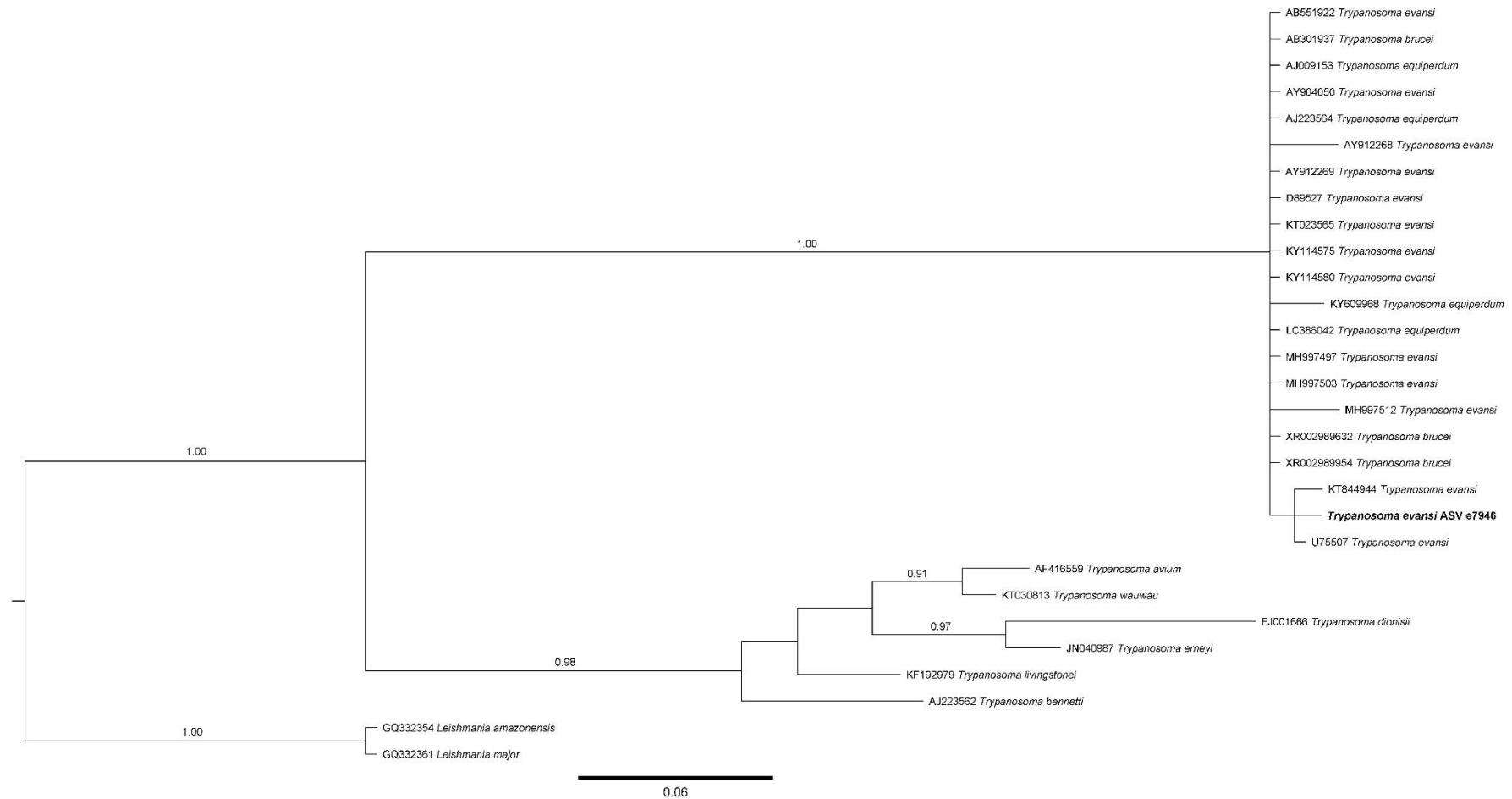


Figure 3.4. *Trypanosoma evansi* phylogenetic analysis by Bayesian inference (BI) of a 170 bp region of the 18S ribosomal RNA (rRNA) gene targeted by our Kinetoplastidae primers. The ASV assigned as *T. evansi* from our NGS data is in bold and identified by a 5-character string code, other sequences are references taken from GenBank, including *T. evansi* and the closely related *Trypanosoma equiperdum* and *Trypanosoma brucei* from different geographical regions. Two *Leishmania* species are used as an outgroup, whilst a range of more distantly related *Trypanosoma* are used for comparison. Posterior probabilities (> 0.85) are indicated on branches. The scale-bar represents the number of substitutions per site. Our *T. evansi* classified ASV, clusters next to *T. evansi*, *T. equiperdum* and *T. brucei* reference sequences, the latter two are known to be indistinguishable at the 18S rRNA gene, however *T. equiperdum* solely infects horses, whilst *T. brucei* is restricted to Africa, meaning only *T. evansi* can be the responsible pathogen (Carnes et al., 2015; Lai et al., 2008).

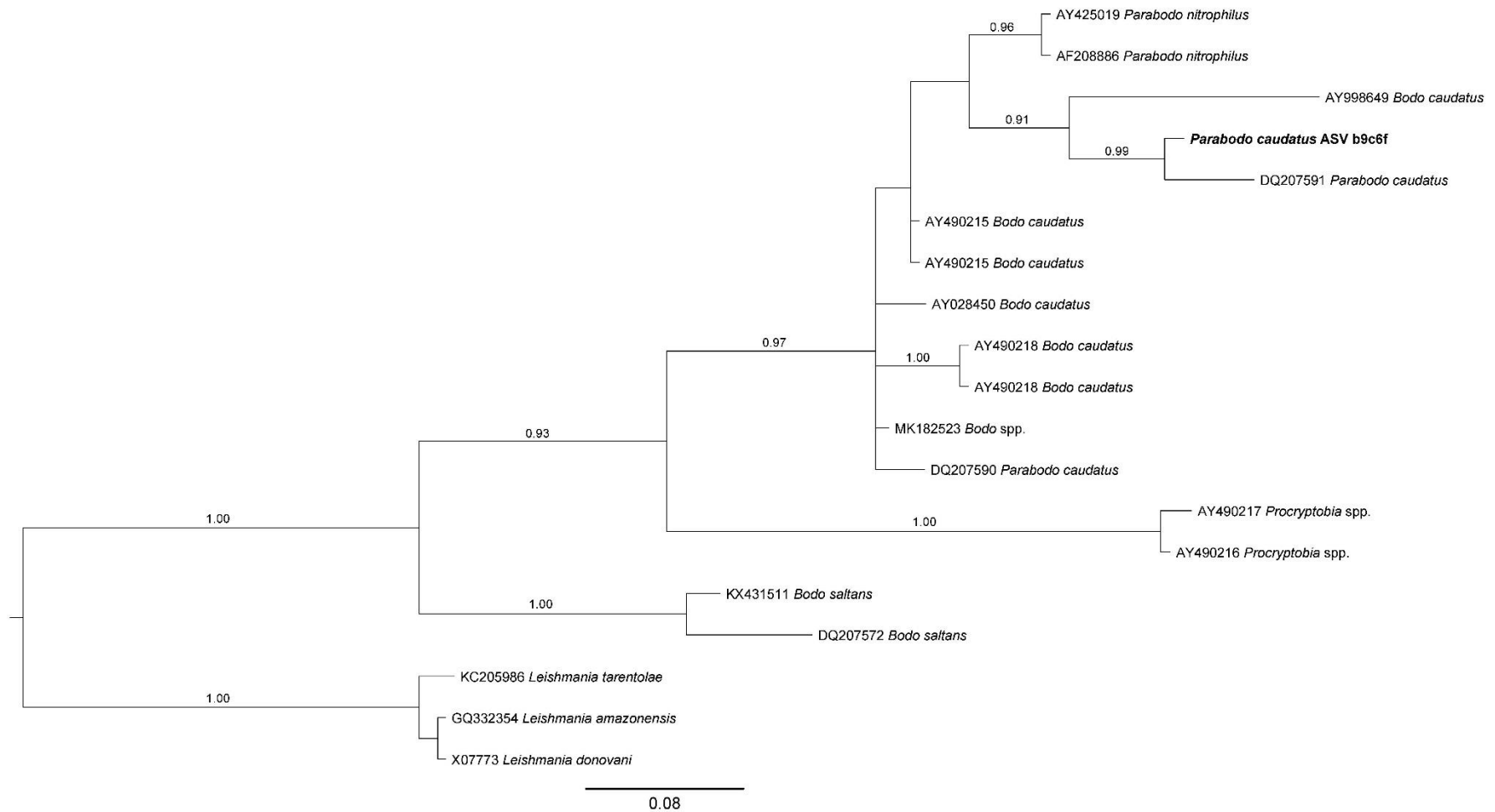


Figure 3.5. *Parabodo caudatus* phylogenetic analysis by Bayesian inference (BI) of a 170 bp region of the 18S ribosomal RNA (rRNA) gene targeted by our Kinetoplastidae primers. The ASV assigned as *P. caudatus* from our NGS data is in bold and identified by a 5-character string code, other sequences are references taken from GenBank, including *P. caudatus* (formerly classified as *Bodo caudatus*) and the closely related *Parabodo nitrophilus* from different geographical regions (Powell and Kohiyar, 1920; Vandersea et al., 2015). Three *Leishmania* species are used as an outgroup. Posterior probabilities (> 0.85) are indicated on branches. The scale-bar represents the number of substitutions per site. Our *P. caudatus* classified ASV, clusters next to *P. caudatus* reference sequences with strong posterior probability support.

3.5. Discussion

Our novel NGS-based diagnostic tool was able to thoroughly characterise the apicomplexan and kinetoplastid haemoparasite microbiome from canines and was demonstrated to be more diagnostically sensitive than conventional PCR on field samples. The developed methodology is not necessarily limited to canine application alone and may show utility in characterising protist pathogens from the blood of a range of animals, including humans. Our diagnostic method's ability to detect unusual haemoparasites from blood, further highlights the power of this methodology to uncover new species or emerging disease threats of veterinary and clinical importance from countries which have so far had limited relevant research done within their borders.

Not only was our NGS method demonstrated to have a large breadth of taxonomic detection ability but was also proven to be highly sensitive. Concordance between the NGS method and *Babesia* specific cPCR was almost 100% with a Kappa statistic, indicating a very good level of agreement between the two methodologies. However, when our method was compared to the *Hepatozoon* specific PCR (Inokuma et al., 2002) it greatly outperformed it finding 21 more single *H. canis* infections with a Kappa statistic demonstrating a fair level of agreement between these tests. This superior sensitivity using an NGS-based method compared to cPCR has been demonstrated previously, in particular when targeting the bacterial pathogen *Anaplasma platys* from canine blood (Huggins et al., 2019).

Babesia species are important canine pathogen capable of producing grave morbidity or mortality in their hosts, particularly in puppies (Irwin, 2009; Irwin and Jefferies, 2004; Vial and Gorenflot, 2006). The 13% of dogs found to be infected in the present study largely supports that of other haemoparasite molecular surveys for the Southeast Asia region. *Babesia* infection rates range from 7.14% of stray dogs in The Philippines (Corales et al., 2014), to 9.4% in Thailand (Liu et al., 2016) and

as many as 32.7% of semi-domesticated dogs in Cambodia (Inpankaew et al., 2016). In comparison, *H. canis* is a less virulent haemoparasite but was found in greater abundance than *B. vogeli* in the present study (Ivanov and Tsachev, 2008). The 38% of dogs infected with *H. canis* by our NGS diagnostic tool was higher than other studies done in the country and SE Asia region. For example, a *H. canis* infection rate of 18.8% was found in Thailand (Liu et al., 2016), whilst 10.9% of community dogs in Cambodia had been found to have this pathogen previously (Inpankaew et al., 2016). In addition, the *B. vogeli* and *H. canis* co-infection rate of 3.6% that was found in Cambodia (Inpankaew et al., 2016) also closely matches the 6% elucidated by NGS in the present study. Future work could benefit from the collection of data on haemoparasite vectors, such as ticks, to assess for potential correlations between local levels of haemoparasite infection and vector abundance.

Our NGS method detected two more *H. canis* and *B. vogeli* mixed infections than cPCR screening. Thorough detection of the complete haemoparasite microbiome is particularly pertinent given that there is a substantial body of evidence demonstrating more severe pathology and increased lethality brought about by multiple VBD infections in a single host (Little, 2010; Low et al., 2018; Rar and Golovljova, 2011; Rojas et al., 2014; Suksawat et al., 2001). The better sensitivity of our NGS method at detecting such co-infections means that particularly at-risk individuals are not missed and can be prioritised. The relative sequence composition of these co-infections can be observed in **Figure 3.1**. This bar graph displays three co-infections as being dominated by over 90% *H. canis* reads, whilst the others comprise over 70% *B. vogeli* reads. Such data may be indicative of the comparative parasitaemia of each VBD at the time of sampling.

Whilst there were only two kinetoplastid infections across the entire sample set, the detection of these was significant as *T. evansi* is a frequently lethal pathogen of dogs (Defontis et al., 2012; Irwin and Jefferies, 2004) and *P. caudatus* has been seldom documented from canines with an unknown role as a potential infectious agent

(Vandersea et al., 2015). *T. evansi* is the aetiological agent of the damaging livestock disease 'Surra' which afflicts horses, cattle and camels but can also infect dogs, and various wildlife species via transmission using tabanid fly vectors (Defontis et al., 2012). *T. evansi* has only been identified from a dog in Thailand once before (Aregawi et al., 2019; Baramechaithanun et al., 2009) and given its severe pathogenicity is thus an important finding, demonstrating potential spill-over transmission from local livestock into canine hosts.

P. caudatus has been previously implicated in a case of gross haematuria in a canine, with these biflagellated protozoans being observed under the microscope from urine samples voided by an infected dog (Vandersea et al., 2015). Similar pathogens have also been documented in human urine samples (Powell and Kohiyar, 1920), whilst closely related *Bodo* species have been found in the blood of the marsupial, *Bettongia penicillate* (Northover et al., 2019) and in bats (Dario et al., 2017). Whether or not the presence of such kinetoplastid DNA demonstrates them to be true pathogens or simply commensal organisms is debatable. In the case of the present study, the detection of *P. caudatus* within the blood microbiome is particularly notable as it is hard to rationalise how this typically environmental organism (Gomaa et al., 2017) could have entered and then persisted in the canine bloodstream. Nonetheless, it is possible that the presence of *P. caudatus* sequences could be the result of environmental contamination, although this is unlikely given the thorough sterilisation and aseptic protocol utilised when collecting blood samples. If we have identified a *P. caudatus* bloodstream infection, the battery of different conventional PCRs that would have needed to be conducted to detect these unusual protozoa using traditional molecular methods would have been great, demonstrating the advantages of NGS-based analysis to find atypical pathogen species.

For the detection of *T. evansi* our 44 read cut-off was not applicable as these reads were unique to one sample and thus could not represent indexing cross-talk errors,

our 18 *T. evansi* reads were also higher than a whole dataset threshold of 10 reads used in similar studies exploring eukaryote diversity via metabarcoding (Cannon et al., 2016). Our *T. evansi* diagnosis was later partially supported by a separate nested PCR developed in the current study with Sanger sequencing that returned a 41 bp run with 95% identity to *Trypanosoma* spp. However, the small size and only genus level taxonomic assignment of this corroboratory cPCR experiment, provides only limited cross-validation for our NGS method's result of a *T. evansi* infection in this individual.

Endpoint PCR cross-validation for *P. caudatus* could not be achieved at all, possibly due to a very low concentration of circulating *P. caudatus* DNA or a dearth of publicly available 18S rRNA sequences for this species, making design of primers to target this organism suboptimal. Future experiments could reconduct NGS at a greater sequencing depth on samples that achieved low read counts for parasite DNA, to assess if results remain consistent and provide further support for the sensitivity of the existing metabarcoding protocol.

No evidence of the important zoonotic pathogen *Leishmania* was found in the canines tested in the present study, despite cases of human leishmaniasis being reported in Thailand caused by both *Leishmania martiniquensis* and *Leishmania siamensis* (Leelayoova et al., 2017). As yet, there is little-to-no evidence of canines acting as a reservoir of human-infecting *Leishmania* species in SE Asia, although in one study it was reported that local medical practitioners indicated a potential for dogs and rats to be acting as a source of human infection (Wiwanitkit and Wiwanitkit, 2015).

The accuracy of species level taxonomic classification by the QIIME2 sci-kit learn classifier was supported by two methods; comparing inter with intraspecific average nucleotide pairwise identities and via phylogenetic analyses, using reference sequences from GenBank. The mean, between species pairwise nucleotide identity

was significantly lower for the Apicomplexa at $71\% \pm 16.4\%$ (mean $\pm$ standard deviation) than the within species means when compared to relevant reference sequences e.g. *H. canis* $98.8\% \pm 0.4\%$ and *B. vogeli* $99.0 \pm 0.5\%$. This substantial difference in nucleotide pairwise identity between and within species, at the 18S rRNA region targeted by our primers, highlights how informative this region is for allowing species level classification. Accuracy of classification by our methodology was further supported by phylogenetic analyses. When classified ASVs were aligned with corroborated species reference sequences, they consistently clustered with references of the same taxonomic identity, highlighting an accurate classification (**Figures 3.2 to 3.5**). Overall, this demonstrates the classifier's ability to recognise high nucleotide homology to reference sequences and accurately assign a species classification to a particular ASV, a tenet that is of particular importance given that such classifiers have been seldom tested on protozoan microbiomes.

During the bioinformatic analysis of the raw NGS data as many as 94.74% of Kinetoplastida and 83% of apicomplexan total reads were filtered out at the quality control, denoising, dereplication, pair-joining and chimera removal stage. The large number of lost reads may in part be due to the high abundance of host DNA relative to a low quantity of haemoparasite template DNA likely contributing to some off-target deep sequencing, despite the specificity of our designed primers (Cannon et al., 2018; Flaherty et al., 2018). This may have also generated some of the ASVs that were taxonomically identified to groups outside of the primer's targets, including hits assigned down to the level of Animalia. Nonetheless, even with the raw reads lost through bioinformatic filtering and occasional off-target amplification the NGS diagnostic tool still proved itself more sensitive than endpoint PCR methods.

Financial considerations still limit the use of NGS based methodologies to some degree, however, as costs of deep sequencing reduce, so too does the difference between these methods and cPCR techniques, particularly for studies exploring

areas with high levels of infection that necessitate more Sanger sequencing and therefore accrue a higher cost.

3.6. Conclusions

Overall, our novel NGS diagnostic tool for the characterisation of the canine haemoparasite microbiome has been demonstrated to be more sensitive and better capable at detecting novel, rare and mixed infections than conventional diagnostic methods. This assay shows much promise in its utility for epidemiological surveys of protozoan haemoparasites, particularly in contexts where parasite diversity and co-infection prevalence is high, such as in developing countries in the tropics. Furthermore, our diagnostic test is not limited to use with canine samples alone and may demonstrate functionality when utilised for the analysis of the haemoparasite microbiome in other animals of veterinary or ecological importance as well as in humans.

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Chapter 4 - A host-specific blocking primer combined with optimal DNA extraction improves the detection capability of a metabarcoding protocol for canine vector-borne bacteria

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4.1. Abstract

Bacterial canine vector-borne diseases are responsible for some of the most life-threatening conditions of dogs in the tropics and are typically poorly researched with some presenting a zoonotic risk to cohabiting people. Next-generation sequencing based methodologies have been demonstrated to accurately characterise a diverse range of vector-borne bacteria in dogs, whilst also proving to be more sensitive than conventional PCR techniques. We report two improvements to a previously developed metabarcoding tool that increased the sensitivity and diversity of vector-borne bacteria detected from canine blood. Firstly, we developed and tested a canine-specific blocking primer that prevents cross-reactivity of bacterial primer amplification on abundant canine mitochondrial sequences. Use of our blocking primer increased the number of canine vector-borne infections detected (five more *Ehrlichia canis* and three more *Anaplasma platys* infections) and increased the diversity of bacterial sequences found. Secondly, the DNA extraction kit employed can have a significant effect on the bacterial community characterised. Therefore, we compared four different DNA extraction kits finding the Qiagen DNeasy Blood and Tissue Kit to be superior for detection of blood-borne bacteria, identifying nine more *A. platys*, two more *E. canis*, one more *Mycoplasma haemocanis* infection and more putative bacterial pathogens than the lowest performing kit.

4.2. Introduction

Despite recent developments in veterinary care and medicine, canine vector-borne diseases (CVBD) continue to inflict a large burden with regard to morbidity and mortality on dogs across the globe (Koh et al., 2016; Maggi and Krämer, 2019; Traub et al., 2015). This is especially true in low-socioeconomic countries, that may have little available resources to invest into disease prevention programs (Koh et al., 2016; Maggi and Krämer, 2019; Traub et al., 2015). In particular, countries spanning the tropics must affront an expansive range of CVBDs that comprise a leading cause of fatality in dogs (Inpankaew et al., 2016; Irwin and Jefferies, 2004; Koh et al., 2016; Rucksaken et al., 2019). Bacterial infections can be some of the deadliest CVBDs in such regions with pathogens such as *Ehrlichia canis*, the causative agent of canine monocytic ehrlichiosis (CME), being the biggest contributor to a raft of life-threatening conditions, including pancytopenia, fever, bleeding tendencies and immunosuppression (Kaewmongkol et al., 2017; Little, 2010; Mylonakis et al., 2019). Other disease-causing species include, *Anaplasma platys* which is a cause of recurrent thrombocytopenia in canines (Carrade et al., 2009), haemotropic *Mycoplasma* that are associated with haemolytic syndrome (Inpankaew et al., 2016) and *Bartonella* spp. that can produce severe endocarditis (Chomel et al., 2001). Rates of canine infection with such pathogenic agents can be very high, especially in tropical countries of Southeast Asia, where, for example 25.5% of Malaysian, 21.8% of Cambodian and 9.9% of Thai dogs have previously been found positive for *E. canis* by PCR (Inpankaew et al., 2016; Kaewmongkol et al., 2017; Koh et al., 2016). Lower but nonetheless significant levels of infection by haemotropic *Mycoplasma* (3.7–12.8%) and *A. platys* (3.7– 4.4%) have also been detected in these same countries (Inpankaew et al., 2016; Kaewmongkol et al., 2017).

Exploration and surveillance of CVBD is not only important from a veterinary perspective but also a public health standpoint, as several bacterial CVBD agents are zoonotic. For example, *Rickettsia felis* and *Rickettsia conorii*, when transmitted to humans, are the causative agents of flea-borne spotted fever and Mediterranean/Indian/Israeli tick typhus, respectively, with both having dogs as reservoir hosts (Hii et al., 2011; Ng-Nguyen et al., 2020). In addition, dogs are considered sentinel hosts for the zoonotic pathogens *Borrelia burgdorferi* sensu lato, which causes Lyme disease and *Anaplasma phagocytophilum* responsible for the

potentially lethal human granulocytic anaplasmosis (Bakken and Dumler, 2015; Fourie et al., 2019; Krämer et al., 2020; Krupka and Straubinger, 2010).

Thorough characterisation and monitoring of CVBD is crucial to protect the health of dogs and humans, especially in regions of Asia where there has been little explorative research done into established, emerging and novel CVBDs (Irwin and Jefferies, 2004; Traub et al., 2015). To address these knowledge gaps, state-of-the-art methodologies could be employed such as next-generation sequencing (NGS) based 16S ribosomal RNA (16S rRNA) metabarcoding that can supersede conventional PCR (cPCR) techniques with regard to their ability to detect and discover rare and/or novel organisms (Takhampunya et al., 2019; Vayssier-Taussat et al., 2013). 16S rRNA metabarcoding is better able to elucidate diversity in explorative research by not relying on likely pathogen prevalence in a region nor on *a priori* knowledge of a pathogen's target genetic sequences, whilst also being better able to characterise coinfection (Greay et al., 2018; Takhampunya et al., 2019). Previous work has already developed a novel 16S and 18S rRNA metabarcoding methodology to characterise the range of blood-borne bacterial, apicomplexan and kinetoplastid organisms infecting canine hosts in Thailand, a country of substantial CVBD diversity (Huggins et al., 2019a, 2019b; Kaewmongkol et al., 2017; Liu et al., 2016; Rucksaken et al., 2019). The current research takes this further, by tackling some of the inherent challenges of NGS microbiome research whilst also improving methods to be better at unearthing pathogen diversity in the context of the canine blood micro-environment (Kim et al., 2017).

Prior research employing NGS metabarcoding analysis of bacterial pathogens from canines suffered from issues of bacterial primer cross-reactivity with the canine mitochondrial 12S ribosomal RNA (12S rRNA) gene (Huggins et al., 2019b). On average, 47% of total reads during previous experiments were from mitochondrial DNA (mtDNA) cross-reactivity despite poor complementarity between the bacterial primers and 12S rRNA gene sequences (Huggins et al., 2019b). This cross-reactivity is likely due to multiple causes, including a dominance of canine host DNA in the blood (Huggins et al., 2019b) in conjunction with the prokaryotic origin of mitochondria producing sequence similarities (Taanman, 1999).

Similar challenges have been tackled before by using blocking primers that selectively bind to and prevent the amplification of DNA sequences that would

otherwise dominate amplicons in contexts where there is a stacked ratio of low copy number DNA of interest compared to overabundant DNA that is ideally excluded (Boessenkool et al., 2012; Goffon et al., 2015; Hino et al., 2016; Leray et al., 2013; Tan and Liu, 2018; Vestheim and Jarman, 2008). Considering this, we designed and tested a 3'-spacer C3 blocking primer that could prevent amplification of the canine 12S rRNA gene by our previously tested bacterial-universal primers (Huggins et al., 2019b). We then compared our bacterial NGS metabarcoding pipeline with and without this blocking primer to assess its blocking efficacy and elucidate whether it improved bacterial detection capability.

In addition, for NGS-based research, the method of DNA extraction employed can play a critical role in the diversity and sensitivity of 16S rRNA detected, particularly in the context of low biomass samples, such as blood (Eisenhofer et al., 2019; Glassing et al., 2016; Podnecky et al., 2013). Commercially available DNA extraction kits differ widely in the physical and chemical systems used from which they produce PCR-ready DNA as well as in their required time and complexity (Podnecky et al., 2013; Yang et al., 2011). Recent attention has been brought to automated DNA extraction systems that simplify the researcher's workload but that may also accrue a cost with regard to DNA quantity and quality for downstream applications (Riemann et al., 2007; Yang et al., 2011). Another variable that must be considered when determining the most appropriate DNA extraction kit for 16S rRNA metabarcoding research is that bacteria frequently contaminate kits and reagents (Eisenhofer et al., 2019; Laurence et al., 2014). Numerous studies have now explored this phenomenon via the use of rigorous negative controls to highlight the prevalence of common kit contaminant bacteria, also demonstrating that levels of kit contamination and the types of contaminants found can vary between kits and even batches within kits (Eisenhofer et al., 2019; Glassing et al., 2016; Velásquez-Mejía et al., 2018).

Taking this into consideration, we tested four DNA extraction systems. Two were a typical spin-column based whole blood DNA extraction procedure and two were automated systems employing the Maxwell® RSC 48 Instrument (Promega, Madison, WI, USA). Of the two automated procedures, one was for extraction of whole blood and the other for extraction from the blood's Buffy Coat layer, which has been reported to increase detection sensitivity of certain vector-borne bacterial species that are typically difficult to detect (Alexandre et al., 2009; Mylonakis et al.,

2003; Werren et al., 2008). Both automated kits use magnetic bead extraction and purification. Kit performance was assessed and compared based on DNA yield, sensitivity of pathogen detection and presence of contaminant DNA.

4.3. Methods

4.3.1. Sampling and DNA extraction

For blocking primer experiments, blood extracted DNA from 50 temple community dogs in Thailand were used as described in Huggins et al. (2019b) under Ethics Permit: OACKU-00758 and extracted using the E.Z.N.A.[®] Blood DNA Mini Kit (Omega Biotek Inc., Norcross, GA, USA). For the comparison of DNA extraction kits in NGS metabarcoding applications, 50 whole blood samples were collected at four sites across Phnom Penh, Cambodia, from a mixture of pagoda temple communities, and locally owned and semi-domesticated dogs. Blood was only taken after obtaining informed consent from the relevant monk in the case of temple community dogs or the owner from local pet dogs. A qualified veterinarian conducted collection of two 1 mL blood samples per dog via cephalic puncture into anti-coagulation EDTA tubes on ice. These were then transferred to a -20 °C freezer upon return from the field. Approximately, 700 µL of blood from one sample, per dog, was then aliquoted to a separate Eppendorf tube and centrifuged at 12,000 rpm for 5 min in a portable LW-ZipCombo-C microcentrifuge (LW Scientific, Lawrenceville, GA, USA). From this, the blood plasma was disposed of and 250 µL of the Buffy Coat layer and surrounding erythrocytes was aliquoted and stored separately at -20 °C. Work in Cambodia was conducted under Ethics Permit: 1814620.1 from the University of Melbourne.

DNA extraction from the same 50 Cambodian canine whole blood or Buffy Coat samples was then carried out via one of four methods. Extractions were conducted according to manufacturer's instructions in the same laboratory using the maximum possible starting quantity of sample for each kit. This was done to elucidate the pathogen detection capability and level of bacterial kit contaminants without subsequent researcher modifications to kit protocol and to assess kit utility for 16S rRNA metabarcoding as developed by the manufacturer. Extraction methods were as follows:

- (1) Extraction of 500 μ L of whole blood using the Maxwell[®] RSC Whole Blood DNA Kit (Promega, Madison, WI, USA) on the automated Maxwell[®] RSC 48 Instrument DNA extraction robot. This was conducted as per the manufacturer's instructions and eluted in 100 μ L of Ambion Nuclease-Free Water (Life Technologies, Carlsbad, CA, USA).
- (2) Extraction of 250 μ L of Buffy Coat layer using the Maxwell[®] RSC Buffy Coat DNA Kit (Promega, Madison, WI, USA) on the Maxwell[®] RSC 48 Instrument, conducted as per the manufacturer's instructions and eluted in 100 μ L of Ambion Nuclease-Free Water.
- (3) Manual extraction of 200 μ L of whole blood, using the ISOLATE II Genomic DNA Kit (Bioline, Memphis, TN, USA), using the manufacturer's protocol with a final elution step in 50 μ L.
- (4) Manual extraction of 100 μ L of whole blood, using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) using the manufacturer's protocol with a final elution step in 50 μ L.

With all DNA extraction methods, two no blood/reagent only, DNA extraction negative controls were run per method to assess for levels of bacterial contamination in kits. Comparison of whole blood vs Buffy Coat based extraction was conducted due to a body of research suggesting that testing of specific blood fractions may improve sensitivity of detection for some blood-borne pathogens (Alexandre et al., 2009; Mylonakis et al., 2003).

4.3.2. DNA quantification

All DNA extractions were quantified on a Qubit[™] 3.0 fluorometer (Life Technologies) using the Qubit[™] dsDNA HS Assay Kit and means determined. Total DNA was also tested for in no blood/reagent only negative controls.

4.3.3. Design of blocking primer

Previous work by Huggins et al. (2019b) testing a variety of bacteria-universal 16S rRNA targeting primer pairs at hypervariable regions V1 to V6 identified cross-reactivity on canine host DNA as a recurrent problem. Even the best performing primer pair; Wehi_Adp_515F (5'-GTGYCAGCAGCCGCGGTAA-3') and Wehi_Adp_806R (5'-GGACTACNVGGGTATCTAAT-3') still generated as many as 97% of total reads in some samples from primer cross-reactivity against canine 12S rRNA sequences, with an average of 47% across all samples found (Huggins et al., 2019b). This was despite prior modifications to both primers to reduce degeneracy and thereby likelihood of cross-reactivity with canine 12S rRNA sequences (Huggins et al., 2019b). Hence, to further prevent this, a *Canis lupus* 12S rRNA specific blocking primer was designed (*Canis-mito-blk*) with a 3'-spacer C3 to prevent DNA polymerase elongation of such mitochondrial sequences when used alongside the Wehi_Adp_515F and Wehi_Adp_806R, 16S rRNA primers. Blocking primer design was conducted in Geneious v. 11.1.5 (Biomatters Ltd.) using 26 16S rRNA sequences from pathogenically relevant bacterial species and an *Escherichia coli* reference sequence. Moreover, eight sequences of the *C. lupus* mitochondrial 12S rRNA gene (which cross-reacted with bacterial primers in prior experiments) were added, alongside the sequences for the bacterial 16S rRNA primers (Huggins et al., 2019b) (**Figure 4.1**). The 5' end of our designed *Canis-mito-blk* primer has an 8 bp overlap with the bacterial forward primer Wehi_Adp_515F, then extending 11 bp downstream of this primer's binding site, ending with a 3' C3 spacer that prevents DNA polymerisation (**Figure 4.1**). The primer's high complementarity to canine 12S rRNA sequences suggests it may block amplification of these sequences. Furthermore, base pair (bp) differences with bacterial 16S rRNA sequences prevent this blocking primer from efficiently binding to this region, allowing amplification of bacterial sequences to continue. The final design of *Canis-mito-blk* was 5'-CCGCGGTCATACGATTAACC/3SpC3/-3'. **Figure 4.1** shows the location of the three bp differences between the Wehi_Adp_515F primer and the canine 12S rRNA mitochondrial sequences, where cross-reactivity can still occur.

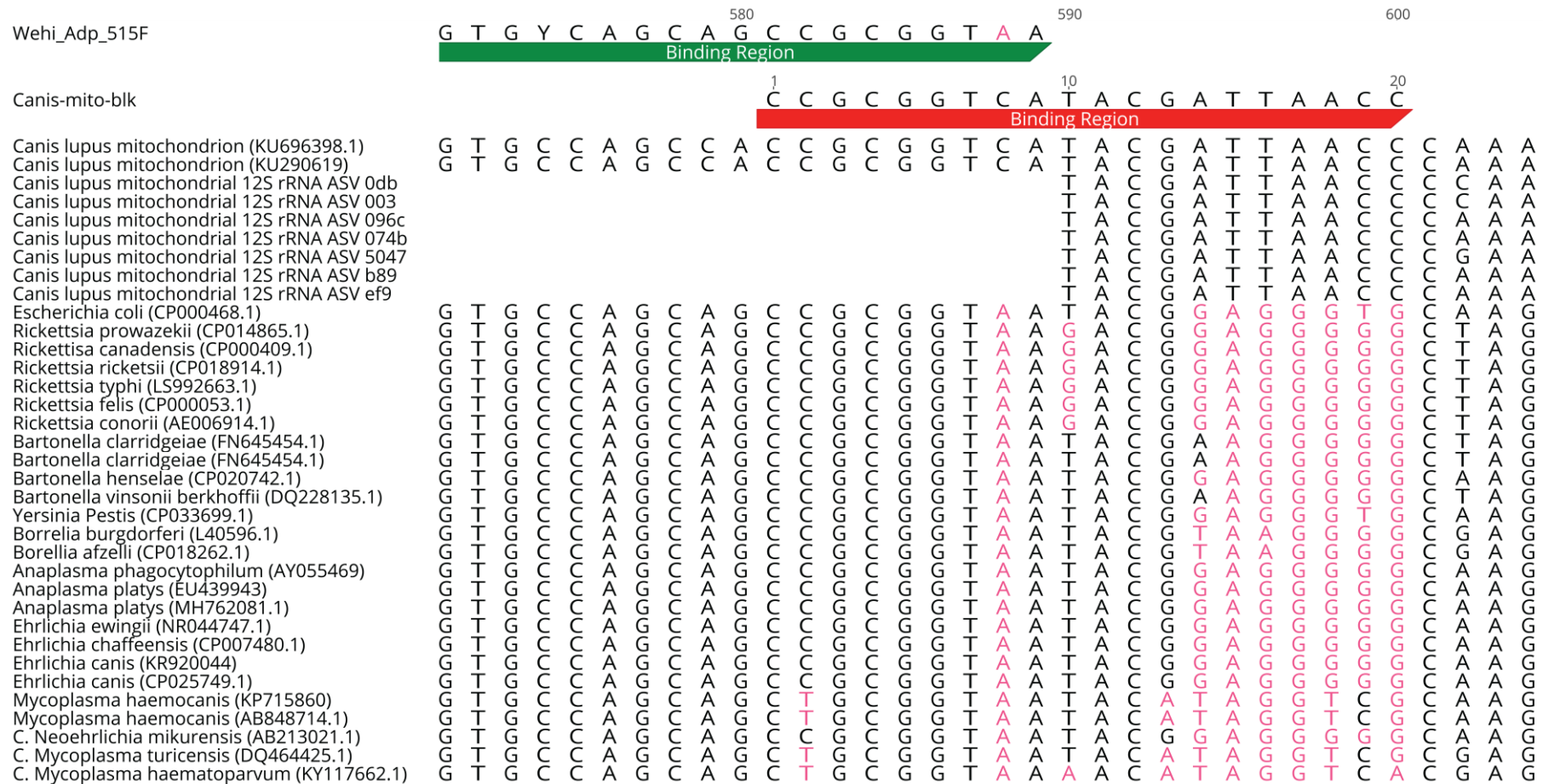


Figure 4.1. Alignment of pathogenic bacterial 16S rRNA sequences, canine 12S rRNA mitochondrial sequences, the forward bacterial primer Wehi_Adp_515F (green bar) and the *Canis*-mito-blk blocking primer (red bar) in the 5' to 3' direction. Nucleotide bases that differ from those at the relevant position in the *Canis*-mito-blk primer are coloured in light red, demonstrating dissimilarity between our designed blocking primer and bacterial 16S rRNA sequences but perfect complementarity with the 12S rRNA mitochondrial counterparts.

4.3.4. Validation of *Canis-mito-blk*

To test for the specificity of *Canis-mito-blk* *in vitro* to canine 12S rRNA sequences, cPCR was conducted with and without the addition of the 3' C3 spacer, plus a reverse canine 12S rRNA specific primer (5'-AATCCCAGTTTGGGTCTTAGC-3') producing a 137 bp product. PCRs were 20 µL in total, comprising 10 µL of OneTaq® 2X Master Mix with Standard Buffer (New England Biolabs, Ipswich, MA, USA) 0.4 µM of both forward and reverse 16S bacterial primers, 1.2 µM of both *Canis-mito-blk* and the 12S rRNA reverse primer, 1 µL of template DNA and Ambion Nuclease-Free Water. The ratio of blocking primer to bacterial primer should be informed by the ratio of cross-reactive 12S rRNA reads to 16S rRNA bacterial sequences (Vestheim et al., 2010), which is at a ratio of approximately 1:1 as calculated using prior data collected by Huggins et al. (2019b). Therefore, to ensure blocking primer was in excess we used a 1:3 ratio of bacterial primer to blocking primer, as reported by similar studies, utilising blocking primers (Gofton et al., 2015; Leray et al., 2013; Tan and Liu, 2018). Thermocycling conditions were taken from Huggins et al. (2019b) and were 95 °C for 3 min, 35 cycles of 95 °C for 45 s, 56 °C for 60 s and 72 °C for 90 s with a final elongation at 72 °C for 10 min. Specific amplification of 12S rRNA sequences was conducted by visual observation of 137 bp bands on a 1.5% agarose gel using a GelDoc™ XR + System (Bio-Rad, Hercules, CA, USA). PCR combinations using the blocking primer with and without bacterial 16S rRNA primers and the 3' C3 spacer on DNA from non-infected and infected blood were conducted to ensure that *Canis-mito-blk* could amplify 12S rRNA sequences in the absence of the 3' C3 spacer and completely block amplification when the 3' C3 spacer was present.

4.3.5. Bacterial 16S rRNA metabarcoding assessment of *Canis-mito-blk* efficacy

Fifty previously characterised blood-extracted DNA samples from Thai dogs (Huggins et al., 2019b) were deep-sequenced with and without the use of the *Canis-mito-blk* primer to assess its efficacy at preventing bacterial primer cross-reactivity and improving pathogen diversity characterisation. All first-step and second-step PCRs for deep-sequencing library preparation were conducted within a PCR hood after UV sterilisation. The first-step PCR consisted of 10 µL of OneTaq® 2X Master Mix, 0.4 µM of both forward and reverse 16S bacterial primers with NGS overhang

sequences, 1.2 µL of *Canis*-mito-blk (for the 50 samples using the blocking primer), 1 µL of template DNA with the whole reaction made up to a total volume of 20 µL with Ambion Nuclease-Free Water. Bacteria primer sequences with the addition of NGS overhangs (underlined) were WehiNGS_Adp_F (5'-GTGACCTATGAACTCAGGAGTCGTGYCAGCAGCCGCGGTAA-3') and WehiNGS_Adp_R (5'-CTGAGACTTGACATCGCAGCGGACTACNVGGGTATCTAAT-3'). The thermocycling profile was used with just 20 amplification cycles, instead of 35. PCR product was then cleaned using 1X Ampure Beads (Beckman Coulter, Brea, CA, USA) and checked for quality on an Agilent 2200 Tape Station (Agilent Technologies, Santa Clara, CA, USA) before proceeding. Two no-template PCR negative controls and four uniquely identifiable positive controls were also included in this first-step PCR. Due to the need to assess for possible cross-contamination between samples during deep-sequencing library preparation or Illumina indexing errors, artificial positive control constructs were designed and synthesised as recommended by Kim et al. (2017). Positive controls were a gBlock synthetic DNA construct (Integrated DNA Technologies, Coralville, IA, USA) comprised of a uniquely identifiable 253 bp 16S rRNA sequence from *Aliivibrio fischeri*, a bacterial endosymbiont found within the Bobtail squid, *Euprymna scolopes* and its environment (Nyholm and McFall-Ngai, 2004; Ruby et al., 2005). The *A. fischeri* sequence is flanked by the relevant 16S rRNA primer binding sites to allow for positive control amplification and is 253 bp long to match the typical length of fragments amplified by the 16S rRNA primers on naturally occurring bacterial species. This bacterial sequence was chosen as there is very little possibility that *A. fischeri* would be found as an environmental or laboratory contaminant, nor would it be found in canine blood, making it uniquely identifiable. **Appendix 4.1** has the sequence of this positive control construct. The second-step PCR for addition of library indices and deep sequencing was then conducted according to Huggins et al. (2019b) using an Illumina MiSeq (Illumina, San Diego, CA, USA) with paired-end 600-cycle v3 chemistry at the Walter and Eliza Hall Institute (WEHI) Proteomics Facility, Parkville, Australia. Paired-end sequencing was conducted to assure read quality as only reads that had identical overlapping R1 and R2 regions were passed on to downstream analysis.

4.3.6. *Bacterial 16S rRNA metabarcoding comparison of DNA extraction kit performance*

The 50 Cambodian dog blood samples and two reagent-only extractions per method were conducted using the previously described protocols on the Maxwell® RSC Whole Blood DNA Kit (WB), the Maxwell® RSC Buffy Coat DNA Kit (BC), the Bioline ISOLATE II Genomic DNA Kit (BL) and the Qiagen DNeasy Blood and Tissue Kit (QG). Samples were then prepared for deep-sequencing in the same manner as those used in the blocking primer comparison, with 112 samples indexed and multiplexed per flow cell. Each batch of 50 DNA extractions per kit included an additional seven controls that were deep sequenced. These were; two reagent-only DNA extraction negative controls, two no-template PCR negative controls, two uniquely identifiable *A. fischeri* positive controls and one ZymoBIOMICS Microbial Community DNA Standard (Zymo Research, Irvine, CA, USA) positive control to assess for PCR amplification bias.

4.3.7. *Bioinformatics*

Raw NGS data was demultiplexed using in-house software at WEHI and then imported into the QIIME 2 (v. 2019.1) environment for bioinformatic analysis. Raw data were processed according to Huggins et al. (2019b) with the only adaptation being that amplicon sequence variants (ASVs) were generated and taxonomically assigned as opposed to sequence clustering and formation of operational taxonomic units (OTUs), reflecting recent research into microbiome analysis best practice (Knight et al., 2018; Langille et al., 2018). Alpha rarefaction plots were generated, using the functions MAFFT (Kato and Standley, 2013) and FastTree 2 (Price et al., 2010) to ensure that OTU diversity plateaued and hence a sufficient sequencing depth had been achieved. All NGS data generated exploring the use of our blocking primer is available from the NCBI BioProject database, with BioProject ID: PRJNA528154 and SRA data accession numbers SRR10895013 to SRR10895109. All data that was obtained to compare the performance of DNA extraction kits is available under BioProject ID: PRJNA601241 and SRA data accession numbers SRR10959940 to SRR10960037 (WB and BC), SRR10972728 to SRR10972776 (BL) and SRR10972824 to SRR10972872 (QG).

The read threshold for a true infection by a bacterial pathogen was determined as the mean reads of non-control samples that were identified as having sequences from *A. fischeri* (the positive control construct) within them. The appearance of these positive control sequences in other samples could be due to occasional index misreading or hybridisation errors during Illumina sequencing as well as low-level cross-contamination during library preparation (Kim et al., 2017). Read threshold values were never higher than 62 reads, which was the NGS run found to have the highest amount of cross-contamination. Appearance of contaminant sequences across the 96-well plate was scattered and not concentrated near the positive control samples, implying that library preparation was unlikely to be the major cause of such cross-contamination.

Taxa found in no-reagent DNA extraction negative controls and no-template PCR negative controls were deducted from the overall dataset used for comparison of pathogen detection capability. This negative control data was used for comparison of contaminant bacterial DNA arising from extraction kits.

The results from the ZymoBIOMICS Microbial Community DNA Standards were compared to the expected read composition. A high level of similarity was found, demonstrating no PCR amplification bias by our chosen bacterial 16S rRNA primers.

4.3.8. Data analysis of blocking primer performance

Data analysis to calculate blocking primer performance, including biodiversity index calculations and blocking efficiency was conducted in Excel 2016 v. 1803 (Microsoft, Redmond, WA, USA). Statistical analyses were carried out using Minitab v. 19.2 (Minitab LLC, State College, PA, USA).

The percentage of relevant vector-borne bacterial reads (*A. platys*, *E. canis* and *Mycoplasma haemocanis*) compared to the total reads for each sample was calculated, to account for differences in sampling effort between samples, observable as differing numbers of total sample reads. Data normality was tested for using the Anderson–Darling test. The mean percentage of bacterial reads compared to total reads, standard error and Wilcoxon paired t-test statistics were calculated to determine if differences in means between paired samples, i.e., the same sample with and without the blocking primer, were statistically significant. Results were

interpreted as significant at the $P < 0.01$ level. The same analysis was carried out to elucidate the average proportion of canine mitochondrial cross-reactivity between the same samples with and without the blocking primer. In addition, blocking primer efficiency was calculated as defined by Tan and Liu (2018) in the equation $(X - Y) \div Y$. Here, X is defined as the ratio of mitochondrial cross-reactive reads to the total of all bacterial reads without the blocking primer for a sample and Y is the ratio of mitochondrial reads to total bacterial reads with the blocking primer for each sample. This equation works on the assumptions that *Canis-mito-blk* is specific to canine mitochondrial sequences and, therefore, that non-mitochondrial sequences will not be inhibited. Following this, the mean and standard error of all sample blocking efficiencies was calculated to get an approximation of overall *Canis-mito-blk* blocking efficiency.

Read cut-off values to ascertain if a sample had a particular vector-borne bacterial species present, i.e., was a true infection were defined according to Huggins et al. (2019b). In brief, the mean number of unique *A. fischeri* positive control construct reads that appeared in non-positive control samples was calculated across all samples, with and without the blocking primer. The means of these reads was taken to demonstrate the average quantity of Illumina indexing errors or potential cross-contamination between samples during library preparation. Therefore, read values under these means were not counted as true infections while those above were counted. The total number of infections was tallied across the same 50 samples with and without the blocking primer to assess sensitivity of pathogen detection.

Biodiversity indices were calculated for all bacterial reads across all samples with and without the blocking primer to elucidate whether their addition improved the ability of our methodology to characterise bacterial species diversity and, therefore, the potential to detect rare or novel species. We used two biodiversity indices; Shannon–Wiener (H) and Simpson's (D) index, which both account for species richness (number of bacterial species) and species abundance; approximated via number of reads of each species (Allaby, 2010; Gill and Joanes, 1979; Hunter and Gaston, 1988; Ogedengbe et al., 2018; Spellerberg and Fedor, 2003).

4.3.9. Data analysis of DNA extraction kit performance

DNA extraction kit performance was assessed using 43 blood samples that were successfully deep-sequenced from all four kit types used. DNA yield, number of vector-borne bacterial infections detected, mean percentages of contaminant bacterial reads and mean percentage of bacterial primer cross-reactivity on canine mitochondrial sequences, relative to total reads were all calculated. Statistical analysis to compare mean percentages of cross-reactivity and mean percentages of bacterial contaminant sequences were calculated using Minitab v. 19.2 using one-way ANOVAs and Tukey pairwise comparisons to assess which means were statistically significant between kit types at the $p < 0.05$ level.

Regarding the assessment of levels of bacterial contamination from kits, i.e., artificial contamination, a bacterial taxon was deemed to be an artificial contaminant if it had a read count of ≥ 100 in either of the no blood/reagent only DNA extraction negative controls, run for that kit type. For every sample, the number of reads for all artificial kit contaminant taxa was totalled and a percentage calculated relative to the total number of reads. Artificial kit contaminants are different from natural contaminants, such as bacteria residing on canine skin, that may be picked up during sampling via venepuncture or the sampling environment, these naturally derived contaminants were not assessed.

4.4. Results

4.4.1. Blocking primer performance

Initial experimentation using cPCR analysis with gel electrophoresis assessed the ability of our blocking primer: *Canis*-mito-blk, to bind to canine 12S mitochondrial sequences when it did not have the 3'-spacer C3 modification, which was observable via production of a 137 bp product (data not shown). When the blocking primer had the 3'-spacer C3 added, this 137 bp product was no longer produced even when the bacterial 16S rRNA primers were present in the PCR, indicating complete blocking of canine 12S rRNA amplification. Bacterial primers were not prevented from amplifying bacterial sequences as an approximately 250 bp product was still produced by these primers when used on canine blood samples positive for the pathogens *E. canis*, *A. platys* and *R. felis* (data not shown).

After bioinformatic processing and filtering of NGS data a total of 4,746,542 reads were accrued, a result within the typical range for amplicon-based Illumina sequencing at a high-quality Phred score of over thirty. From the total reads, 58% were from samples amplified without blocking primers and 42% from samples with blocking primers.

Analysis of the mean percentage of canine mitochondrial reads relative to total reads on a per sample basis was 59% ($\pm 5.1\%$) for samples amplified without the blocking primer compared to 19% ($\pm 3.1\%$) with the primer (**Figure 4.2**). The difference between these two groups was statistically significant at the $P < 0.01$ level, indicating that our blocking primer significantly reduced the amount of canine mitochondrial reads amplified. Mean blocking efficiency across our canine DNA samples was 25% ($\pm 3.0\%$) according to the formula defined by Tan and Liu (2018), with individual sample blocking efficiencies ranging from as low as 2.2% in some samples with low total sequence counts to as high as 100% blocking efficiency in others.

Pathogenic vector-borne bacterial species were detected from the species *E. canis*, *A. platys*, *M. haemocanis*, '*Candidatus Mycoplasma haematoparvum*', *Mycoplasma turicensis*, *Bartonella* spp. as had been previously found by Huggins et al. (2019b). However, only the high prevalence of *E. canis*, *A. platys* and *M. haemocanis*, permitted statistically comparable results between the ability of the metabarcoding methodology to detect vector-borne bacterial DNA with and without the use of *Canis-mito-blk*. When our blocking primer was used more *E. canis* and *A. platys* infections were detected by our bacterial metabarcoding method (**Table 4.1**). For *E. canis*, 24 infections were found using the blocking primer compared to 19 when they were not used, a similar increase in detection capability was found for *A. platys* with 20 infections detected using the blocking primer compared to 17 without. For detection of *M. haemocanis*, 19 infections were detected both with and without the blocking primer. The mean percentage of vector-borne bacterial reads with the blocking primer was always higher than when no blocking primers was used for; *E. canis* ($15\% \pm 4.0\%$ vs $5.4\% \pm 2.0\%$), *A. platys* ($35\% \pm 8.5\%$ vs $23\% \pm 7.0\%$) and *M. haemocanis* ($53\% \pm 8.7\%$ vs $35\% \pm 7.5\%$) with all differences being statistically significant (**Figure 4.3**).

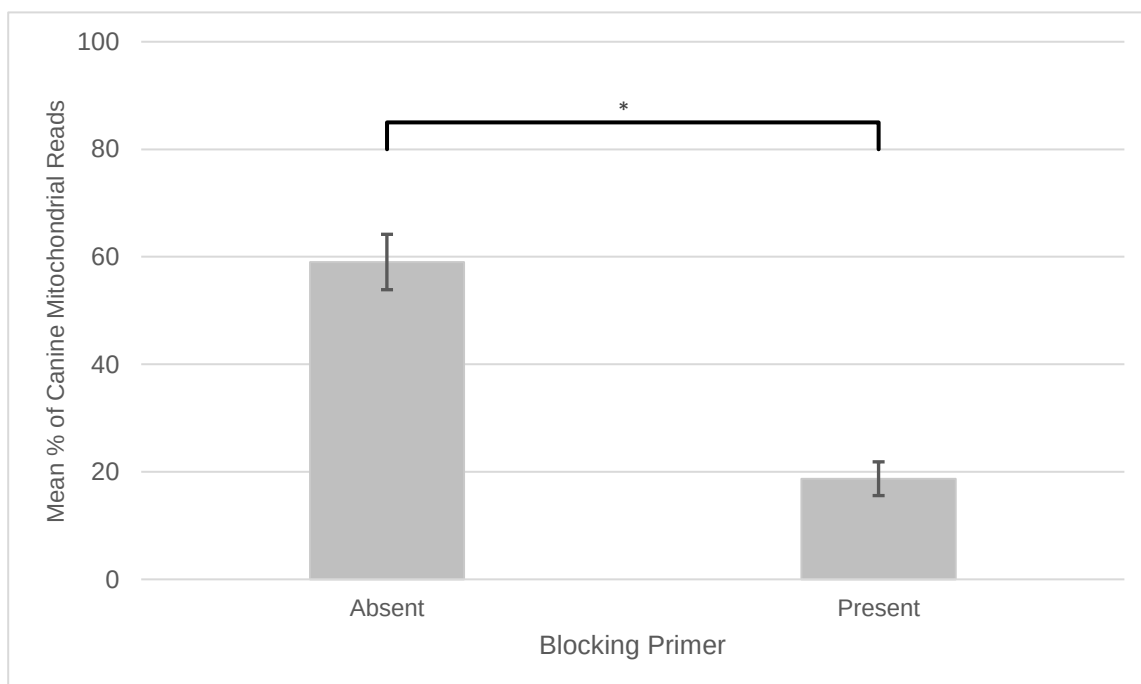


Figure 4.2. Mean percentage of canine mitochondrial reads across all samples in the absence and presence of the blocking primer. Vertical error bars display standard error, horizontal bar highlights a statistically significant difference at the $p < 0.01$ level (*).

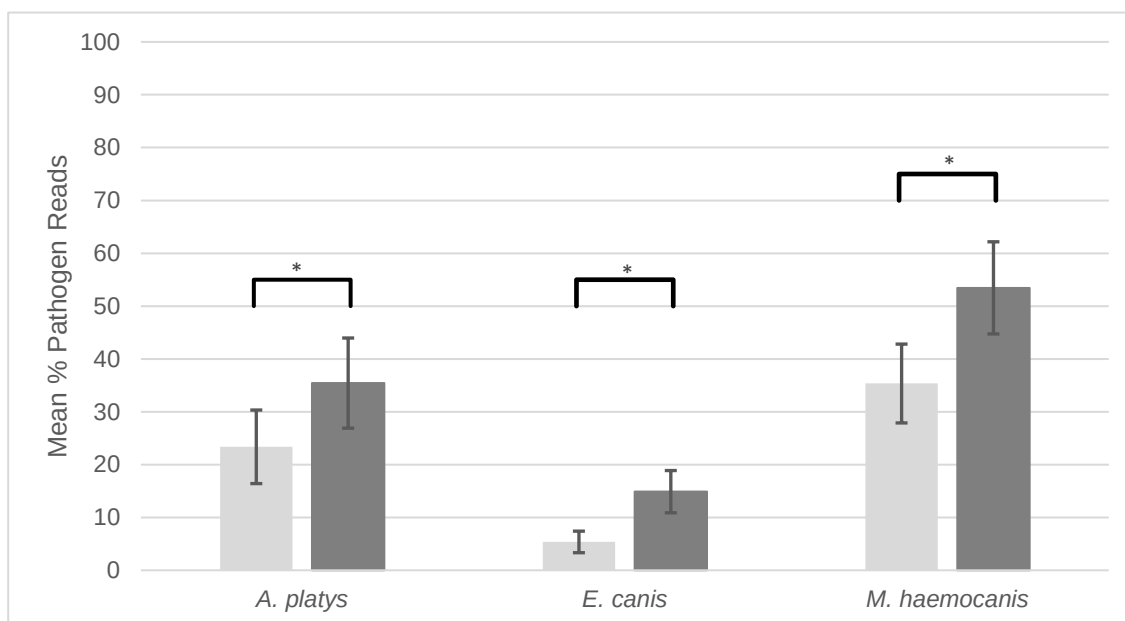


Figure 4.3. Mean percentage of three different pathogen reads across all pathogen positive samples in the absence of the blocking primer (light grey) and with the blocking primer (dark grey). Vertical error bars display standard error and horizontal bars highlight differences that are significant at the $p < 0.01$ level (*).

Table 4.1. Comparison of total number of infections detected when *Canis-mito-blk* was absent or present. Infections were deemed true when the total number of reads of a particular pathogen were over the read cut-off value as defined in the “Methods”.

Vector-Borne Bacteria	Total Infections:	Total Infections:
	Blocking Primer Absent	Blocking Primer Present
<i>A. platys</i>	17	20
<i>E. canis</i>	19	24
<i>M. haemocanis</i>	19	19

Table 4.2. Comparison of biodiversity indices when blocking primers (BP) were absent or present. The higher the index, the greater the relative diversity.

Bacterial Diversity: Shannon–Wiener (H)		Bacterial Diversity: Simpson (D)	
Index		Index	
BP Absent	BP Present	BP Absent	BP Present
1.81	2.3	3.15	4.07

For samples with less common vector-borne bacteria, the percentage of pathogen reads compared to total reads was always higher for the samples that utilised the blocking primer. For example, for the two samples that were found to have a *Bartonella* spp. infection and two with a *Mycoplasma turicensis* infection, the average percentage of reads for the former pathogen was 1.5% vs 0.42% (with blocking primer vs without) and 16% vs 2.1% for the latter pathogen. A single sample with ‘*Candidatus Mycoplasma haematoparvum*’ reads had 54% of reads from this pathogen when using *Canis-mito-blk*, compared to 27% without.

Comparing the number of bacterial taxonomic identifications across all samples with the blocking primer, it was found that there were 353 different sequences vs 327 without the blocking primer. Moreover, diversity indices calculated on just the bacterial portion of total sequences for all samples that used blocking primers vs all samples that did not, indicated that using the blocking primer improved characterisation of bacterial diversity. For both indices, a greater species diversity is

demonstrated by a larger number. Using Simpson's index, a value of 4.07 was acquired for samples using the blocking primer compared to 3.15 without (**Table 4.2**). With the Shannon–Wiener index, which takes into greater consideration rarer species with low read numbers, the index value was 2.3 when the blocking primer was used in contrast to 1.81 for samples without it (Morris et al., 2014), see **Table 4.2**.

4.4.2. DNA extraction kit performance

DNA extraction kit yield was compared which found that the automated extraction methods; Promega Buffy Coat (BC) = 680.2 ng/μL and Promega whole blood (WB) = 483.8 ng/μL obtained much higher average DNA yields than the non-automated kits Qiagen whole blood (QG) = 13.9 ng/μL and Bioline whole blood (BL) = 3.4 ng/μL (**Table 4.3**). At the same time, the range of DNA quantified in the extraction negative controls was also higher for the automated methods 0.005–0.024 ng/μL, while for QG, a lower range of 0.001–0.014 ng/μL was found and BL had a DNA yield below the lower limit at which the Qubit™ can detect (**Table 4.3**). Such findings demonstrate a potentially higher initial quantity of bacteria or bacterial DNA in the automated extraction kits.

Following bioinformatic processing, differing numbers of total raw and filtered reads were obtained for the datasets from each extraction method, an inevitable result of samples from each extraction method having to be run on separate NGS flow cells (**Table 4.3**). After filtering, the number of reads between datasets varied by a maximum of 1,157,220 reads. The total ASVs found across each kit's dataset also differed greatly with QG kits finding the largest diversity of ASVs at 6683, followed by BL at 1401, WB at 435 and BC at 428 (**Table 4.3**). Counterintuitively, total reads post-filtering was not a good predictor of ASV diversity as QG, which had the lowest number of post-filtering reads and also had the highest ASV diversity. Additionally, BC had the highest post-filtering read total and the lowest ASV diversity.

In a similar manner to the data from Thai dogs, the main vector-borne bacterial DNA found was from the species *E. canis*, *A. platys* and *M. haemocanis*, with differences in the numbers of infections elucidated between extraction kit types (**Table 4.4**). Across the 43 Cambodian blood samples compared, the QG kit found the highest

number of infections for all three main pathogenic bacterial species at 35, followed by WB and BC with 28 and BL finding the least infections at 23 (**Table 4.4**). In addition, the QG kits found ASVs taxonomically classified as putative pathogens within the genera *Rickettsia* spp. and *Coxiella* spp., which were not detected by any of the other kit types. These taxonomic classifications could not be resolved to species level through either QIIME2 or a BLASTn search in GenBank due to very high sequence similarities between different species in the same genus.

Table 4.3. Comparison of mean DNA quantity and standard error (S.E.) from the same blood samples extracted with different kits and comparison of contaminant DNA in kit reagents alone. Includes post-bioinformatic analysis results of total raw and filtered reads accrued between different kit types as well as total Amplicon Sequence Variants (ASVs), i.e. diversity of DNA sequences found.

DNA Extraction Kit	Mean DNA Quantity ± S.E. (ng/μL)	DNA Quantity		Total Raw Reads	Reads Post- Filtering	Total ASVs
		in Extraction	Negative Controls (ng/μL)			
Promega Whole Blood (WB)	483.8 (±150.9)	0.005–0.017		5,351,987	3,857,213	435
Promega Buffy Coat (BC)	680.2 (±394.7)	0.012–0.024		5,916,458	4,716,590	428
Bioline Whole Blood (BL)	3.4 (±0.6)	<0.001		5,935,103	4,188,285	1401
Qiagen Whole Blood (QG)	13.9 (±1.1)	0.001–0.014		4,462,788	3,559,370	6683

Table 4.4. Comparison of total number of infections detected of the three main bacterial pathogens by different extraction kits. Infections were deemed true when the total number of reads of a pathogen were over the read cut-off value as defined in the “Methods”.

Vector-Borne Bacteria	Promega Whole Blood (WB)	Promega Buffy Coat (BC)	Bioline Whole Blood (BL)	Qiagen Whole Blood (QG)
<i>A. platys</i>	18	17	13	22
<i>E. canis</i>	7	7	6	8
<i>M. haemocanis</i>	3	4	4	5

The amount of artificial kit-derived contaminant DNA detected by our NGS methodology differed significantly depending on the extraction kit used (**Figure 4.4**). Both automated extraction kits had a significantly higher percentage of bacterial contaminant DNA (BC 86% and WB 80% of total reads) compared to 34% of reads for QG and 19% for BL. All differences between kits were significant at the $p < 0.05$ level apart from the mean contaminant reads between the two automated kits; WB and BC.

The number of different ASVs assessed as being artificial kit contaminants due to them appearing with a read count of ≥ 100 in DNA extraction negative controls also varied with WB and BC kits having just four kit-contaminant ASVs, BL ($n = 18$) and QG ($n = 57$). Furthermore, the WB and BC kits demonstrated a distinct bacterial contaminant ‘fingerprint’, (**Table 4.5**). For example, with the WB kits, results, on average, had a total of 51% of reads classified as *Massilia* spp. and 28% of reads as *Pseudomonas* spp. whilst BC kits on average had a composition of 61% *Massilia* spp. and 24% *Cellulomonas* spp. reads. In comparison, the composition of contaminant bacterial reads was less marked for the non-automated kits with QG having 7.9% of sequences classified as belonging to chloroplast DNA and 4.3% as *Burkholderia* spp., whilst BL kits had 4.8% of total reads on average belonging to *Burkholderia* spp. (**Table 4.5**).

The amount of cross-reactive amplification between the bacterial primers on canine mitochondrial sequences also differed significantly, depending on the DNA extraction kit used, despite blocking primers being present in all reactions. When samples were extracted using BL kits a statistically significant increase ($p < 0.05$) in the mean percentage of canine mitochondrial reads relative to total reads was observed with an average of 31% of reads being host mitochondrial sequences (**Figure 4.5**). This contrasted with much lower percentages of host mitochondrial sequences when samples were extracted with QG (1.5%), WB (1.1%) and BC (0.2%).

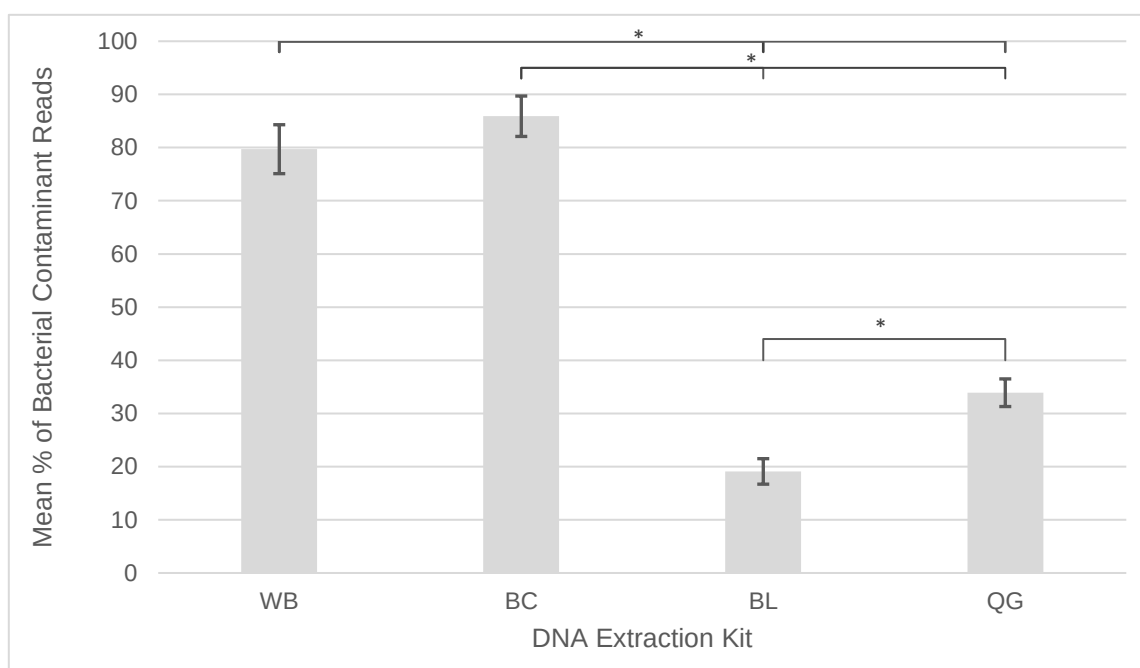


Figure 4.4. Mean percentages of kit-derived artificial bacterial contaminant reads relative to total reads across four different DNA extraction kits: Promega Whole Blood (WB), Promega Buffy Coat (BC), Bioline Whole Blood (BL), Qiagen Whole Blood (QG). Vertical bars display standard error and asterisks show differences between kit types that are significant at the $p < 0.05$ level (*).

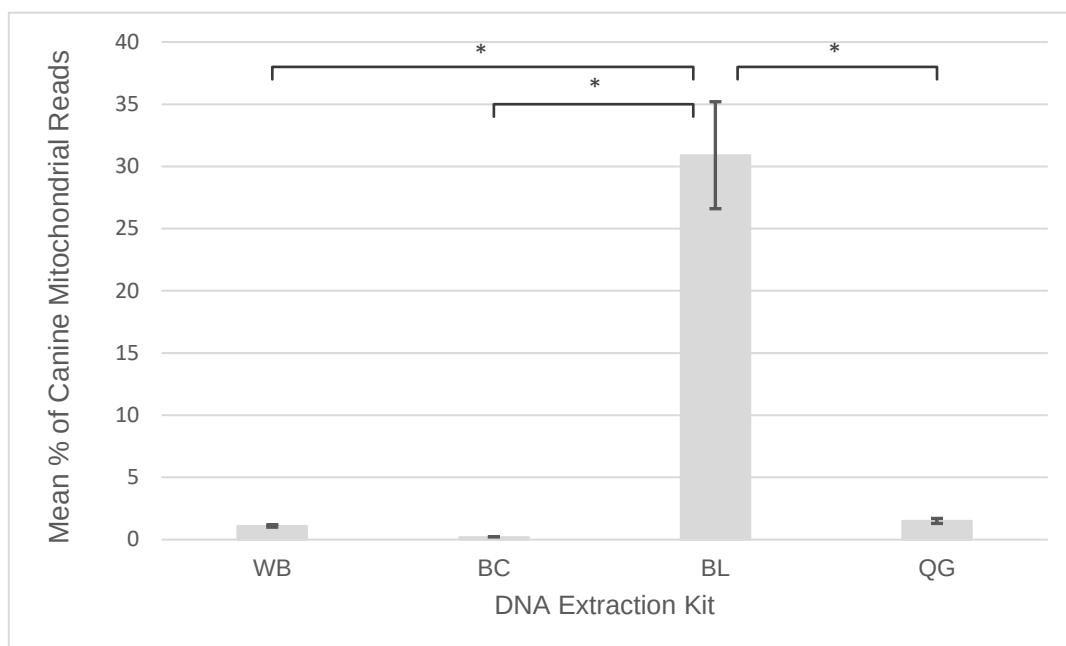


Figure 4.5. Mean percentages of canine mitochondrial reads relative to total reads across four different DNA extraction kits: Promega Whole Blood (WB), Promega Buffy Coat (BC), Bioline Whole Blood (BL), Qiagen Whole Blood (QG). Vertical bars display standard error and asterisks show differences between kit types that are significant at the $p < 0.05$ level (*).

Table 4.5. Most abundant bacterial ASVs found within four different DNA extraction kits' reagents. Only bacterial ASVs that comprise over 0.1% of total reads for the respective extraction kit's NGS run and that were able to be classified below the rank of class are included. Maxwell® RSC Whole Blood DNA Kit = WB, Maxwell® RSC Buffy Coat DNA Kit = BC, Bioline ISOLATE II Genomic DNA Kit = BL, Qiagen DNeasy Blood & Tissue Kit = QG.

WB Kit	Reads	QG Kit	Reads
Contaminant	(% of Total)	Contaminant continued	(% of Total)
<i>Massilia</i> spp.	51.36	<i>Prevotella</i> spp.	1.42
<i>Pseudomonas</i> spp.	28.16	<i>Candidatus Nomurabacteria</i>	1.24
<i>Leifsonia</i> spp.	0.17	<i>Flavobacterium</i> spp.	1.20
BC Kit Contaminant	Reads	<i>Acinetobacter</i> spp.	1.13
(% of Total)	(% of Total)	<i>Fodinicola</i> spp.	1.08
<i>Massilia</i> spp.	61.20	Saccharimonadales	1.03
<i>Cellulomonas</i> spp.	24.33	<i>Legionella</i> spp.	0.77
<i>Leifsonia</i> spp.	0.36	<i>Sphingomonas</i> spp.	0.62
BL Kit Contaminant	Reads	<i>Aquicella</i> spp.	0.41
(% of Total)	(% of Total)	<i>Gracilibacteria</i> spp.	0.34
Burkholderiaceae	4.79	Candidatus	0.27
<i>Pseudomonas</i> spp.	4.66	<i>Zambryskibacteria</i>	
<i>Acinetobacter</i> spp.	1.02	Candidatus	0.25
<i>Janthinobacterium</i> spp.	0.82	<i>Magasanikbacteria</i>	
<i>Sphingomonas</i> spp.	0.67	<i>Oligoflexales</i> spp.	0.25
Enterobacteriaceae	0.61	Saprospiraceae	0.24
<i>Massilia</i> spp.	0.36	Sphingomonadaceae	0.24
<i>Aquabacterium</i> spp.	0.26	Lachnospiraceae	0.22
<i>Pedobacter</i> spp.	0.25	<i>Methylobacterium</i> spp.	0.17
<i>Undibacterium</i> spp.	0.15	<i>Faecalibacterium</i> spp.	0.16
QG Kit Contaminant	Reads	<i>Polynucleobacter</i> spp.	0.15
(% of Total)	(% of Total)	Weeksellaceae	0.13
Chloroplast	7.95	Babeliales	0.12
Burkholderiaceae	4.33	<i>Arcicella</i> spp.	0.11
<i>Parcubacteria</i> spp.	3.38		
Mitochondria	1.63		

4.5. Discussion

4.5.1. Blocking primer performance

The use of our blocking primer (*Canis*-mito-blk) was demonstrated to significantly reduce the amount of bacterial primer cross-reactivity on host canine mitochondrial sequences, significantly increase the relative proportion of vector-borne bacterial DNA sequenced and increase the detection capability for elucidating infections by our NGS metabarcoding method. The increase in detection capability between our metabarcoding method with and without our blocking primer is particularly important as five more *E. canis* infections and three more *A. platys* infections were detected from canines when using the blocking primer in the first-step PCR. Accurate detection and characterisation of such pathogens is key when conducting a range of studies from large-scale surveillance investigations where reductions in sensitivity may impair the ability to provide apparent estimates that match the true prevalence of disease, through to clinical diagnostics on infirm canines where a missed infection may be fatal (Greiner and Gardner, 2000; Little, 2010; Mylonakis et al., 2019). An increase in detected infections when using the blocking primer is similar to several studies which have reported exposition of previously occult DNA of interest using various blocking primers, in the context of both pathogen detection (Gofton et al., 2015) and ancient DNA (Boessenkool et al., 2012).

The number of detected infections by *M. haemocanis* was the same for samples regardless of whether the blocking primer was used. *M. haemocanis* infections were typically observed to have high numbers of reads, frequently over 10,000 per sample but reaching as high as 149,000 in one sample. This may result in *M. haemocanis* infections being easier to detect than other vector-borne bacterial species and thus minimising the possibility of missed infections even in the absence of blocking primers.

Not only were more samples detected using our blocking primer but the relative proportion of canine vector-borne bacterial reads, relative to the total number of reads, was always higher when using this blocking primer. These results provide greater support for an improved metabarcoding sensitivity when utilising our blocking primer as if more pathogen 16S rDNA is amplified and sequenced relative to total amplified DNA, then the probability that infections with even low levels of circulating

bacteria are detectable is also increased. Similar results were obtained by Tan and Liu (2018) who were able to increase the relative abundance of desired protist reads when using metazoan-specific blocking primers in marine environments.

Conversely, the relative proportion of canine mitochondrial sequences that were amplified by our bacterial primers was lower by an average of three times when using our blocking primer, a result that is further supported by the primer's mean blocking efficiency of 25%. Not only do such results demonstrate the blocking primer's ability to prevent amplifying primer cross-reactivity on host 12S rRNA sequences but they also signify an increase in relevant data obtainable when deep sequencing with our blocking primer. A typical MiSeq v3 chemistry run outputs between 3.3 to 15 Gb of data, (Ravi et al., 2018) and if, as found in the current research, as much as 59% of the data output is the result of primer cross-reactivity on host mitochondrial DNA (mtDNA), then a substantial cost is accrued in terms of labour, time and money to the researcher. Furthermore, if a large proportion of sampling effort is due to cross-reactive amplification then there is the potential for a significant masking effect whereby bacterial pathogen DNA may be missed as disproportional quantities of canine mtDNA are sequenced instead.

The concentration of blocking primer used throughout this study was informed by prior experimentation elucidating a roughly 1:1 ratio of target to non-target DNA amplification. When this information was assessed, in conjunction with comparison from studies that had dealt with similar proportions of non-target DNA (Gofton et al., 2015; Tan and Liu, 2018; Vestheim et al., 2010), a ratio of 3:1 blocking primer to amplifying primers was deemed sufficient. Experimentation using cPCR and evidence from a reduction of canine mitochondrial sequences demonstrates our chosen ratio to be effective, however, even with the addition of a blocking primer at this ratio there was still some continued amplification of canine mtDNA. On average 18% of each sample's total reads was comprised of canine mitochondrial reads, with great variability on a sample by sample basis ranging from 0% to 64%. Taking this into consideration, an even higher ratio of blocking primer to amplifying primers may have reduced this cross-reactivity even more. Nonetheless, there is also a possibility that blocking primers could be generating off-target blocking effects on bacterial sequences, potentially reducing the detection of certain bacterial groups. For example, the 5' end of our blocking primer has a run of at least seven bp that are

capable of binding to bacterial 16S rRNA sequences, indicating there may be some potential for this blocking primer to anneal to bacterial sequences and reduce Wehi_Adp_515F efficiency. Therefore, it was important to keep blocking primer concentration as low as possible, whilst remaining effective at blocking desired targets (Boessenkool et al., 2012; Tan and Liu, 2018).

4.5.2. DNA extraction kit performance

Differences between results acquired throughout the range of parameters investigated were substantial between DNA extraction kit types, despite the same canine blood samples being used. Considering all kit performance results, the Qiagen DNeasy Blood and Tissue Kit performed the best within our NGS metabarcoding framework as it was able to detect the highest number of vector-borne bacterial infections (**Table 4.4**). This kit also elucidated a greater diversity of bacterial sequences, including some from putative pathogens, such as *Rickettsia* spp. and *Coxiella* spp., that were not found using the other extraction kits. Such results are of importance to our study given that the strength of our NGS-methodology is its ability to detect unusual or novel pathogens without *a priori* information regarding likely pathogens or their respective 16S rRNA sequences. Our findings, mirror those of other NGS kit comparison assessments that have found that DNA extraction kit employed can have a significant effect on the abundance and diversity of bacterial groups found (Hart et al., 2015; Velásquez-Mejía et al., 2018). For example, similar to our current results, Hart et al. (2015) found that a Qiagen spin-column based extraction method was better able to detect rare phyla when compared to four other extraction methods (Hart et al., 2015).

The considerably higher number of ASVs detected when using the QG kits was over 15 times the amount found by the automated WB and BC kits and almost five times that found by the BL kits, demonstrating the strength of the QG kits to liberate a higher diversity of bacterial sequences and allow for better blood microbiome characterisation (**Table 4.3**). Moreover, of all the kit types tested, the QG kit used the lowest quantity of initial starting material at just 100 µL of whole blood compared to 200 µL for both the BL and BC kits and 500 µL for the WB kit. Such findings provide weight to the argument that quantity of starting material alone does not

necessarily improve sensitivity of pathogen detection and that DNA extraction methodology and chemistry may play a more significant role. This is further supported by the data on the number of raw and filtered reads obtained for the different kits, which found that the QG kits had less than that obtained for WB, BC and BL, an inevitable result of the samples from different kits having to be run on separate NGS flow cells to permit an adequate sampling depth (**Table 4.3**). Therefore, despite an initially smaller sample volume and a lower quantity of NGS read data, the superiority of the QG kit extraction process appears to have overcome such potential disadvantages and highlight the greatest number of infections and bacterial diversity of the four kits tested.

A range of similar studies have explored the impacts of DNA extraction kits, either manual or automated, for pathogen detection using various downstream molecular methods, including cPCR, real-time PCR, restriction digest analysis and deep sequencing (Hart et al., 2015; Phillips et al., 2012; Podnecky et al., 2013; Riemann et al., 2007; Schiebelhut et al., 2017; Wiesinger-Mayr et al., 2011; Yang et al., 2011). Overall, there are few consistent DNA extraction types that perform well in all contexts, with optimal extraction methods typically being very host, pathogen and sample-type specific (Podnecky et al., 2013; Schiebelhut et al., 2017). For example, large differences in kit performance may be observed depending on whether the source material contains lots of PCR inhibitors, such as in faeces and blood, or on the biological properties of the pathogen of interest, e.g., viruses that are easy to lyse vs resilient spore-forming bacteria (Phillips et al., 2012; Podnecky et al., 2013; Riemann et al., 2007; Velásquez-Mejía et al., 2018). Moreover, the ability of different extraction kits to remove PCR-inhibitory molecules may play a large impact on obtainable downstream results. Blood contains potent PCR inhibitors such as haemoglobin that affects DNA polymerase activity and hence observed differences in kit performance could be due to how well different extraction processes remove such molecules (Sidstedt et al., 2018).

In the current research, differences in extraction chemistry may be responsible for differences in bacterial pathogen detection between kits (Yang et al., 2011). For example, neither of the automated WB and BC kits used a proteinase K digestion step, whilst both manual QG and BL kits did use this digestion process. Proteinase-based degradation may release DNA sequestered by peptides or from within

bacterial cell walls, thus liberating more pathogen DNA, making it more readily available for PCR amplification (Riemann et al., 2007). Nonetheless, whilst both manual kits used proteinase K digestion only, the QG kit identified more infections than the automatic kits, meaning that this biochemical variable is likely just one of many responsible for differences in pathogen detection capability between extraction kits.

Depending on the extraction method utilised, the average DNA yield obtained also demonstrated marked differences. The automated BC and WB methodologies consistently extracted DNA yields in the range of hundreds of ng/ μ L with large variability between samples in the amount of DNA extracted (**Table 4.3**). In contrast, QG kits extracted an average of 13.9 ng/ μ L and BL kits extracted the lowest quantities at 3.4 ng/ μ L, with both spin-column based methods displaying much lower variability in nucleic acid yield. Such substantial differences in yield are due to the physical extraction methodology and chemistry employed by a given kit (Hall et al., 2019; Schiebelhut et al., 2017). QG and BL kits are dependent on retention of DNA in a silica column matrix which becomes saturated, capping the maximum amount of DNA obtainable (Hart et al., 2015; Schiebelhut et al., 2017). The Promega WB and BC automated methods use magnetic-bead based extraction whereby the large quantity of bead surface area available permits much larger amounts of DNA to be carried over between extraction stages (Wiesinger-Mayr et al., 2011; Yang et al., 2011). Nonetheless, as previously explored, the higher DNA yields gained by the WB and BC kits did not necessarily translate to a greater number of vector-borne bacterial infections found. One explanation for this may be that the larger quantity of DNA extracted by the WB and BC kits meant total host DNA was in great excess, making efficient blocking by our *Canis*-mito-blk primer ineffective, resulting in bacterial infections being missed.

With the advent of 16S rRNA bacterial metabarcoding, there has been a growing number of studies investigating the existence of endogenous bacterial contamination within DNA extraction kits and laboratory reagents (Eisenhofer et al., 2019; Glassing et al., 2016; Laurence et al., 2014). Such findings underscore the importance of conducting no sample/reagent only negative controls within metabarcoding studies to correctly identify such contaminant species and thereby discern true species present within samples from those introduced by extraction kits (Kim et al., 2017;

Tedersoo et al., 2019; Velásquez-Mejía et al., 2018; Zinger et al., 2019). In NGS studies that use a source material with a high density of bacteria, such as soil or faeces, this contamination typically has little effect as kit contaminant bacterial DNA is masked by that extracted from the source material (Eisenhofer et al., 2019; Glassing et al., 2016). However, when the source material has a low bacterial biomass, such as in blood, then kit contaminant bacterial DNA may be amplified and sequenced at similar levels to that of the source material, potentially competing and obscuring the microbiome signature of true bacterial inhabitants of the blood compartment (Eisenhofer et al., 2019; Glassing et al., 2016). An ever-growing list of common contaminant bacteria of kits highlights the tenacity of such organisms to survive and grow in a variety of environments, including ultra-pure water systems and other reagent manufacturing equipment and processes (Eisenhofer et al., 2019; Laurence et al., 2014). This creates a necessity to explore and identify differences in bacterial contamination between extraction kits which may influence the detection of true sample bacteria and demonstrate whether some kits are better suited to NGS applications.

In this study, bacterial taxa were considered an artificial kit contaminant if they had a read count of 100 or over in either of the two no-sample/reagent only negative controls, extracted alongside the blood samples for each kit type. Large differences in the average percentage of all contaminant bacterial reads compared to total reads were observable between kit types with the automated BC (86%) and WB (80%) kits having on average over two times as much contamination as the QG kits and just under four times as much contamination as the BL kits (**Figure 4.4**). Such findings are supported by the DNA yield data found within extraction negative controls, from which WB and BC kits had the highest levels of negative control DNA, especially compared to the BL controls which had levels below the detection limit of the Qubit™ 3.0 fluorometer. Kit-derived bacterial contaminant DNA was dominated by just a few key genera in the WB (51% *Massilia* spp. and 28% *Pseudomonas* spp.) and BC kits (61% *Massilia* spp. and 24% *Cellulomonas* spp.) whilst the QG and BL kits demonstrated a larger diversity of kit contaminants, none of which comprised more than 9% of total reads (**Table 4.5**).

Disparate levels of kit contamination may be due to the physical properties of the extraction protocol used by each kit type. The automated WB and BC extraction

methods utilise a series of seven wells all filled with between 700 to 1000 μL of lysis and wash buffers providing a large reagent pool from which contaminant bacteria could be introduced. In contrast, less than 600 μL of each reagent are added at any one time to the spin-column based QG and BL protocols with fewer wash steps from which endogenous bacteria could be added, lysed and their DNA extracted. The preponderance of a few key bacteria in the automated kit types may point to the growth or introduction of these species within the manufacturing process of these kits, whilst a larger diversity of less prevalent bacterial species in the QG and BL kits may be the product of small contamination events from the laboratory environment (Eisenhofer et al., 2019). The large percentage of contaminant reads from the WB and BC kits may explain, in some part, the reduced number of vector-borne bacterial infections elucidated when compared to those found with the QG kits. Endogenous bacterial DNA may be concealing the detection of pathogen DNA when using these kits, particularly in blood samples with low levels of circulating pathogens (Eisenhofer et al., 2019; Glassing et al., 2016).

An unexpected result identified by our assessment of DNA extraction kit performance was a significant difference in the amount of bacterial primer cross-reactivity on canine mitochondrial sequences between the kit types utilised. The BL kit obtained results that had a significantly higher amount of canine mitochondrial reads compared to the total, despite the use of the *Canis*-mito-blk primer, whilst the other three kit types had as low as 0.2% to 1.5% of total reads generated from cross-reactivity (**Figure 4.5**). An explanation may be the much lower DNA yields from the BL kits, that had, on average, just 3.4 ng/ μL of DNA, a large proportion of which would be canine DNA. Such low sample yields may not have provided sufficient bacterial DNA template for 16S rRNA amplification, leading to more off-target amplification and sequencing of host mitochondrial sequences. Moreover, such low starting DNA yields may also be the reason for the poorer performance of the BL kits with respect to the number of vector-borne bacterial infections found, compared to the other kit types (Hall et al., 2019; Vijayvargiya et al., 2019).

4.6. Conclusions

Taken together, the current study provides two complementary methodological amendments to improve the vector-borne bacterial detection capability of a previously developed metabarcoding protocol (Huggins et al., 2019b). Firstly, employment of a 3'-spacer C3 blocking primer (*Canis-mito-blk*) to prevent bacterial amplification primers from cross-reacting on host mtDNA was demonstrated to improve the number of infections detected and the relative quantity of pathogen DNA relative to total DNA. Secondly, use of Qiagen's DNeasy Blood and Tissue Kit appears superior to two automated methods utilising Promega's Maxwell® RSC 48 Instrument and Bioline's manual ISOLATE II Genomic DNA Kit. This superiority was demonstrated via the DNeasy Blood and Tissue Kit's ability to detect more infections as well as its lower quantities of kit contaminant bacterial DNA and generation of less off-target cross-reactivity on host mtDNA. Such methodological optimisation improves the power of our developed technique to unearth potentially latent vector-borne bacterial infections, a common phenomenon of many CVBDs where clinical signs may be followed by cyclical periods of remission when infections are hard to detect (Beck et al., 2019; Mylonakis et al., 2019). Furthermore, both amendments increased the diversity of different bacterial sequences elucidated, augmenting our protocol's possibility of identifying rare or novel bacterial pathogens. To conclude, together these are key improvements to our metabarcoding method allowing it to mine and more fully characterise the range of bacterial pathogens in regions of the world where there is a plethora of different CVBDs alongside a dearth of applicable research (Colella et al., 2020; Irwin and Jefferies, 2004; Traub et al., 2015).

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4.8. Appendix

Appendix 4.1. Sequence of our unique positive control gBlock DNA construct.

Positive control sequence is comprised of WehiNGS_AdP primer binding sites (underlined) and the V4 region of the *Aliivibrio fischeri* 16S rRNA gene.

5'-

GTGCCAGCAGCCGCGGTAATTACGGAGGGTGCGAGCGTTAATCGGAATTACTGGG
CGTAAAGCGCATGCAGGTGGTTCATTAAGTCAGATGTGAAAGCCCGGGGCTCAAC
CTCGGAACCGCATTGTGAAACTGGTGAAGTAGAGTGCTGTAGAGGGGGGTAGAATT
TCAGGTGTAGCGGTGAAATGCGTAGAGATCTGAAGGAATACCAGTGGCGAAGGCG
GCCCCCTGGACAGACACTGACACTCAGATGCGAAAGCGTGGGGAGCAAACAGGAA
TTAGATACCCTGGTAGTCC - 3'

Chapter 5 - A multipronged next-generation sequencing metabarcoding approach unearths hyper-diverse and abundant dog pathogen communities in Cambodia

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5.1. Abstract

Recent surveys in Southeast Asia, including Cambodia, have identified canine vector-borne pathogens (VBPs), including those with zoonotic potential, as highly prevalent. The lack of veterinary care alongside the close association semi-domesticated dogs have with humans in the region exacerbates these zoonotic risks. Nonetheless, the number of studies investigating such pathogens and the threats they pose to dog and human health is limited. Here, we utilise a next-generation sequencing (NGS) based metabarcoding protocol to conduct an assumption-free characterisation of the bacterial, apicomplexan and kinetoplastid blood-borne pathogens of free-roaming dogs from across Cambodia. From 467 dogs at five field sites, 62% were infected with one of eight confirmed pathogens, comprising *Anaplasma platys* (32%), *Ehrlichia canis* (20%), *Hepatozoon canis* (18%), *Babesia vogeli* (14%), *Mycoplasma haemocanis* (13%), the zoonotic pathogen *Bartonella clarridgeiae* (3%), *Candidatus Mycoplasma haematoparvum* (0.2%) and *Trypanosoma evansi* (0.2%). Coinfections of between two and four VBPs were common with 28% of dogs found to have a mixed infection. Moreover, DNA from putatively infectious agents belonging to the bacterial family and genera *Coxiella*, *Mycobacterium*, *Neisseria*, Rickettsiaceae, *Treponema* and two uncharacterised *Mycoplasma* species were identified, in addition to protozoan genera *Colpodella*, *Parabodo* and *Bodo*. Using a multiple logistic regression model, the presence of ectoparasites, abnormal mucous membranes, anaemia and total protein were found as predictors of canine VBP exposure. This study represents the first time an NGS metabarcoding technique has been used to holistically detect the bacterial and protozoan haemoparasites communities of dogs through an in-depth survey, highlighting the power of such methods to unearth a wide spectrum of pathogenic organisms in an unbiased manner.

5.2. Introduction

Vector-borne pathogens (VBP) or those predominantly transmitted by blood-feeding arthropods, inflict a significant toll with regard to morbidity and mortality on dogs, and nowhere is this more pronounced than in the tropics. In Southeast Asia, uncontrolled populations of stray and semi-domesticated itinerant dogs harbor a substantial diversity of ectoparasitic vectors, found infesting over a third of dogs sampled in countries such as Indonesia, Vietnam and the Philippines (Colella et al., 2020; Irwin and Jefferies, 2004). This high-level of ectoparasite infestation in stray dogs simultaneously acts as a VBP reservoir for well-cared-for pet dogs in the local area (Inpankaew et al., 2016; Koh et al., 2016; Liu et al., 2016). Moreover, the close association of dogs with humans via shared environments presents a significant zoonotic risk to people. For example, dogs can act as reservoirs of zoonotic vector-borne pathogens such as *Rickettsia felis*, *Rickettsia conorii* or *Bartonella* spp. that can be transmitted via arthropod bites to cohabiting humans, emphasising the importance of veterinary pathogen surveillance for safeguarding human health as well (Williams et al., 2011; Álvarez-Fernández et al., 2018; Ng-Nguyen et al., 2020).

Some of the most common vector-borne diseases of dogs in the tropics are caused by members of the bacterial Anaplasmataceae family, including *Anaplasma platys* and *Ehrlichia canis* (Little, 2010; Ng-Nguyen et al., 2020). *Ehrlichia canis* causes canine monocytic ehrlichiosis (CME) a frequently lethal multisystemic disease. In contrast, *A. platys* is less pathogenic, although clinical signs can be exacerbated when dogs are coinfecting with other VBPs (Hii et al., 2015; Huggins et al., 2019a; Inpankaew et al., 2016). Apicomplexan VBPs, such as *Babesia* spp. and *Hepatozoon canis* are also endemic to Southeast Asia (Huggins et al., 2019b; Inpankaew et al., 2016; Irwin and Jefferies, 2004; Liu et al., 2016) and generate disease that varies in pathogenicity from relatively mild in the case of hepatozoonosis, to severe, for example in the case of acute haemolytic anaemia caused by babesiosis, in particular when *Babesia gibsoni* is implicated (Ivanov and Tsachev, 2008; Kirk et al., 2017). With the exception of *B. gibsoni*, the aforementioned VBP are all vectored by the brown-dog tick, *Rhipicephalus sanguineus* that is ubiquitous in tropical Southeast Asia (Ng-Nguyen et al., 2020).

However, unlike many VBPs, *H. canis* is transmitted via ingestion of the infected brown dog tick, as opposed to blood-feeding (Baneth et al., 2003).

Additionally, kinetoplastid protozoans such as *Leishmania infantum* also pose a risk to dogs and humans (Alvar et al., 2012; Medkour et al., 2019). In Southeast Asia, *Trypanosoma evansi*, the cause of ‘surra’ in ruminants, also infects dogs through biting flies or ingestion of infected raw meat to produce severe clinical signs and death (Baramechaithanun et al., 2009; Bui et al., 2021; Rjeibi et al., 2015).

Detection of VBPs has historically relied on microscopic examination of blood smears, a technique now recognised as notoriously insensitive and incapable of characterising many VBP to a species level (Megat Abd Rani et al., 2010). Molecular methods e.g. conventional PCR (cPCR) or real-time PCR (qPCR) obviate these challenges by providing highly sensitive species level classification at a relatively modest price (Gasser, 2006; Goodwin et al., 2016). Nonetheless, such methods rely on *a priori* knowledge of the VBP in a given population (Huggins et al., 2020). From here, appropriate diagnostic methods must be selected to target expected VBPs, with little-to-no ability to detect novel, rare or unexpected pathogens in a region (Takhampunya et al., 2019).

Next-generation sequencing (NGS)-based methodologies permit surveillance with no *a priori* knowledge of VBPs as they can be designed to target whole communities of organisms via sequencing of conserved genes such as the small ribosomal RNA subunit (16S rRNA) gene in bacteria or 18S rRNA gene in eukaryotes (Flaherty et al., 2021; Huggins et al., 2020). Metabarcoding or the collective amplification and sequencing of all taxonomically informative genetic regions from a group of interest can be used as a powerful tool for characterising pathogen communities in both vector and mammalian hosts (Chaudhry et al., 2019; Flaherty et al., 2018; Greay et al., 2018; Huggins et al., 2019b). These methods are being increasingly utilised to detect known, as well novel bacterial and protozoan haemoparasites from domestic animals and a range of other mammalian species (Ghafar et al., 2021, 2020; Glidden et al., 2020; Huggins et al., 2019a, 2019b; Mohamed et al., 2021; Takhampunya et al., 2019).

Cambodia, like other countries in Asia, harbours a large population of free-roaming or poorly-cared for semi-domesticated dogs, at an estimated rate of one dog per every three

people, equating to approximately 5 million dogs (Sor et al., 2018). These dogs are often infected by multiple canine VBPs due to limited veterinary service availability (Huggins et al., 2020; Inpankaew et al., 2016). In light of such data, we used recently developed NGS-based metabarcoding assays (Huggins et al., 2020, 2019b, 2019a) to holistically detect i.e. detect DNA sequences of all relevant bacterial and protozoan haemoparasites, in an unbiased manner from dogs across Cambodia.

5.3. Methods

5.3.1. Study sites and sample collection

Dog blood samples were collected from five different study areas across Cambodia each containing between three and six study sites. Samples were collected in the months of April, May and September 2019, thereby covering Cambodia's drier, warmer northeast monsoon season and the wetter southwest monsoon season (Brook et al., 2008). Study areas included metropolitan Phnom Penh, the Cambodian capital (11°31'N, 104°55'E), Kampong Chhnang town (12°15'N, 104°39'E), Siem Reap city (13°26'N, 103°45'E), Battambang city (13°25'N, 103°73'E) and Tbong Khmum district (12°01'N, 105°30'E) as displayed in **Figure 5.1**. Study sites were selected based on trying to achieve a cross-section of densely populated and lightly populated urban areas, including rural towns as well as urban areas surrounded by both farmland and rainforest. Climate across all study sites is similar. Study site selection was also influenced by the existence of local rabies eradication programs, whereby opportunistic canine sampling could be conducted at the same time as administration of rabies vaccinations. Dogs sampled were from a mixture of locally owned animals and pagoda community dogs cared for by monks. Most sampled dogs from all field areas lacked access to adequate veterinary care, including anti-parasiticial treatments, desexing and vaccination. There were no exclusion criteria for dog sampling apart from whether the individual was too aggressive to capture and restrain.

A total of 467 dogs were sampled across the study's entirety; Phnom Penh (PP), $n = 135$, Kampong Chhnang (KC), $n = 48$, Siem Reap (SR), $n = 92$, Battambang (BB), $n = 92$ and Tbong Khmum (TK), $n = 100$. Dogs were humanely restrained, and blood was only taken

following informed consent from the relevant monk in the case of pagoda dogs or the owner for semi-domesticated community and pet dogs. Two ml whole blood was collected by a veterinarian via cephalic or jugular puncture into two anti-coagulation EDTA tubes and temporarily kept on ice. Blood samples subjected to NGS assays were stored at -20 °C until further use, whilst the second EDTA tube was refrigerated at 4 °C and processed within 48 hours for packed cell volume (PCV), total protein and subjected to the commercial SNAP 4Dx Plus Test (IDEXX, Westbrook, ME, USA) which detects antibodies to *Anaplasma platys/phagocytophilum*, *Ehrlichia canis/ewingii*, *Borrelia burgdorferi sensu lato* (all serotypes) and antigens of *Dirofilaria immitis*.

Relevant dog metadata including age, sex, breed, whether neutered, reproductive status and sampling location coordinates, were recorded and findings of clinical examinations performed at time of sample collection were noted. Clinical examinations were conducted by a qualified veterinarian with rectal temperature taken, body score, dog demeanour, haircoat and mucous membrane condition all visually evaluated. An abdominal palpation was also conducted alongside assessment of lymph node enlargement and respiration rate. The presence of ectoparasites, such as fleas, ticks and lice, was also recorded.

Field work in Cambodia was conducted under the University of Melbourne Animal Ethics Committee approved Permit: 1814620.1.

5.3.2. DNA extraction

Dog whole blood samples were couriered frozen to the University of Melbourne, Australia for processing. 200 µl of defrosted whole blood was extracted using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) using the manufacturer's protocol with a 30-minute proteinase K digestion at 56 °C and two final elution steps, the first in 30 µl and second in 20 µl (50 µl total eluent). Extracted DNA was kept at -20 °C until use.

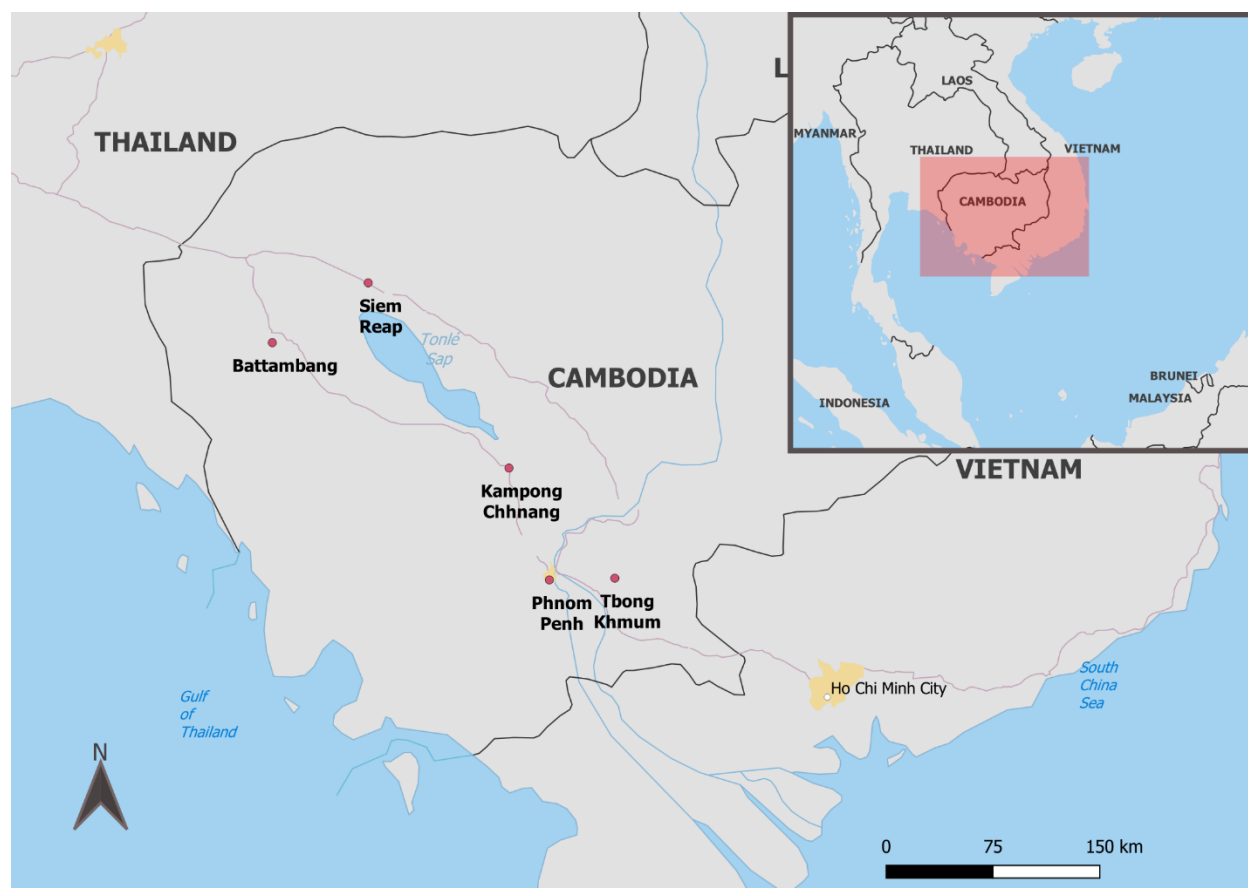


Figure 5.1. Map of field areas from which canine samples were collected. Phnom Penh (PP, $n = 148$), Kampong Chhnang (KC, $n = 48$), Siem Reap (SR, $n = 155$), Battambang (BB, $n = 165$) and Tbong Khmum (TK, $n = 156$). Map created in QGIS 3.4 via QGIS.org, 2021. QGIS Geographic Information System. QGIS Association.

5.3.3. Bacterial 16S rRNA gene metabarcoding

The 467 blood samples collected were analysed using our previously developed bacterial 16S rRNA gene targeting metabarcoding protocol (Huggins et al., 2020, 2019a). In brief, our methodology involved first-step amplification of 100 blood extracted DNA samples plus 12 controls, per batch. This required use of 1 μ L of DNA template, 10 μ L of OneTaq® 2X Master Mix with Standard Buffer (New England Biolabs, Ipswich, MA, USA), 0.4 μ L of primer WehINGS_AdP_F and WehINGS_AdP_R at 10 μ M, alongside 1.2 μ L of the canine mitochondrial DNA blocking primer; Canis-mito-blk (Huggins et al., 2020) with the reaction made up to 20 μ L with Ambion Nuclease-Free Water (Life Technologies, Carlsbad, CA, USA). All sample batches were also run with four positive controls, four no DNA template

PCR controls and four blood-omitted sample extraction controls i.e. DNA extracted from extraction reagents only at the same time as blood DNA extractions. Positive controls were uniquely identifiable synthetic DNA constructs as defined in Huggins et al. 2020, diluted to 1×10^{-7} ng/ μ l synthesised by Integrated DNA Technologies (Coralville, IA, USA), see **Appendix 5.1** for sequences. Thermocycling conditions were 95 °C for 3 min followed by 20 cycles of 95 °C for 45 s, 56 °C for 60 s and 72 °C for 90 s with a final elongation at 72 °C for 10 min conducted on a MiniAmp™ Thermal Cycler (Thermo Fisher Scientific, Waltham, MA, USA). PCR product was then cleaned using 1X Ampure Beads (Beckman Coulter, Brea, CA, USA) and checked for quality on an Agilent 2200 Tape Station (Agilent Technologies, Santa Clara, CA, USA) before proceeding. Finally, the second-step PCR addition of library indexes, followed by deep sequencing was conducted according to Huggins et al. (2019a) using an Illumina MiSeq (Illumina, San Diego, CA, USA) with 600-cycle v3 chemistry at the Walter & Eliza Hall Institute Proteomics Facility, Parkville, Australia.

5.3.4. Apicomplexan and kinetoplastid 18S rRNA gene metabarcoding

The same subsample used for the bacterial 16S rRNA gene metabarcoding was also analysed using our previously developed 18S rRNA gene protocol designed to target parasitic apicomplexan and kinetoplastid organisms (Huggins et al., 2019b). In brief, batches of 104 samples were amplified using 2 μ l of DNA template and the previously reported ApicomplexF and ApicomplexR primers modified to include indexing overhangs (Huggins et al., 2019b) alongside two no DNA template PCR controls, two DNA extraction controls and two synthetic DNA construct positive controls (**Appendix 5.1**). For kinetoplastid detection the same samples were also separately amplified using the Kinetof and Kinetor primers, with indexing overhangs plus four negative and two positive controls (**Appendix 5.1**). For apicomplexan PCR amplification thermocycling conditions were 94 °C for 5 min, followed by 25 cycles of 94 °C for 30 s, 52 °C annealing for 30 s and 72 °C for 30 s with a final elongation at 72 °C for 5 min, this was the same for kinetoplastid PCR except for that annealing was conducted at 56 °C instead. PCR product was then processed in the same manner as that for bacterial 16S rRNA gene

metabarcoding apart from that the second step indexing PCR used just 20 cycles of amplification to add on multiplexing indexes as opposed to the 24 cycles originally reported in Huggins et al. 2019b. After addition of indexes, batches of 104 apicomplexan targeting PCR products were pooled with 104 kinetoplastid targeting PCR products, producing multiplexed pools of 208 samples achievable via 16 unique forward indexes and 13 unique reverse indexes. Finally, pools were run on an Illumina MiSeq using 300-cycle v2 chemistry (2 x 150 bp paired-end reads).

5.3.5. Bioinformatics

Raw NGS data was processed according to Huggins et al. 2020 with all bioinformatic steps conducted in QIIME2 version 2020.11. DADA2 truncation parameters were chosen via inspection of read quality plots and subsequent exploration of parameters to find those that generated the most post-filter sequences for each batch of samples (Callahan et al., 2016). Amplicon sequence variants (ASVs) were again chosen over operational taxonomic units (OTUs) so as to minimise data loss at each processing step (Knight et al., 2018; Langille et al., 2018) and sequences were classified using sci-kit learn built classifiers working from the latest available SILVA reference database (version 138.1) (Pedregosa et al., 2011). Additionally, ASVs were taxonomically identified using the BLASTn program in GenBank (NCBI) to corroborate classifications and in some cases classify down to a lower taxonomic level than possible using QIIME2 classifiers. Alpha rarefaction plots were also generated, using MAFFT and FastTree 2 to confirm that ASV diversity plateaued and hence a sufficient sequencing depth had been achieved (Kato and Standley, 2013; Price et al., 2010). All NGS data produced in the present study are available from the NCBI BioProject database, BioProjectID: PRJNA601241, BioSampleIDs: SAMN13870739 to SAMN13870796 and SAMN18323289 to SAMN18323754. Raw NGS read data for bacteria, apicomplexans and kinetoplastids is available from the NCBI sequence read archive (SRA) under the aforementioned BioSample accession numbers.

Sequences of taxa found in no-reagent DNA extraction negative controls and no-template PCR negative controls were removed from the overall dataset using the QIIME2

taxonomy-based filtering command which can be used to exclude negative control taxa from further downstream analysis. These taxa were excluded as they likely represent organism's DNA introduced via DNA extraction and PCR amplification materials as well as from the environment. High read counts of these taxa in non-control samples may have been indicative of their endogenous presence in blood samples, however none of such taxa were from pathogenic or putatively pathogenic genera and hence further analysis was not deemed necessary.

Positive control samples consisted of uniquely identifiable synthetic DNA for a short 130 - 253 bp stretch of the 16S rRNA gene from the bacterial species *Aliivibrio fischeri* flanked with the complementary sequences to the relevant bacterial, apicomplexan or kinetoplastid primer sequence, see **Appendix 5.1** (Huggins et al., 2020). *A. fischeri* is highly host specific to the Bobtail squid *Euprymna scolopes* and its environment (Nyholm and McFall-Ngai, 2004; Ruby et al., 2005), with no possibility that such sequences arose endogenously from dog blood samples, thus providing a unique positive control sequence that could be identified at the end of bioinformatic processing. Reads of these unique positive control sequences were used to calculate each batch of samples' unique read threshold. The read threshold signifies how many pathogen reads need to be identified in a sample for it to be considered a true infection so as to accurately discern true presence from occasional index misreading or hybridisation errors during Illumina sequencing as well as low-level cross-contamination during library preparation (Kim et al., 2017). The read threshold used was the highest number of uniquely identifiable positive control reads i.e. reads for *A. fischeri*, found in a sample that was not a positive control for that batch.

5.3.6. Conventional PCR and Sanger sequencing

For some samples with reads that could not be assigned to species level classification, endpoint PCR assays were run to amplify larger gene regions or more informative genes for better characterisation (**Table 5.1**). These cPCRs were conducted using HotStarTaq DNA Polymerase (Qiagen) according to the relevant author's methods (**Table 5.1**). Positive amplicons were then cleaned using ExoSAP-IT™ PCR Product Cleanup Reagent (Thermo Fisher Scientific) and sent to Macrogen (Seoul, South Korea) for

Sanger sequencing. Results were then run through BLASTn, to compare them to existing GenBank reference sequences and corroborate with NGS findings, i.e. to ascertain whether cPCR Sanger sequences returned the same taxonomic classification in GenBank as NGS reads classified using the SILVA database.

Table 5.1. Corroboratory qPCR and cPCR assays used, only those that were successful at cross-validating NGS results are shown.

Taxon targeted	PCR type (primers & probe)	Gene targeted	Product size (bp)	Reference
<i>Bartonella</i> spp. specific	qPCR (ssrA-F, ssrA-R & ssrA-probe)	Transfer-mRNA (ssrA)	301	(Diaz et al., 2012)
	cPCR (prAPT0257 & prAPT0258)	Filamenting Temperature-Sensitive Mutant Z (ftsZ)	500	(Belkhiria et al., 2017)
	cPCR (BhCS.781p & BhCS.1137n)	Citrate synthase (gltA)	380 - 400	(Norman et al., 1995)
<i>Mycoplasma</i> spp. specific	cPCR (HBT-F & HBT-R)	16S rRNA	600	(Criado-Fornelio et al., 2003)
	cPCR (RNaseP30s & RNaseP200as)	Ribonuclease P (RNase P)	165	(Maggi et al., 2013)

5.3.7. Statistical & risk indicator analyses

The 95% confidence intervals (CIs) were calculated using the Wilson score interval via the open source software Epitools (<https://epitools.ausvet.com.au>). Chi-square (χ^2) statistical methods calculated using IBM SPSS Statistics 27 (SPSS, Chicago, IL, USA) were used to compare measured categorical variables against NGS positivity for VBPs. A multiple logistic regression model was then used to assess dog risk factors to VBP exposure in GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA). VBP exposure was here defined as dogs found positive to a pathogen via NGS or a SNAP 4Dx Plus Test (IDEXX). Variables that were tested were sampling seasons (wet or dry), dog age, sex, husbandry, temperature, presence of ectoparasites, haircoat condition, body

score, dog demeanour, PCV and total protein (as a continuous variable). Additional variables tested were for the presence of enlarged lymph nodes, abdominal palpation condition e.g. hepatosplenomegaly, respiration rate for example panting as well as mucous membrane condition i.e. normal or abnormal with abnormal considered as signs of pallor, jaundice or presence of petechiae. Variables with a significance of < 0.2 found via univariate analysis were included in the regression model. PCV was converted to the binary variable anaemic i.e. PCV $< 35\%$ or non-anaemic PCV $> 35\%$ for this model.

A significance level of $P < 0.2$ was then used again for iterative backward elimination of variables that were not significant until the final multivariate model was obtained. Associations were considered statistically significant if $P < 0.05$ or very close to it. The best-fit model was selected using Akaike's corrected Information Criterion value, multicollinearity was checked using variance inflation factors to check for strongly dependent predictors. Area under the ROC curve was used to measure the ability of the model in classifying negative and positive events.

Kappa statistics were used to compare agreement of NGS positivity for *A. platys* and *E. canis* vs results from SNAP 4Dx Plus (IDEXX) in SPSS Statistics 27. We used the formula defined in McHugh (2012) with the agreement between tests considered poor if $k \leq 0.20$, fair if $0.21 \leq k \leq 0.40$, moderate if $0.41 \leq k \leq 0.60$, substantial if $0.61 \leq k \leq 0.80$ and high if $k > 0.81$ (McHugh, 2012).

5.4. Results

5.4.1. Sample collection

Our study sample of 467 dogs assessed for vector-borne pathogens consisted of 213 (45.6%) females, 246 (52.7%) males and 8 (1.7%) with unreported data (**Table 5.2**). Dog ages ranged from one month to seven years and older with 142 (30.4%) dogs in the youngest age category of six months and younger, 96 (20.6%) dogs between six months and a year, 200 (42.8%) dogs between one and seven years old, 18 (3.9%) dogs over seven years of age and 11 (2.3%) with unreported data. Only 12 (2.6%) dogs were pure bred, including an Alsatian, a Golden Retriever, an English Bulldog, a Labrador, Huskies

and German Shepherds. Just two dogs (0.4%) were found to be neutered. It was more common for dogs to have at least one ectoparasite present, with 344 (73.7%; 95% CI = 69% - 77%) of dogs found infested with ticks, fleas and/or lice (**Table 5.2**).

Table 5.2. Distribution, sex, age, breed and presence of ectoparasites across the 467 canine samples assessed for vector-borne pathogens by our NGS-based methodology (PP = Phnom Penh, KC = Kampong Chhnang, SR = Siem Reap, BB = Battambang, TK = Tbong Khmum). At some study sites category totals do not equal the relevant sample area total due to unreported data.

Study Area	Sex		Age (years)				Breed		Ectoparasites	
	F	M	< 0.5	> 0.5 to < 1	> 1 to < 7	> 7	Mongrel	Pure	Present	Absent
PP (n= 135)	57	77	66	22	39	5	131	4	96	39
KC (n= 48)	25	23	12	5	31	0	46	2	37	11
SR (n= 92)	33	56	23	16	49	3	91	1	59	33
BB (n= 92)	45	44	21	15	49	6	87	5	65	27
TK (n= 100)	53	46	20	38	32	4	100	0	87	13
Total (n=467)	213	246	142	96	200	18	455	12	344	123

5.4.2. Bioinformatic processing

A total of five separate 600-cycle MiSeq runs were used to deep-sequence all 467 samples for bacterial pathogens as well as 60 negative and positive control samples, with the highest raw reads obtained for any run at 14,880,659 and the lowest at 9,636,742 (mean $\pm$ SE = 11,446,984 $\pm$ 907,014) reads. After bioinformatic filtering and quality control steps, the mean total filtered reads per batch was 9,106,146 $\pm$ 818,499 (**Table 5.3**). This translated to an average of 9,191 $\pm$ 1,610 ASVs or unique reads per batch and an average median read per sample of 74,640 $\pm$ 9,454.

For the protozoan pathogen characterisation five separate 300-cycle MiSeq runs were used to deep-sequence 672 apicomplexan amplicons, 672 kinetoplastid amplicons, alongside 30 positive and negative control samples. For the apicomplexan NGS-assessment the highest raw reads for any run was 9,467,041 and the lowest 6,044,754 (mean $\pm$ SE = 7,398,546 $\pm$ 671,281). Comparatively, the kinetoplastid characterisation accrued fewer raw reads per batch with the highest at 4,090,659 and lowest 988,199 (mean $\pm$ SE = 2,142,296 $\pm$ 563,110). Post-bioinformatic processing, the average filtered reads per apicomplexan batch was 4,039,592 $\pm$ 546,136 and for kinetoplastids was 1,231,726 $\pm$ 368,998 (**Table 5.3**). This generated a mean of 870 $\pm$ 99 unique ASVs per apicomplexan batch and 400 $\pm$ 110 per kinetoplastid batch as well as an average median read per sample of 11,003 $\pm$ 2,741 for the apicomplexan analysis and 3,592 $\pm$ 1,881 for the kinetoplastids.

After bioinformatic processing and taxonomic classification all uniquely identifiable positive controls were detected in every batch. The highest number of control reads found in a sample other than a positive control was identified and used as a read threshold to discern true positives for that specific batch from index cross-talk and/or low-level cross-contamination. Reads were always detected in no blood DNA extraction controls and no DNA template PCR negative controls, however, total reads in such negative controls were always substantially lower than for biological samples, as previously found by Huggins et al. (2020). ASVs and taxa found in negative control samples were subtracted from the relevant batch's overall dataset.

Table 5.3. NGS read data across MiSeq batches pre and post-filtering. Bacterial batches show results from 600-cycle MiSeq kits, whilst apicomplexan and kinetoplastid samples were pooled and run on 300-cycle MiSeq runs, hence the lower raw and filtered read values. The median number of reads per sample in each batch and number of amplicon sequence variants (ASVs), i.e. unique reads, per batch are also shown, alongside relevant means and standard errors (S.E.).

<u>Bacteria</u>					
Batch	Study areas	Raw reads	Filtered reads	Median sample reads	ASVs
1	PP, KC	9,636,742	7,737,311	65,920	15,139
2	SR	14,880,659	12,247,954	110,944	10,059
3	PP, SR	10,339,939	7,896,377	67,681	6,547
4	BB	11,306,097	8,698,046	56,193	7,117
5	TK	11,071,481	8,951,040	72,461	7,091
Mean		11,446,984	9,106,146	74,640	9,191
S.E.		907,014	818,499	9,454	1,610
<u>Apicomplexans</u>					
Batch	Study areas	Raw reads	Filtered reads	Median sample reads	ASVs
6	PP, KC	6,192,572	1,903,119	1,903	700
7	SR	9,467,041	4,419,860	19,099	1,118
8	PP, SR, BB	8,458,399	4,396,449	10,557	775
9	BB, TK	6,829,962	5,012,882	12,281	659
10	TK	6,044,754	4,465,650	11,176	1,098
Mean		7,398,546	4,039,592	11,003	870
S.E.		671,281	546,136	2,741	99
<u>Kinetoplastids</u>					
Batch	Study areas	Raw reads	Filtered reads	Median sample reads	ASVs
6	PP, KC	2,667,973	1,641,348	6,780	784
7	SR	4,090,659	2,475,039	9,362	465
8	PP, SR, BB	1,664,452	932,855	1,206	362
9	BB, TK	1,300,197	512,854	316	152
10	TK	988,199	596,534	298	236
Mean		2,142,296	1,231,726	3,592	400
S.E.		563,110	368,998	1,881	110

5.4.3. NGS-based detection of confirmed canine vector-borne pathogens

Key species and genera of known canine pathogens were identified as *Anaplasma platys*, *Bartonella* spp., *Babesia vogeli*, *Ehrlichia canis*, *Hepatozoon canis*, *Candidatus Mycoplasma haematoparvum*, *Mycoplasma haemocanis* and *Trypanosoma evansi*, which were then extracted from the overall datasets for each batch (**Table 5.4**). Across all field sites 62% (95% CI = 57% - 66%) of dogs were found to be infected with at least one of these eight pathogens with the highest apparent prevalence of infection found in Battambang at 75% (95% CI = 65% - 83%) and lowest in Tbong Khmum at 47% (95% CI = 37% - 56%).

From the eight known pathogens detected by our methodology *A. platys* was the most prevalent across all field sites with 32% (95% CI = 28% - 36%) of dogs infected, followed by *E. canis* at 20% (95% CI = 17% - 24%), *H. canis* at 18% (95% CI = 15% - 22%), *B. vogeli* at 14% (95% CI = 11% - 18%), *M. haemocanis* at 13% (95% CI = 10% - 16%), *Bartonella* spp. at 3% (95% CI = 1% - 4%) and single infections found for *C. M. haematoparvum* and *T. evansi*; both at 0.2% (95% CI = 0% - 1%) (**Table 5.4**).

Mixed infections, with between two to four pathogens detected simultaneously, were common with 28% (95% CI = 24% - 32%) of dogs found to be coinfecting across all field sites (**Table 5.4**). The most common coinfection was *A. platys* and *E. canis* in 4% (95% CI = 3% - 6%), followed by *A. platys* and *H. canis* in 3% (95% CI = 2% - 5%) of dogs. The most common coinfection comprising three pathogens was *A. platys*, *B. vogeli* and *H. canis* in 2% (95% CI = 1% - 4%) of dogs. The most common coinfection with four pathogens was *A. platys*, *B. vogeli*, *E. canis* and *H. canis* in 1.2% (95% CI = 1 - 3) of dogs. **Figure 5.2** shows the relative proportion of infection-free, single infection and coinfecting dogs at the five field sites. Battambang had the most coinfecting dogs at 42% (95% CI = 32% - 52%), followed by Phnom Penh at 29% (95% CI = 22% - 37%), whilst Tbong Khmum had the least; 15% (95% CI = 9% - 23%).

Table 5.4. Number of positive dogs (+) and apparent pathogen prevalence (%) with 95% confidence intervals (CI) found for both single and mixed infections, as detected by our bacterial, apicomplexan and kinetoplastid targeting NGS-method at five different study areas in Cambodia (PP = Phnom Penh, KC = Kampong Chhnang, SR = Siem Reap, BB = Battambang, TK = Tbong Khmum). Table shows results from only known canine-pathogenic genera and species.

	PP (n= 135)		KC (n= 48)		SR (n= 92)		BB (n= 92)		TK (n= 100)		All Sites (n= 467)	
	+	% (CI)	+	% (CI)	+	% (CI)	+	% (CI)	+	% (CI)	+	% (CI)
Single infection												
<i>Anaplasma platys</i> (Ap)	21	16 (10 - 23)	5	10 (5 - 22)	11	12 (7 - 20)	9	10 (5 - 18)	20	20 (13 - 29)	66	14 (11 - 18)
<i>Bartonella</i> spp. (Ba)	0	0 (0 - 3)	0	0 (0 - 7)	5	5 (2 - 12)	0	0 (0 - 4)	3	3 (1 - 8)	8	2 (1 - 3)
<i>Babesia vogeli</i> (Bv)	7	5 (3 - 10)	1	2 (0 - 11)	1	1 (0 - 6)	0	0 (0 - 4)	0	0 (0 - 3)	9	2 (1 - 4)
<i>Ehrlichia canis</i> (Ec)	11	8 (5 - 14)	6	13 (6 - 25)	6	7 (3 - 14)	7	8 (4 - 15)	7	7 (3 - 14)	37	8 (6 - 11)
<i>Hepatozoon canis</i> (Hc)	4	3 (1 - 7)	0	0 (0 - 7)	5	5 (2 - 12)	8	9 (4 - 16)	0	0 (0 - 3)	17	4 (2 - 6)
<i>C. M. Haemaphysalis</i> (Cmh)	0	0 (0 - 3)	0	0 (0 - 7)	0	0 (0 - 4)	0	0 (0 - 4)	0	0 (0 - 3)	0	0 (0 - 1)
<i>Mycoplasma haemocanis</i> (Mh)	3	2 (1 - 6)	4	8 (3 - 20)	8	9 (4 - 16)	6	7 (3 - 14)	2	2 (1 - 7)	23	5 (3 - 7)
<i>Trypanosoma evansi</i> (Te)	0	0 (0 - 3)	0	0 (0 - 7)	1	1 (0 - 6)	0	0 (0 - 4)	0	0 (0 - 3)	1	0.2 (0 - 1)
	PP (n= 135)		KC (n= 48)		SR (n= 92)		BB (n= 92)		TK (n= 100)		All Sites (n= 467)	
	+	% (CI)	+	% (CI)	+	% (CI)	+	% (CI)	+	% (CI)	+	% (CI)
Mixed infections												
Ap & Bv	7	5 (3 - 10)	2	4 (1 - 14)	1	1 (0 - 6)	0	0 (0 - 4)	1	1 (0 - 5)	11	2 (1 - 4)
Ap & Ec	7	5 (3 - 10)	2	4 (1 - 14)	4	4 (1 - 11)	4	4 (1 - 11)	2	2 (1 - 7)	19	4 (3 - 6)
Ap & Hc	2	1 (0 - 5)	1	2 (0 - 11)	5	5 (2 - 12)	6	7 (3 - 14)	0	0 (0 - 3)	14	3 (2 - 5)
Ba & Ap	0	0 (0 - 3)	0	0 (0 - 7)	0	0 (0 - 4)	0	0 (0 - 4)	1	1 (0 - 5)	1	0.2 (0 - 1)
Bv & Hc	2	1 (0 - 5)	3	6 (2 - 17)	1	1 (0 - 6)	5	5 (2 - 12)	0	0 (0 - 3)	11	2 (1 - 4)
Ec & Bv	1	1 (0 - 4)	1	2 (0 - 11)	1	1 (0 - 6)	0	0 (0 - 4)	2	2 (1 - 7)	5	1 (0 - 2)
Ec & Hc	2	1 (0 - 5)	0	0 (0 - 7)	0	0 (0 - 4)	1	1 (0 - 6)	0	0 (0 - 3)	3	1 (0 - 2)
Mh & Ap	2	1 (0 - 5)	0	0 (0 - 7)	3	3 (1 - 9)	1	1 (0 - 6)	2	2 (1 - 7)	8	2 (1 - 3)
Mh & Ba	0	0 (0 - 3)	0	0 (0 - 7)	3	3 (1 - 9)	0	0 (0 - 4)	0	0 (0 - 3)	3	1 (0 - 2)
Mh & Cmh	0	0 (0 - 3)	0	0 (0 - 7)	0	0 (0 - 4)	0	0 (0 - 4)	1	1 (0 - 5)	1	0.2 (0 - 1)
Mh & Ec	1	1 (0 - 4)	0	0 (0 - 7)	0	0 (0 - 4)	1	1 (0 - 6)	1	1 (0 - 5)	3	1 (0 - 2)
Mh & Hc	1	1 (0 - 4)	0	0 (0 - 7)	2	2 (1 - 8)	3	3 (1 - 9)	2	2 (1 - 7)	8	2 (1 - 3)

Ap & Bv & Hc	6	4 (2 - 9)	2	4 (1 - 14)	0	0 (0 - 4)	3	3 (1 - 9)	0	0 (0 - 3)	11	2 (1 - 4)
Ap & Ec & Bv	2	1 (0 - 5)	0	0 (0 - 7)	0	0 (0 - 4)	0	0 (0 - 4)	2	2 (1 - 7)	4	1 (0 - 2)
Ap & Ec & Hc	0	0 (0 - 3)	0	0 (0 - 7)	1	1 (0 - 6)	2	2 (1 - 8)	0	0 (0 - 3)	3	1 (0 - 2)
Ec & Bv & Hc	3	2 (1 - 6)	0	0 (0 - 7)	0	0 (0 - 4)	1	1 (0 - 6)	0	0 (0 - 3)	4	1 (0 - 2)
Mh & Ap & Ec	0	0 (0 - 3)	1	2 (0 - 11)	0	0 (0 - 4)	3	3 (1 - 9)	1	1 (0 - 5)	5	1 (0 - 2)
Mh & Ap & Hc	0	0 (0 - 3)	0	0 (0 - 7)	0	0 (0 - 4)	2	2 (1 - 8)	0	0 (0 - 3)	2	0.4 (0 - 2)
Mh & Bv & Hc	0	0 (0 - 3)	0	0 (0 - 7)	1	1 (0 - 6)	2	2 (1 - 8)	0	0 (0 - 3)	3	1 (0 - 2)
Mh & Ec & Bv	1	1 (0 - 4)	0	0 (0 - 7)	1	1 (0 - 6)	0	0 (0 - 4)	0	0 (0 - 3)	2	0.4 (0 - 2)
Mh & Ec & Hc	0	0 (0 - 3)	0	0 (0 - 7)	1	1 (0 - 6)	0	0 (0 - 4)	0	0 (0 - 3)	1	0.2 (0 - 1)
Ap & Ec & Bv & Hc	1	1 (0 - 4)	0	0 (0 - 7)	0	0 (0 - 4)	5	5 (2 - 12)	0	0 (0 - 3)	6	1.2 (1 - 3)
Mh & Ec & Bv & Hc	1	1 (0 - 4)	0	0 (0 - 7)	0	0 (0 - 4)	0	0 (0 - 4)	0	0 (0 - 3)	1	0.2 (0 - 1)
Total mixed infections	39	29 (22 - 37)	12	25 (15 - 39)	24	26 (18 - 36)	39	42 (32 - 52)	15	15 (9 - 23)	129	28 (24 - 32)
	PP (n= 135)		KC (n= 48)		SR (n= 92)		BB (n= 92)		TK (n= 100)		All Sites (n= 467)	
	+	% (CI)	+	% (CI)	+	% (CI)	+	% (CI)	+	% (CI)	+	% (CI)
Single & mixed infections												
Ap	48	36 (28 - 44)	13	21 (12 - 34)	25	27 (19 - 37)	35	38 (29 - 48)	29	29 (21 - 39)	150	32 (28 - 36)
Ba	0	0 (0 - 3)	0	0 (0 - 7)	8	9 (4 - 16)	0	0 (0 - 4)	4	4 (2 - 10)	12	3 (1 - 4)
Bv	31	23 (17 - 31)	9	19 (10 - 32)	6	7 (3 - 14)	16	17 (11 - 26)	5	5 (2 - 11)	67	14 (11 - 18)
Ec	30	22 (16 - 30)	10	27 (17 - 41)	14	15 (9 - 24)	24	26 (18 - 36)	15	15 (9 - 23)	93	20 (17 - 24)
Hc	22	16 (11 - 23)	6	13 (6 - 25)	16	17 (11 - 26)	38	41 (32 - 52)	2	2 (1 - 7)	84	18 (15 - 22)
Cmh	0	0 (0 - 3)	0	0 (0 - 7)	0	0 (0 - 4)	0	0 (0 - 4)	1	1 (0 - 5)	1	0.2 (0 - 1)
Mh	9	7 (4 - 12)	5	10 (5 - 22)	19	21 (14 - 30)	18	20 (13 - 29)	9	9 (5 - 16)	60	13 (10 - 16)
Te	0	0 (0 - 3)	0	0 (0 - 7)	1	1 (0 - 6)	0	0 (0 - 4)	0	0 (0 - 3)	1	0.2 (0 - 1)
Total positive	85	63 (55 - 70)	28	58 (44 - 71)	61	66 (56 - 75)	69	75 (65 - 83)	47	47 (37 - 56)	290	62 (57 - 66)
Total negative	50	37 (29 - 45)	20	42 (29 - 56)	31	34 (25 - 44)	23	25 (17 - 35)	53	53 (43 - 62)	177	38 (34 - 42)

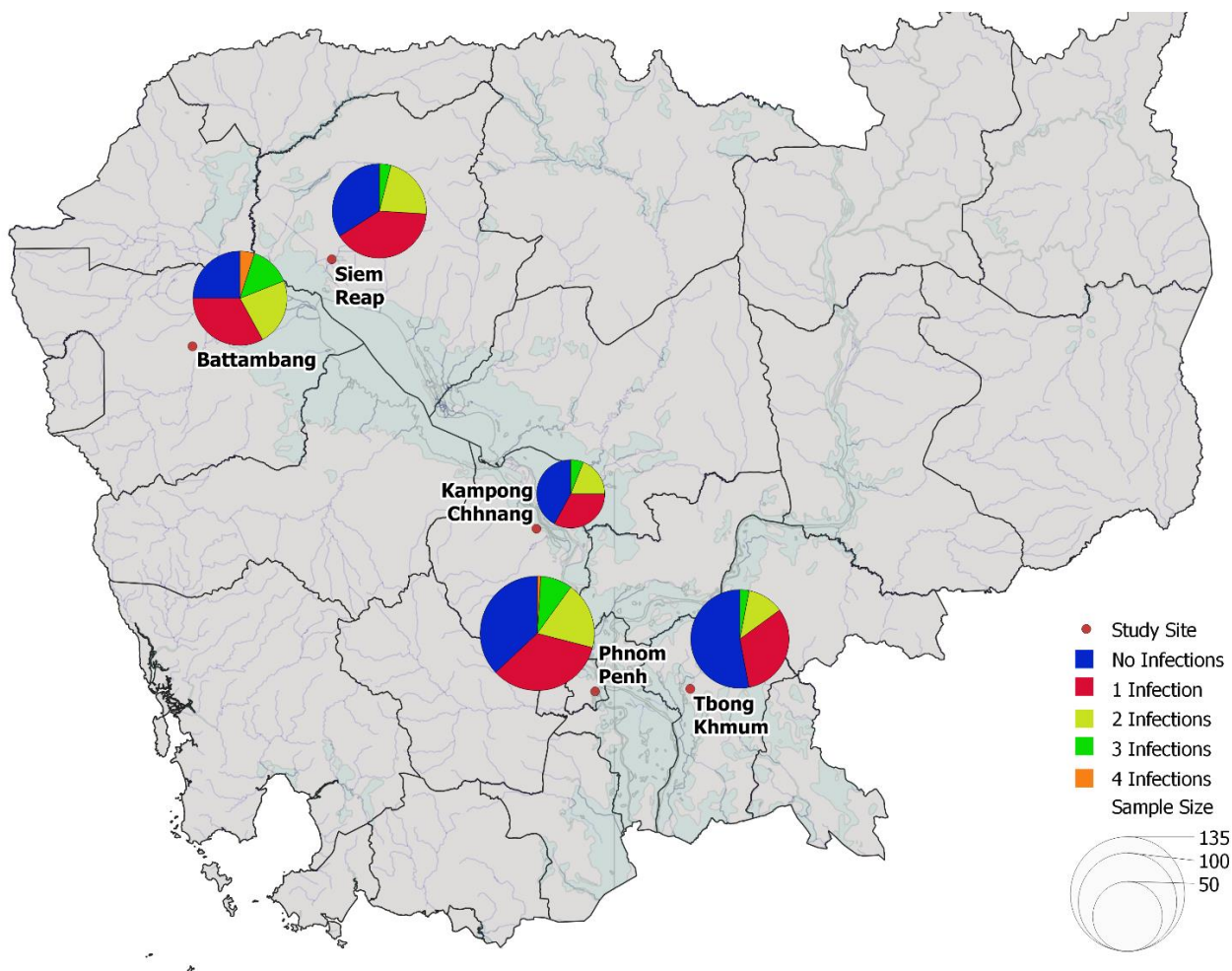


Figure 5.2. Map with the relative proportions of uninfected, singularly infected and coinfecting dogs at five different Cambodian field sites. Pie chart size is proportional to sample size for that study site. Dark blue lines depict rivers whilst light blue areas demarcate lakes and regions prone to inundation. Map created in QGIS 3.4 via QGIS.org, 2021. QGIS Geographic Information System. QGIS Association.

5.4.4. Serology

Of 467 dog blood samples subjected to NGS metabarcoding, 457 samples were also subjected to the SNAP 4Dx Plus Test (IDEXX). Across all field sites 42% (95% CI = 37% - 46%) of dogs were found exposed to *Anaplasma* spp. and 32% (95% CI = 27% - 36%) to *Ehrlichia* spp., whilst 23% (95% CI = 20% - 27%) had been exposed to both (**Table 5.5**). One dog from Tbong Khmum was serologically positive to *B. burgdorferi* sensu lato (and *Ehrlichia* spp.), whilst no dogs were found positive for *D. immitis*.

Table 5.5. Number of serologically positive dogs (+) and pathogen prevalence (%) with 95% confidence intervals (CI) found for mixed and overall infections, as detected by SNAP 4Dx Plus Test at five different study areas in Cambodia (PP = Phnom Penh, KC = Kampong Chhnang, SR = Siem Reap, BB = Battambang, TK = Tbong Khmum). No dogs were *D. immitis* antigen positive and 10 tests had a failed internal control and were therefore excluded from the overall results.

	PP = 134		KC = 46		SR = 86		BB = 92		TK = 99		All Sites = 457	
	+	% (CI)	+	% (CI)	+	% (CI)	+	% (CI)	+	% (CI)	+	% (CI)
Single & mixed infection												
<i>Anaplasma</i> spp.	61	46 (37 - 54)	24	52 (38 - 66)	34	40 (30 - 50)	38	41 (32 - 52)	34	34 (26 - 44)	191	42 (37 - 46)
<i>Ehrlichia</i> spp.	45	34 (26 - 42)	23	50 (36 - 64)	27	31 (23 - 42)	34	37 (28 - 47)	15	15 (9 - 24)	144	32 (27 - 36)
<i>Borrelia burgdorferi</i>	0	0 (0 - 3)	0	0 (0 - 8)	0	0 (0 - 4)	0	0 (0 - 4)	1	1 (0 - 6)	1	0.2 (0 - 1)
Mixed infection												
<i>Anaplasma</i> + <i>Ehrlichia</i>	34	25 (19 - 33)	16	35 (23 - 49)	21	42 (17 - 34)	24	26 (18 - 36)	11	11 (6 - 19)	106	23 (20 - 27)
<i>Ehrlichia</i> + <i>B. burgdorferi</i>	0	0 (0 - 3)	0	0 (0 - 8)	0	0 (0 - 4)	0	0 (0 - 4)	1	1 (0 - 6)	1	0.2 (0 - 1)
Total positive	72	54 (45 - 62)	31	67 (53 - 79)	40	47 (36 - 57)	48	52 (42 - 62)	38	38 (29 - 48)	229	50 (46 - 55)
Total negative	62	46 (38 - 55)	15	33 (21 - 47)	46	53 (43 - 64)	44	48 (38 - 58)	61	62 (52 - 71)	228	50 (45 - 54)

5.4.5. Statistical and risk indicator analyses

Univariate analysis identified study site as having a significant effect on NGS positivity for a confirmed vector-borne pathogen ($P < 0.05$). Specifically, dogs in Battambang were more likely to be found exposed to VBPs than those in Phnom Penh, Kampong Chhnang and Siem Reap, whilst dogs in Tbong Khmum were less likely to be found exposed than those at the other field sites. Other variables that were found to exert a significant effect on VBPs positivity and were included in our multiple logistic regression model were presence of ectoparasites, haircoat condition, body score, dog demeanour, mucous membrane condition i.e. normal or abnormal, PCV (anaemic or non-anaemic) and total protein.

Our multiple logistic regression model identified presence of ectoparasites, abnormal mucous membrane condition, increase in total protein, and PCV (as an indicator of anaemia) as significant predictors of VBP exposure in dogs (**Table 5.6**). Via our model, we estimate that the odds ratio of VBP exposure for dogs with ectoparasites to be between 1.33 and 3.59, whilst holding PCV, total protein and mucous membrane variables constant. The odds ratio for VBP exposure in dogs with abnormal mucous membrane condition ranged between 1.29 and 4.92, when the other variables were kept constant. We also estimate that as total protein increases by one unit the odds of VBP infection increases by 1.35 to 1.92, holding other variables constant. Moreover, the odds ratio of VBP exposure for dogs with a PCV indicative of anaemia (below 35%) was between 1.01 and 3.085, if total protein, ectoparasite presence and mucous membrane condition variables were kept constant.

Kappa statistics, used to compare agreement between two diagnostic tests, assessed the level of concordance between detection of antibodies using the SNAP 4Dx Plus Test and detection of pathogen DNA using our NGS method (**Table 5.7**). Agreement for detection of *E. canis* was 0.517 (SE 0.044, $P < 0.05$) indicating moderate agreement, with 81.2% total agreement, whilst for *A. platys* the kappa value was 0.162 (SE 0.046, $P < 0.05$) indicating poor agreement, with 60.6% total agreement (**Table 5.7**).

Table 5.6. Clinical parameter estimates and odds ratios (95% profile likelihood) for exposure to vector-borne pathogens in 467 dogs from Cambodia.

Parameter estimates	Estimate	Standard error	Odds ratios	p value
Intercept	-3.33	0.726		<0.0001
PCV [anaemic]	0.554	0.2852	1.74 (1.01-3.085)	0.0522
Total protein	0.469	0.089	1.60 (1.35-1.92)	<0.0001
Mucous membranes [abnormal]	0.889	0.34	2.43 (1.29-4.92)	0.0089
Presence of ectoparasites	0.779	0.2529	2.18 (1.33-3.59)	0.002

Table 5.7. NGS vs SNAP 4Dx Plus test agreement statistics for the canine VBPs *E. canis* and *A. platys*. POS = positive, NEG = negative, SE = standard error. Kappa agreement level defined as poor if coefficient (k) is ≤ 0.20 , fair agreement if $0.21 \leq k \leq 0.40$, moderate agreement if $0.41 \leq k \leq 0.60$, substantial agreement if $0.61 \leq k \leq 0.80$ and high agreement if $k > 0.81$.

VBP	NGS	SNAP 4Dx Plus		Total agreement (%)	Kappa statistic* (agreement)	Kappa SE
		POS	NEG			
<i>E. canis</i>	POS	296	17	81.2	0.517 (moderate)	0.044
	NEG	69	75			
<i>A. platys</i>	POS	199	67	60.6	0.162 (poor)	0.046
	NEG	113	78			

5.4.6. Confirmatory Sanger sequencing and suspected pathogen detection

All *Bartonella* NGS positive samples were successfully characterised via either an *ssrA* targeting qPCR (Diaz et al., 2012), *ftsZ* targeting cPCR (Belkhiria et al., 2017) or a *gltA* targeting cPCR (Norman et al., 1995). All NGS positive *Bartonella* samples were identified as the zoonotic species *Bartonella clarridgeiae* with the Sanger sequenced product of the *ssrA* qPCR achieving a 100% query cover and identity match with *B. clarridgeiae* isolate BFP-41219-9 (accession #: MK298159.1) using the GenBank BLASTn tool. The *ftsZ* targeting assay achieved a 99% query cover and 100% identity match with *B. clarridgeiae* strain 73 (accession #: FN645454.1), whilst the *gltA* cPCR returned a 99% query cover and 99% identity match with *B. clarridgeiae* citrate synthase (accession #: MK977726.1).

Using a confirmatory cPCR assay (Criado-Fornelio et al., 2003) all of the four haemotropic *Mycoplasma* positive samples found via NGS that could not be identified to species level (1% of sampled population; 95% CI = 0% - 2%) had a 575bp long amplicon match with 100% query cover and identity to an uncharacterised *Mycoplasma* species (accession

#: HE577612.1), previously identified in dogs from Indigenous communities in Australia (Barker et al., 2012).

A subsample of *M. haemocanis* positive dogs, as determined by our NGS method, were cross-validated by an RNaseP gene targeting cPCR (Maggi et al., 2013) as *M. haemocanis* shares high 16S rRNA gene nucleotide identity with *M. haemofelis*. These assays confirmed sequences as belonging to *M. haemocanis* (accession #: KU743386.1) with 100% query cover and 99% identity.

In addition to the detection of confirmed canine pathogens by our metabarcoding method, the bacterial 16S rRNA gene deep-sequencing identified several samples with reads from bacterial genera that contain canine pathogens or species of zoonotic importance. Nonetheless, from the NGS data alone, reads could only be classified to genus-level due to the conserved nature of the 16S rRNA gene region targeted for some bacterial taxa. Bacterial genera of potential pathogenic significance found across all dogs sampled included *Leptospira* spp. in 3% (95% CI = 2% - 4%) of dogs with the NGS reads most similar to existing GenBank entries returning a 100% query cover and 98.4% identity with *Leptospira interrogans* (accession #: DQ522190.1).

One dog was also positive for *Wolbachia* spp. the reads of which were found to have 100% identity match and query cover to *Wolbachia* endosymbiont of *Dirofilaria repens* (accession #: KY114937.1) and 99.2% to an endosymbiont of *Dirofilaria immitis* (accession #: AF487892.1) in GenBank. A second uncharacterised *Mycoplasma* species was also identified in 1% of sampled dogs (95% CI = 0% - 2%) found most similar to a species previously identified from tortoises (accession #: HQ326170.1) at 89% identity.

Other genera of pathogenic relevance were *Mycobacterium* spp. found in 7% (95% CI = 5% - 9%) of dogs, reads from members of the Rickettsiaceae family that includes the VBP-relevant genus *Rickettsia* in 3% (95% CI = 2% - 5%) of canines, *Coxiella* spp. in 8% (95% CI = 6% - 10%), *Neisseria* spp. in 2% (95% CI = 1% - 3%), *Rhodococcus* spp. in 5% (95% CI = 3% - 8%), and *Treponema* spp. in 3% (95% CI = 1% - 4%). Moreover, a substantial diversity of reads classified as *Prevotella* spp. were also found in dogs from all field sites, including one dog with a high number of reads identified as *Prevotella colorans*, a species previously characterised from a human wound (Buhl et al., 2016).

Bordetella spp. reads were also identified from one dog, BLASTn revealed this sequence to share 99.2% identity with *Bordetella bronchiseptica* and *Bordetella petrii*, the former of which is a canine pathogen and one of the agents responsible for canine infectious respiratory disease (CIRD) (Schulz et al., 2014).

The apicomplexan and kinetoplastid 18S rRNA gene metabarcoding also returned reads classified from putative pathogens, which were then identified against the NCBI database using BLASTn, see **Table 5.8**.

Table 5.8. BLASTn results from putative pathogens detected by the apicomplexan and kinetoplastid-targeting NGS metabarcoding protocol, including prevalence with confidence intervals (CI) of these sequences found across all study sites. Query cover and identity ranges are included as multiple ASVs were identified belonging to the same genus in some cases.

<u>Apicomplexan-targeting NGS</u>				
Highest similarity NCBI BLASTn results	Accession #	Query Cover	Identity	% of dogs (95% CI)
<i>Colpodella</i> spp.	MW261750.1	100	95	0.2 (0 - 1)
<i>Goussia balatonica</i> & <i>Goussia chalupskyi</i>	GU479659.1, GU479653.1	100	100	0.2 (0 - 1)
<u>Kinetoplastid-targeting NGS</u>				
Highest similarity NCBI BLASTn results	Accession #	Query Cover	Identity	% of dogs (95% CI)
<i>Parabodo</i> spp.	DQ207591.1, AY425019.1	100	99	7 (5 - 10)
<i>Bodo</i> spp.	AY425015.1, AY753601.1, AY490230.1, AY998648.1	98 - 100	92 - 99	4 (3 - 6)
<i>Neobodo</i> spp.	AY998653.1, MT355132.1	100	83 - 100	6 (5 - 9)
<i>Herpetomonas</i> spp. & <i>Blechnomonas pulexsimulantis</i>	AJ843897.1, KF054129.1	99 - 100	88	0.2 (0 - 1)

5.5. Discussion

With the aid of a multipronged NGS metabarcoding approach, we report highly diverse and hyperendemic dog pathogen communities in Cambodia. We found that almost three quarters of dogs were infested with either ticks, fleas or lice and 62% of dogs were found to have a confirmed VBP. The most common pathogen detected was *A. platys*, followed by *E. canis*, *H. canis* and *B. vogeli*, all of which are known to be vectored by the tick *R. sanguineus*, a species that is highly prevalent across Southeast Asia (Kernif et al., 2012; Colella et al., 2020; Nguyen et al., 2019). Our elucidation of the pathogens *A. platys*, *T. evansi* and *B. clarridgeiae* represents the first molecular-based identification of these pathogens in Cambodian dogs, whilst many other suspected pathogens were also detected in dog blood.

Apparent prevalence of the most common VBPs identified here is consistent with previous studies in the region. A cPCR-based study conducted in Cambodia's central-northeastern province of Preah Vihear found canine VBPs in 71.3% of dogs, specifically *B. vogeli* in 32.7% of dogs vs 14% in our data, *E. canis* 21.8% vs 20%, *H. canis* 10.9 % vs 18% and *M. haemocanis* 9.9% vs 13% reported here. The only discrepancy between studies was the lack of *A. platys* found by Inpankaew et al. (2016), nonetheless, we found apparent *A. platys* prevalence to be lowest at our Siem Reap and Kampong Chhnang study sites i.e. those which are geographically closest to Preah Vihear. Our data also mirrors results found in nearby countries, for example *Hepatozoon* spp. (18.8%), *Babesia* spp. (9.4%) and *Mycoplasma* spp. (19.9%) in stray dogs in Thailand (Liu et al., 2016) as well as *E. canis* (25.8%) and *A. platys* (13.3%) serological exposure demonstrated in Vietnam (Colella et al., 2020).

Vector-borne pathogen coinfection was common with 28% of dogs across all field sites found to have between two and four confirmed VBPs. Coinfection rates peaked in Battambang, followed by Phnom Penh and were lowest in Tbong Khmum (**Table 5.4 & Figure 5.2**). The most common coinfection identified was *A. platys* and *E. canis* in 4% of dogs – this is unsurprising given that the two pathogens share a common vector and have been consistently found as one of the most prevalent coinfections identified in Asian dogs before (Colella et al., 2020; Díaz-Regañón et al., 2020; Huggins et al., 2019a; Rucksaken

et al., 2019). Mixed infections comprising three and four pathogens were also detected which is important from both an epidemiological and clinical standpoint, as simultaneous vector-borne pathogen infection is associated with more severe pathology, such as exacerbated immune-mediated hemolytic anemia or lower PCV (Rawangchue and Sungpradit, 2020; Rojas et al., 2014; Suksawat et al., 2001)

The detection of *B. clarridgeiae* at two of our study sites is significant given that this species along with several closely related *Bartonella* are zoonotic. Like *Bartonella henselae*, this flea-borne agent utilises domestic cats as primary sources of infection and is a cause of cat scratch disease (CSD) in humans (Breitschwerdt, 2014; Silva et al., 2019). Typically, both immunosuppressed humans and dogs suffer similar clinical signs including lymphadenitis, endocarditis, peliosis hepatis, granulomatous hepatitis and other pathology (Breitschwerdt et al., 2010; Chomel and Kasten, 2010; Silva et al., 2019). *B. clarridgeiae* has previously been detected in the blood of dogs from Thailand (Huggins et al., 2019a) as well as from fleas in Laos (Calvani et al., 2020) and the Thai-Myanmar border (Parola et al., 2003).

Identification of a single dog infected with the kinetoplastid pathogen *T. evansi* is significant given that canine trypanosomiasis is often lethal, generating severe pathology including oedema, fever, anaemia, weight loss and ocular damage, such as keratitis and corneal opacity (Bui et al., 2021; Defontis et al., 2012; Panigrahi et al., 2015; Rjeibi et al., 2015). *T. evansi* has a wide mammalian host range and has been reported in dogs in nearby Thailand and Vietnam previously (Bui et al., 2021; Huggins et al., 2019b). *T. evansi* is more frequently found infecting water buffalo, *Bubalus bubalis*, a species that is common around Siem Reap where our infected dog was found. Infection in companion animals is typically associated with spillover from such reservoir hosts, via tabanid fly vectors and the species is zoonotic with infrequent reports of human infection e.g. in Vietnam and India (Aregawi et al., 2019; Van Vinh Chau et al., 2016). However, given the low apparent prevalence and high mortality rates of *T. evansi* in dogs it is unlikely they play a role in the transmission of this pathogen to livestock or humans.

DNA of *Leptospira* spp. was also identified in 3% of dog blood samples via NGS. BLASTn cross-validation of NGS taxonomic classification found one ASV to be similar to *L.*

interrogans (accession #: DQ522190.1), sharing 98.4% identity, whilst other ASVs ranged down to 95% identity with the same species. Endpoint PCR for different *Leptospira* genes (*gyrB*, *secY*) could not successfully amplify DNA from these samples. This may be due to leptospiral load being very low in these individuals and possibly below the limit of detection of the cPCRs used, potentially highlighted by a lower sensitivity of some cPCR assays when compared to NGS methods (Huggins et al., 2019b, 2019a). Therefore, whether the ASVs found were from an uncharacterised strain of *L. interrogans* or whether they represent a novel species could not be concluded. Nonetheless, *Leptospira* spp. are important zoonotic pathogens with approximately 500,000 globally reported cases annually, with high lethality depending on species, strain and case severity (Esson et al., 2019; Song et al., 2021). Exposure to *Leptospira* has been demonstrated in humans with unexplained sepsis in Cambodia and in people with undifferentiated febrile illness in Northern Thailand (Rozo et al., 2020; Takhampunya et al., 2019). Moreover, the latter study demonstrated the presence of *Leptospira* DNA in small mammal, vector and human populations, highlighting the significance of vector species to provide a conduit for animal-to-human transmission in Southeast Asia (Takhampunya et al., 2019).

Across all sample batches analysed by 16S rRNA gene deep-sequencing the variety of bacterial sequences unearthed was exceedingly high, with per batch diversity reaching peaks of 15,139 unique ASVs over 100 samples. ASVs belonging to genera with known pathogenic species were further explored via corroboratory BLASTn classification and cross-validators cPCR, as the metabarcoding protocol in many cases could only confidently taxonomically classify down to the level of genus. One of the most noteworthy findings was the identification from four dogs (1%) of a haemotropic *Mycoplasma* spp. species 100% identical to one previously only found in dogs from remote aboriginal communities in central and western Australia (accession #: HE577612.1) (Barker et al., 2012). The identification of this species in the same host in Cambodia significantly increases the geographical range of this organism, although little inference can be made as to its pathogenic potential given the small number of cases identified. A second novel *Mycoplasma* species was also identified that had low similarity to any existing GenBank entries with just 89% identity to HQ326170.1.

Further significant bacterial taxonomic groups identified were Rickettsiaceae (3% of samples) which includes the genus *Rickettsia* of which many species are vector-borne pathogens, including the zoonosis *R. felis* which uses dogs as a natural asymptomatic reservoir (Ng-Nguyen et al., 2020). *Mycobacterium* (7%) and *Coxiella* (8%) DNA was also detected across all study sites, a relevant finding given that serology suggests dogs can harbour the human pathogens *Mycobacterium tuberculosis* and *Coxiella burnetii* (Ma et al., 2020; Parsons et al., 2012; Shapiro et al., 2016). Previous work has molecularly identified *Coxiella*-like endosymbiont DNA of ticks in both the vector and mammalian host, providing an alternative explanation for the *Coxiella* DNA found in this study (Khoo et al., 2016). *Neisseria* species found in 2% of our samples can be part of the normal canine oral microbiome, however dog bite wounds have been implicated in fatal cases of *Neisseria* infection in dogs and people (Cantas et al., 2011; Cobiella et al., 2019). *Rhodococcus* species, such as *Rhodococcus equi* can generate serious pathology in dogs, especially those that are immunocompromised, whilst *Treponema* spp. have been implicated in canine periodontal disease, the former pathogen being found in 5% and the latter in 3% of dogs in this study (Bryan et al., 2017; Nises et al., 2018; Takai et al., 2003). Nonetheless, with all classifications that could only be made to family or genus level, pathogenicity could not be confirmed given that all these taxonomic groupings also contain non-pathogenic environmental species. In many cases high 16S rRNA gene homology across the genus prevented species level discrimination, an issue highlighted extensively before with regard to *Rickettsia* classification (Greay et al., 2018; Portillo et al., 2017; Santib   ez et al., 2013).

Rarer ASVs found in a limited number of dog samples included the human-wound isolated *Prevotella colorans* (Buhl et al., 2016) and an ASV sharing 99.2% identity to *Bordetella bronchiseptica* which can cause canine infectious respiratory disease (CIRD). The detection of *Wolbachia* DNA sharing 99.2% to 100% identity from *Wolbachia* found in different species of the parasitic filarial worm genus *Dirofilaria* is also significant as *D. immitis*, the aetiological agent of heartworm, is endemic to northeast Cambodia and Southeast Asia more generally, whilst *D. repens* has not been found in the region before (Colella et al., 2020; Inpankaew et al., 2016). Heartworm in particular, is a commonly

lethal canine infection and in this case, the potential detection of its bacterial endosymbiont's DNA could be indicative of an active infection, as has been previously demonstrated (Huggins et al., 2019a; Laidoudi et al., 2020; Taylor et al., 2005).

Our 18S rRNA gene deep-sequencing protocols also elucidated a plethora of sequences from organisms detected in dog blood (**Table 5.8**). The apicomplexan method detected an ASV in one dog from *Colpodella* spp. a genus which has typically been associated with free-living species, but that has been increasingly found in the bloodstream and haemolymph of various mammals and arthropod vectors including humans, cattle, *Ixodes* and *Rhipicephalus* ticks (Jiang et al., 2018; Matsimbe et al., 2017; Sam-Yellowe et al., 2020; Squarre et al., 2020; Yuan et al., 2012). In cases of previously identified human infections, pathology was generated including fever, neurological symptoms and haemolytic anaemia (Jiang et al., 2018; Yuan et al., 2012). Here, the detection of *Colpodella* spp. DNA from the blood of a dog represents the first report of its kind, with our sequence sharing 95% identity with a sequence previously detected in horse blood (accession #: MW261750.1).

The detection of *Goussia* spp. in one dog is also very unexpected given that this is a genus typically associated with parasites of fish and amphibians (Gestal and Azevedo, 2005; Jirků et al., 2009; Molnár et al., 2012). Our ASV had a 100% query cover and identity match with both *Goussia balatonica* (accession #: GU479659.1) and *Goussia chalupskyi* (accession #: GU479653.1) - two species associated with fish parasitism (Molnár, 1989; Xavier et al., 2020). Therefore, whether detection of their DNA in dog samples demonstrates biologically viable *Goussia* cells or another explanation, such as environmental contamination or fish consumption leading to trace quantities of circulating DNA is impossible to conclude (Hornung et al., 2019; Tamburini et al., 2018).

The kinetoplastid targeting protocol, apart from the single *T. evansi* infection, discovered a large number of samples across all field sites with diverse ASVs classified to genera in the order Bodonida, including *Parabodo* (7%), *Bodo* (4%) and *Neobodo* (6%) (Moreira et al., 2004). These genera are typically recognised as being free-living organisms, however, a growing body of literature has identified them in a range of different animals, occasionally associated with pathology. For example, ASVs in the present study were

found to be 99% identical to *Parabodo caudatus* (accession #: DQ207591.1) a species that has been found, via cystocentesis, in the urine of a dog with haematuria (Vandersea et al., 2015) as well as in the blood of a Thai dog (Huggins et al., 2019b). In addition, DNA from the genus *Bodo* has been previously found in the blood of an Algerian dog (Medkour et al., 2020), Brazilian bats (Dario et al., 2017) and the Australian marsupial *Bettongia penicillate* (woylie) (Northover et al., 2019). In the present study negative controls were free from Bodonida DNA and the number of reads from Bodonida organisms was in some cases much higher than would be expected if skin/needle-stick contamination was the cause. Additionally, further indications on the potential vector-borne transmission of Bodonida organisms comes from Szöke et al. (2017) which found larval ticks that had fed exclusively on cohabiting bats to be PCR positive for *Bodo* organisms (Szöke et al., 2017). Nonetheless, identification of DNA presence alone is not sufficient to ascertain the role of an arthropod in the transmission of a given pathogen. Another sequence had a low similarity to any entries in GenBank, with the highest being an 88% identity match with *Herpetomonas* spp. (accession #: AJ843897.1) and *Blechnomonas pulexsimulantis* (accession #: KF054129.1), both genera are associated with parasites of arthropod vectors, such as fleas and midges, as well as free-living forms (Podlipaev et al., 2004; Votýpka et al., 2013).

Our study's statistical modelling identified the presence of ectoparasites, abnormal mucous membrane condition, elevated total protein, and anaemia (low PVC) to be significant predictors for canine VBP exposure. Of these, the odds ratio for VBP exposure in dogs was highest in those that had an abnormal mucous membrane condition (odds ratio 2.43, CI 1.29-4.92), with pallor the most common abnormality identified. This is not unexpected given that many canine VBPs generate anaemia (Harrus and Waner, 2011; Otranto et al., 2009). This indicator was further corroborated by the higher odds ratios in VBP exposed dogs that were found with ectoparasites (odds ratio 2.18, CI 1.33-3.59) or classified as anaemic (odds ratio 1.74, CI 1.01-3.085), based on PCV results (Hayes et al., 2011; Riond et al., 2014). In addition, we identified that a total protein increase of one unit was associated with a higher odds ratio for VBP exposure (odds ratio 1.60, CI 1.35-1.92). Hyperproteinemia is a common consequence of a chronic immune response to

VBP as total protein increasing with host immunoglobulin production in response to infection (Hayes et al., 2011; Shaw et al., 2001).

Comparison of serological testing with NGS analysis identified moderate agreement ($K = 0.517$) for *Ehrlichia* spp. and poor agreement ($K = 0.162$) for *Anaplasma* spp. with serology identifying a greater number of positives for both pathogens than our metabarcoding method (**Table 5.7**). This is not surprising given that serology measures pathogen exposure via the production of antibodies that can remain in the blood for months to years, whilst circulating pathogen DNA can be reduced below the limit of detection for many molecular diagnostics (Kidd, 2019; Little, 2010; Mahmoud et al., 2015; Wong et al., 2011). Cyclical production of antibodies against Rickettsiales pathogens has been identified in dogs before, specifically for the species *R. felis* (Ng-Nguyen et al., 2020). Ng-Nguyen et al. (2020) identified an inverse and fluctuating relationship between levels of IgG antibody against *R. felis* and PCR positivity in experimentally infected dogs. Peaks of IgG production coincided with buffy coat PCR negativity, whilst reductions in IgG titers correlated with PCR positivity. Such mechanisms could provide some explanation as to why NGS methods in the current study failed to detect rickettsial pathogens that were identified as positive using SNAP 4Dx tests.

In contrast, some dogs were found to be NGS positive and SNAP 4Dx negative for both *Anaplasma* spp. (67 samples) and *Ehrlichia* spp. (17 samples), see **Table 5.7**. Such results could be due to dog sampling having been conducted too soon after infection by the relevant VBP and thus production of host antibodies may not have yet commenced, generating SNAP 4Dx negative results, whilst circulating pathogen DNA was detectable via our NGS method (Wong et al., 2011).

The discrepancy between methods highlights the need for multiple diagnostic methods to be used in an integrated manner so as to more accurately characterise the apparent prevalence and exposure to different VBPs (Kidd, 2019). The identification of one sample serologically positive to *B. burgdorferi* sensu lato was unexpected, particularly given that no *Borrelia* DNA was detected by deep-sequencing. However, seropositive dogs to this important zoonotic bacterium have been identified in Indonesia and the Philippines before (Colella et al., 2020). The lack of *D. immitis* antigen positive dogs was also surprising,

particularly as 15.8% of dogs were previously found positive to this filarial pathogen via cPCR in northern Cambodia (Inpankaew et al., 2016).

5.6. Conclusions

To the best of the authors' knowledge this study represents a first of its kind, in-depth, NGS-based survey for all bacterial, apicomplexan and kinetoplastid canine VBPs. There is a significant dearth of information on the presence and epidemiology of such diseases in dogs in Cambodia and Southeast Asia more generally, especially when compared to equivalent research focused on Europe and North America. The extreme diversity of simultaneously detected bacterial and protozoan organisms found in dog blood, a bodily compartment at one point thought to be sterile (Païssé et al., 2016), demonstrates the power of NGS methodologies to characterise poorly researched microbiomes and uncover novel pathogens. Future work may take this further by also using deep-sequencing to test for rare or emerging VBPs in the ectoparasites found infesting the dogs herein sampled, allowing us to better disentangle and understand pathogen transmission cycles. Overall, the data accrued through our techniques provides us with a key baseline understanding of both canine VBP and potential zoonotic threats in Cambodia, provisioning us with critical information from which future surveillance and intervention studies can be built upon.

5.7. References

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5.8. Appendix

Appendix 5.1. Sequences of our unique positive control gBlock DNA constructs.

1) Bacterial 16S rRNA metabarcoding analysis. Positive control sequence is comprised of WehiNGS_Adp primer binding sites (underlined) and the V4 region of the *Aliivibrio fischeri* 16S rRNA gene. All gBlock constructs were synthesised by Integrated DNA Technologies (Coralville, IA, USA).

5'-GTGCCAGCAGCCGCGGTAATACGGAGGGTGCGAGCGTTAATCGGAATTACTGG
GCGTAAAGCGCATGCAGGTGGTTCATTAAGTCAGATGTGAAAGCCCGGGGCTCAA
CCTCGGAACCGCATTGAACTGGTGAAGTAGAGTGCTGTAGAGGGGGGTAGAAT
TTCAGGTGTAGCGGTGAAATGCGTAGAGATCTGAAGGAATACCAGTGGCGAAGGC
GGCCCCCTGGACAGACACTGACACTCAGATGCGAAAGCGTGGGGAGCAAACAGG
ATTAGATACCCTGGTAGTCC - 3'

2) Apicomplexan 18S rRNA metabarcoding analysis. Positive control sequence is comprised of ApicomplexF and ApicomplexR primer binding sites (underlined) and a section of the V4 region of the *Aliivibrio fischeri* 16S rRNA gene.

5'-CAAGGAAGTTTAAGGCAATAACAGTACGGAGGGTGCGAGCGTTAATCGGAATTA
CTGGGCGTAAAGCGCATGCAGGTGGTTCATTAAGTCAGATGTGAAAGCCCGGGGC
TCAACCTCGGAACCGCATTGAACTGGTGAAGTAGAGTGCTGTAAATCGTGAACG
AGGAATGCCTAG - 3'

3) Kinetoplastid 18S rRNA metabarcoding analysis. Positive control sequence is comprised of Kinetof and Kinetor primer binding sites (underlined) and a section of the V4 region of the *Aliivibrio fischeri* 16S rRNA gene.

5'-
CAAACGATGACACCCATGAAGGAACCGCATTGAACTGGTGAAGTAGAGTGCTG
TAGAGGGGGGTAGAATTCAGGTGTAGCGGTGAAATGCGTAGAGATCTGAAGGAA
TACCAGTGGCGAAGGCGGCCCCCTGGACAGACACTGACACTCAGATGCGAAAGC
GTGGGGAGCAAACAGGGAGGTTACAGTCTCAGGGGG - 3'

Chapter 6 - Novel high-throughput multiplex qPCRs for the detection of canine vector-borne pathogens in the Asia-Pacific

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6.1. Abstract

The Asia-Pacific hosts a large diversity of canine vector-borne pathogens (VBPs) with some of the most common and most pathogenic, generating significant mortality as well as a spectrum of health impacts on local dog populations. The VBPs *Anaplasma platys*, *Babesia gibsoni*, *Babesia vogeli*, *Ehrlichia canis*, *Hepatozoon canis* and haemotropic *Mycoplasma* spp. are all endemic throughout the region, with many exhibiting shifting geographical distributions that warrant urgent attention. Moreover, many of these species cause similar clinical signs when parasitising canine hosts, whilst knowledge of the exact pathogen is critical to ensure treatment is effective. This is complicated by frequent coinfection that can exacerbate pathology. Here, we describe the development, optimisation and validation of two novel quadruplex Taq-Man based real-time PCRs (qPCRs) for the specific and sensitive detection of the aforementioned VBPs. To ensure accurate evaluation of diagnostic performance, results of our qPCRs were evaluated on field samples from Thai dogs and compared with both conventional PCR (cPCR) results and next-generation sequencing (NGS) metabarcoding. Our qPCRs were found to be more sensitive at detecting canine VBP than cPCR and generated results similar to those achieved by NGS. These qPCRs will provide a valuable high-throughput diagnostic tool available to epidemiologists, researchers and clinicians for the diagnosis of key canine VBPs in the Asia-Pacific and further afield.

6.2. Introduction

A plethora of pathogenic agents ranging in size from minute arboviruses to bacteria, protozoa, and multicellular parasites, such as filarial nematodes, can all be transmitted by blood-feeding arthropods and are collectively known as vector-borne pathogens (VBPs) (Beugnet and Marié, 2009; Day, 2011). VBPs due to their vectors' ubiquitous nature are found globally and affect a vast range of animals including humans. This has been highlighted through outbreaks of diseases like Zika (Gardy and Loman, 2018), Dengue Fever (Wang et al., 2016), leishmaniasis (Miró et al., 2017), Chagas disease (Saldaña et al., 2015) and tick-borne diseases, for example Lyme disease (Petersen et al., 2019). Dogs are a host species that are known to acquire an expansive variety of VBPs, producing a spectrum of disease from acute and potentially fatal infections, to chronic and recrudescent illness that may be carried by the canine host for the duration of its life (Kidd, 2019). Pathogenesis typically centres around non-specific clinical signs, including fever, anaemia, thrombocytopenia, anorexia and lethargy that make precise diagnosis of the aetiological agent essential for administration of effective treatment (Kaur et al., 2020; Kidd, 2019; Little, 2010).

In the tropics canine VBP prevalence can be especially high, due to climatic conditions that encourage the fast growth and reproduction of the one-host brown dog tick, *Rhipicephalus sanguineus* sensu lato, an important vector of numerous pathogens (Irwin and Jefferies, 2004). One key phylum from which many canine VBPs belong to is the Apicomplexa, which includes the species *Babesia vogeli*, *Hepatozoon canis* and less commonly in the tropics, *Babesia gibsoni* (Colella et al., 2020; Huggins et al., 2019a; Irwin and Jefferies, 2004). *Babesia* parasites infect erythrocytes after arthropod blood-feeding, whilst *H. canis* is transmitted by host ingestion of an infected tick (Irwin, 2009; Ivanov and Tsachev, 2008; Solano-Gallego and Baneth, 2011). Apicomplexan VBPs produce a range of disease severity in parasitised dogs, from the potentially fatal *B. gibsoni* to the less virulent *B. vogeli* that is more pathogenic in younger canines. In contrast, hepatozoonosis is often asymptomatic, although in a small percentage of individuals unable to control the infection, high levels of parasitaemia can develop, accompanied by cachexia, anaemia

and even death (Baneth et al., 2003; Muhlnickel et al., 2002; Solano-Gallego and Baneth, 2011).

Bacterial vector-borne disease, such as those produced by the rickettsial organisms *Anaplasma platys* and *Ehrlichia canis* are also highly abundant in dogs in the tropics causing canine cyclic thrombocytopenia (CCT) and canine monocytic ehrlichiosis (CME), respectively (Harrus and Waner, 2011; Irwin and Jefferies, 2004; Little, 2010; Rymaszewska and Grenda, 2008). CME can often be lethal in canines if left untreated, generating fever, anorexia, myalgia, bleeding tendencies, epistaxis and neurological complications (Harrus and Waner, 2011; Little, 2010). Fatal outbreaks of *E. canis* have recently been discovered in an expanding range of arid and tropical regions of central and northern Australia, a country that had previously benefited from an *Ehrlichia*-free status until 2020 (Australian Government, 2020a; Harrus and Waner, 2011). Haemotropic *Mycoplasma* species are also important VBPs of dogs and although their exact mechanisms of spread are still debated, tick-transmission is suspected to play a role, with these pathogens typically generating disease in splenectomised or immunocompromised dogs (Barker et al., 2010; Hii et al., 2012; Hulme-Moir et al., 2010; Wengi et al., 2008). Moreover, many of these VBPs are commonly found coinfecting the same host, which can result in exacerbated disease and worse prognosis (Calzada et al., 2015; Eddlestone et al., 2007; Gaunt et al., 2010; Snellgrove et al., 2020).

Given the breadth of potential canine VBPs, accurate diagnosis of the responsible species is critically important as each may require different treatment protocols to cure the infection (Kidd, 2019; Maggi et al., 2014). Diagnosis has historically been conducted by microscopic examination of stained blood smears, a method notorious for its poor sensitivity, that also requires substantial expertise on the part of the microscopist to distinguish morphologically similar pathogens (Buddhachat et al., 2020; Kaur et al., 2020; Megat Abd Rani et al., 2010). Some of these issues are also encountered when conducting serological diagnosis, e.g., low sensitivity or poor specificity due to antibody cross-reactivity (Calzada et al., 2015). Today, molecular techniques such as conventional and real-time PCR (qPCR) are regularly used for canine VBP diagnoses given their ability to detect low levels of circulating pathogen DNA, whilst at the same time accurately

discerning different species (Hii et al., 2018; Massetti et al., 2020). Taq-Man based assays have been proven to be superior to conventional PCR and SYBR-based assays, especially in detecting mixed species infections, whilst also representing an ideal high-throughput diagnostic method suitable for large epidemiological studies (Espy et al., 2006; Massetti et al., 2020; Maurin, 2012; Peleg et al., 2010).

In this study, we developed two multiplex Taq-Man qPCR assays to simultaneously detect the common canine VBPs circulating in the Asia-Pacific, i.e., *A. platys*, *B. gibsoni*, *B. vogeli*, *E. canis*, *H. canis* and haemotropic *Mycoplasma* spp. (Barker et al., 2012; Carslake et al., 2017; Colella et al., 2020; Irwin and Jefferies, 2004; Muhlnickel et al., 2002; Shapiro et al., 2017). Herein, we define the Asia-Pacific as East Asia, including China, South Asia, including India, Southeast Asia and Oceania.

6.3. Methods

6.3.1. Multiplex qPCR development

Our multiplex qPCR was designed to consist of two quadruplex reactions, i.e. one targeting the bacterial canine vector-borne pathogens *A. platys*, *E. canis*, haemotropic *Mycoplasma* spp. and a mammal mitochondrial DNA (mtDNA) as a DNA extraction control, whilst the other designed to target the apicomplexans *B. gibsoni*, *B. vogeli* and *H. canis* and DNA of Equine Herpes Virus (EHV) as an internal qPCR reaction control.

For the apicomplexan quadruplex designs 18S ribosomal RNA (18S rRNA) gene sequences were downloaded from NCBI and aligned in Geneious Prime v. 2021.0.3. (Biomatters Ltd., Auckland, New Zealand), alongside sequences from other non-target canine apicomplexan pathogens to ensure specificity. All reference sequences of target and non-target organisms are shown in **Table 6.1**. A range of parasite isolate sequences found in different geographic regions were used to design probes against, whilst common non-target pathogens and environmental contaminant species' sequences were also included. Given that some regions of the 18S gene are highly conserved across the phylum Apicomplexa, regions that were identical across the three target species were investigated as potential primer binding sites, particularly if they flanked regions of

diversity that could be used as probe binding sites. Putative regions were manually assessed, and then exact primer binding sites were determined using the open-source software Primer3 v. 0.4.0. (<https://bioinfo.ut.ee/primer3-0.4.0/> (accessed on 13/07/2020)). Following this, primer and probe designs were further analysed and modified to ensure suitability in a multiplex format using the OligoAnalyzer™ Tool (Integrated DNA Technologies, Coralville, IA, USA), which permitted assessment of optimal annealing temperatures as well as removal of potential homo- and hetero-dimers from the designs. Finally, primers and probes were tested for specificity *in silico* via running NCBI's BLASTn tool to confirm that high nucleotide identity was only achieved with the relevant target species. The same design process was conducted for the bacterial pathogen quadruplex assay, with the 16S rRNA gene targeted instead. Reference sequences of target and non-target organisms are shown in **Table 6.1** with final designs for both quadruplex assays in **Figure 6.1**.

All primer and probe were designed using locked nucleic acid (LNA) technology to increase thermal stability and hybridization specificity. All qPCRs were run on a four-channel Magnetic Induction Cycler (Bio Molecular Systems, Upper Coomera, QLD, Australia).

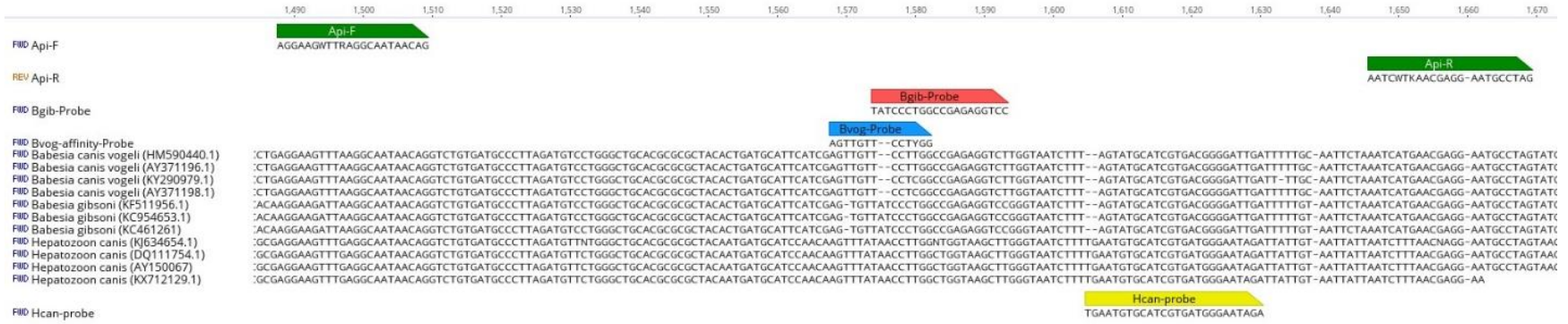
For the apicomplexan quadruplex assay one primer pair Api-F and Api-R was used to amplify a common 180 bp stretch of the 18S rRNA gene from *B. gibsoni*, *B. vogeli* and *H. canis*, with three separate Taq-Man probes (**Table 6.2**) as well as a previously designed EHV-4 primer pair and probe internal control amplifying an 80 bp region of the EHV-4 glycoprotein B (gB) gene (Bialasiewicz et al., 2009). For the bacterial quadruplex the primer pair Ehr/Ana-F and Ehr/Ana-R were designed to amplify a 145–149 bp region from both *A. platys* and *E. canis*, alongside two unique probes, whilst the primer pair Myco-F-D1 and Myco-R-Mod1 was designed to amplify a conserved 134 bp region of various haemotropic *Mycoplasma* spp. 16S rRNA genes.

Table 6.1. Target and non-target bacterial and protozoan 16S rRNA and 18S rRNA sequence accessions used in multiplex qPCR designs. To ensure specificity of Taq-Man probes to the target pathogen species a range of non-target sequences representing common pathogenic species as well as reagent and environmental bacterial contaminants were included.

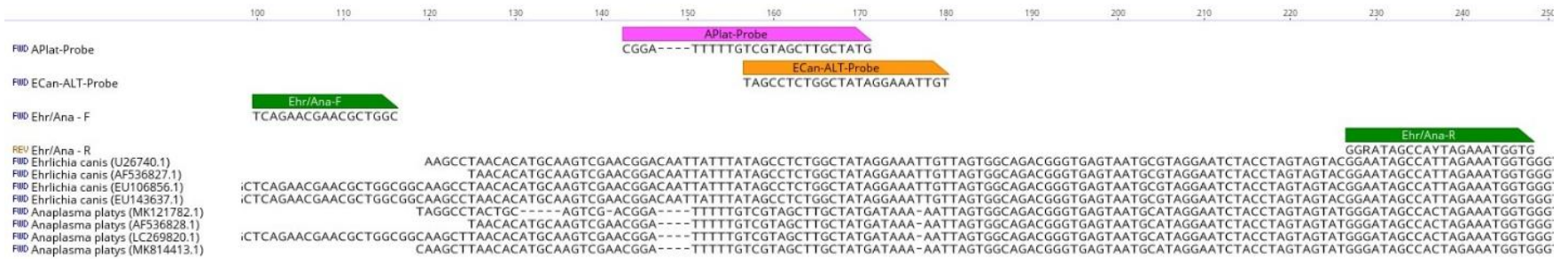
Bacterial quadruplex design		Apicomplexan quadruplex design	
Target species	NCBI accession #	Target species	NCBI accession #
<i>Anaplasma platys</i>	EF139459.1, LC269822.1, KU586001.1, MK814413.1, LC269820.1, AF536828.1, MK121782.1	<i>Babesia gibsoni</i>	KC461261, KF511956.1, KC954653.1
<i>Ehrlichia canis</i>	U26740.1, AF536827.1, EU106856.1, EU143637.1	<i>Babesia vogeli</i>	AY371198.1, KY290979.1, AY371196.1, HM590440.1
<i>Mycoplasma haemocanis</i>	KY117659.1, AY150973.1, EF416568.1	<i>Hepatozoon canis</i>	KX712129.1, AY150067, DQ111754.1, KJ634654.1
<i>Candidatus Mycoplasma haematoparvum</i>	AY532390.1, KY117661.1, GQ129114.1		
<i>Mycoplasma haemofelis</i>	EU145745.1		
<i>Candidatus Mycoplasma haemominutum</i>	AY150981.1		
<i>Candidatus Mycoplasma turicensis</i>	DQ464423.1		
<i>Mycoplasma coccoides</i>	AY171918.1		
<i>Mycoplasma suis</i>	EU603330.1		
Non-target species	NCBI accession #	Non-target species	NCBI accession #
<i>Acinetobacter viviani</i>	NR148847.1	<i>Babesia canis</i>	L19079.1
<i>Anaerobacillus alkalilacustris</i>	NR115854.1	<i>Babesia conradae</i>	AF158702.1
<i>Anaplasma phagocytophilum</i>	DQ458808.2	<i>Babesia divergens</i>	LC477143.1
<i>Bartonella clarridgeiae</i>	NR036961.1	<i>Babesia microti</i>	AB241631.1
<i>Bartonella henselae</i>	L35101.1	<i>Babesia ovis</i>	AY998123.1

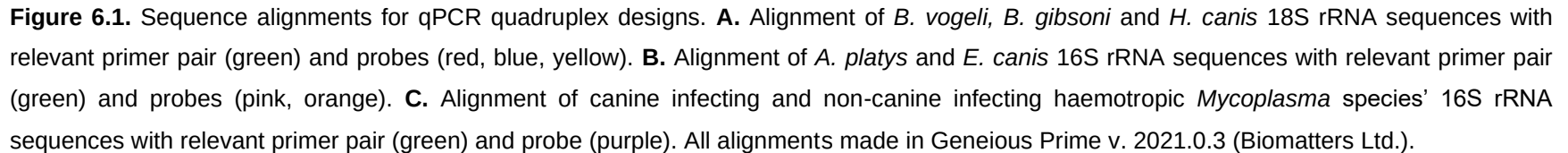
<i>Bartonella vinsonii</i>	DQ228135.1	<i>Hammondia hammondi</i>	KT184369.1
<i>Borrelia burgdorferi</i>	GQ478290.1	<i>Hammondia heydorni</i>	KT184370.1
<i>Bradyrhizobium centrosematis</i>	KC247115.1	<i>Hepatozoon americanum</i>	AF176836
<i>Candidatus Neoehrlichia mikurensis</i>	AB213021.1	<i>Hepatozoon felis</i>	KM435071
<i>Candidatus Rickettsia asemboensis</i>	JN315967.1	<i>Hepatozoon sipedon</i>	JN181157
<i>Corynebacterium aurimucosum</i>	AY536426.1	<i>Leishmania donovani</i>	XR_002966730.1
<i>Ehrlichia chaffeensis</i>	AF147752.2	<i>Leishmania infantum</i>	AJ634343.1
<i>Ehrlichia ewingii</i>	NR044747.1	<i>Plasmodium berghei</i>	AJ243513
<i>Enhydrobacter aerosaccus</i>	AB641400.1	<i>Plasmodium ovale</i>	KF018656
<i>Pseudomonas syringae</i>	AB001448.1	<i>Sarcocystis neurona</i>	KT184371.1
<i>Rickettsia conorii</i>	NR041934.1	<i>Theileria annulata</i>	EU083801
<i>Rickettsia felis</i>	DQ102712.1	<i>Theileria buffeli</i>	DQ104611
<i>Rickettsia rickettsii</i>	L36217.1	<i>Theileria ovis</i>	AY260172
<i>Rickettsia typhi</i>	NR118679.1	<i>Theileria sergenti</i>	EU083802
<i>Sediminibacterium aquarii</i>	NR152667.1	<i>Theileria sinensis</i>	KF559355
<i>Sphingomonas ginsengisoli</i>	AB245347.1	<i>Toxoplasma gondii</i>	L24381.1
<i>Staphylococcus aureus</i>	EF463060.1	<i>Trypanosoma evansi</i>	AJ009154.1
<i>Stenotrophomonas maltophilia</i>	FJ657669.1		
<i>Streptococcus lutetiensis</i>	NR037096.1		

A



B





A separate primer pair and probe taken and modified from (Hii et al., 2018), that target a 92 bp region of the canine mitochondrial 16S rRNA gene were included in the bacterial quadruplex to act as a DNA extraction control (**Table 6.2**). Upon *in silico* examination of this primer pair and probe the mtDNA 16S rRNA gene region targeted is conserved across a range of vertebrate species, including bears, monkeys, whales, otters, seals and snakes. Both quadruplex assays are designed to be run as a single panel; one includes the EHV internal reaction control, capable of detecting the presence of qPCR inhibitors, whilst the mtDNA extraction control in the bacterial quadruplex assesses whether the DNA extraction procedure has been successful and therefore if template DNA is present.

Optimisation to find ideal primer and probe concentrations was conducted using gBlock synthetic double-stranded DNA fragments (Integrated DNA Technologies) of the target region for each singleplex reaction. Serial dilution series of gBlock fragments used alongside iterative and stepwise testing of primer and probe concentrations permitted elucidation of the optimal concentration of each reagent (**Table 6.2**) to give the highest assay efficiency and greatest sensitivity.

We used QuantiNova Probe PCR Master Mix (Qiagen, Hilden, Germany) in the recommended 10 μ L reactions for all quadruplex assays. Overall, for each quadruplex 10 μ L reaction we used 1 μ L of template with the addition of 1 μ L of 1×10^{-7} ng/ μ L EHV-4 gDNA in the apicomplexan quadruplex, plus the relevant quantities of qPCR probes and primers listed in **Table 6.2**. Ambion Nuclease-Free Water (Life Technologies, Carlsbad, CA, USA) was used to achieve the final 10 μ L reaction volume.

All reactions were conducted using the following thermocycling profile: 2 min at 95 °C, then 15 s at 95 °C and 1 min at 60 °C for 40 cycles, for both quadruplex assays. Synthetic gBlock fragments diluted to 1×10^{-7} ng/ μ L were included as positive controls for all pathogens tested as well as nuclease-free water as negative controls. Samples and controls were run in duplicate for all optimisation and testing. Cycling analysis parameters were set at a fluorescent threshold of 5% and the first five cycles for all pathogen targets were ignored. Twelve-fold serial dilutions of gBlock fragments for each pathogen with a known DNA concentration were made and used to assess the efficiency and the intra-assay reproducibility of singleplex reactions vs when they were layered in a single

multiplex reaction. The correlation coefficients (R^2) from standard curves were produced and assessed using MIC PCR software (BioMolecular Systems, Upper Coomera, QLD, Australia).

Table 6.2. Oligonucleotide primers and probes, including fluorophores, for two quadruplex assays for canine VBP detection, with a + denoting a proceeding locked nucleic acid (LNA) base. Final reagent concentrations in reactions are shown.

Target	Primers and Probes	Sequence (5'–3')	Gene Target	Size (bp)	Conc (nm)	Source
<i>B. gibsoni</i> , <i>B. vogeli</i> & <i>H. canis</i>	Api-F	AGGAAGWTT RAG GCAATAACAG	18S rRNA	180	700	This study
	Api-R	CTA GGCATT CCT CGT TMAWGATT	18S rRNA	180	700	This study
<i>B. gibsoni</i>	Bgib-Probe	/56-FAM/TATCCCTG G/ZEN/CCGAGAGGTC C/3IABkFQ/	18S rRNA		60	This study
<i>B. vogeli</i>	Bvog- affinity- Probe	/5HEX/AG +T+T+G +TTC +C+TYGG/3IABkFQ/	18S rRNA		400	This study
<i>H. canis</i>	Hcan-probe	/56-ROXN/TGAATGTG CATCGTGATGGGAAT AGA/3IAbRQSp/	18S rRNA		100	This study
Equine Herpes Virus 4	EHV-F	GATGACACTAGCG ACTTCGA	gB gene	80	80	(Bialasiewicz et al., 2009)
	EHV-R	CAGGGCAGAAACC ATAGACA	gB gene	80	80	(Bialasiewicz et al., 2009)
	EHV-Probe	/5Cy5/TTTCGCGT G/TAO/C CTC CTCCAG/3IAbRQSp/	gB gene		200	(Bialasiewicz et al., 2009)
<i>A. platys</i> & <i>E. canis</i>	Ehr/Ana-F	TCA GAA CGA ACGCTGGC	16S rRNA	145–149	200	This study

	Ehr/Ana-R	CACCATTTCTAR TGGCTATYCC	16S rRNA	145–149	200	This study
<i>A. platys</i>	APlat-Probe	/56-FAM/CG GAT TT+T +TGTCGTAGCTTG CT+ATG/3IABkFQ/	16S rRNA		50	This study
<i>E. canis</i>	ECan-ALT-Probe	/56-ROXN/TA GCC TCT GGCTAT A+G+G AAA TTGT/3IAbRQSp/	16S rRNA		150	This study
Haemotropic <i>Mycoplasma</i> spp.	Myco-F-D1	CAM GTC AAG TCATCATGCCCC	16S rRNA	134	250	This study
	Myco-R-Mod1	CGA ATT GCA GCC TYYTAT CC	16S rRNA	134	250	This study
	Myco-ALT-Probe	/5HEX/TG +CAAA+C+G TGCTACAATGG/3IABkFQ/	16S rRNA	134	200	This study
Mammalian mtDNA	Mam-F	CGACCTCGATGT TGGATCAG	mtDNA	92	100	(Hii et al., 2018)
	Mam-R	GAAGTCAGA TCA CGT AGG ACT TT	mtDNA	92	100	(Hii et al., 2018)
	Mam-Probe	/5Cy5/CCTAATGGTGC AGCAGC+TA+TTAA GG/3IAbRQSp/	mtDNA	92	50	This study

6.3.2. Sensitivity and specificity of quadruplex qPCRs

Analytical sensitivity as defined by Saah and Hoover (1997) of the optimised multiplex assays was evaluated using 12-fold serial dilutions of gBlock synthetic DNA for each pathogen targeted (Saah and Hoover, 1997). Starting concentrations were 11.9 ng/μL for *B. gibsoni*, 10.8 ng/μL for *B. vogeli*, 7.5 ng/μL for *H. canis*, 2.86 ng/μL for *A. platys*, 7.78 ng/μL for *E. canis*, 5.24 ng/μL for *M. haemocanis* and 5.2 ng/μL for mammalian mtDNA with serial dilutions being made down to concentrations of 1×10^{-12} ng/μL.

The ability for our assay to exclusively detect our target pathogens and not detect similar but different organisms' DNA, i.e., its analytical specificity (Saah and Hoover, 1997), was achieved by testing the multiplex qPCRs against a bank of related and relevant blood-borne pathogens of zoonotic and veterinary importance. Potential cross-reactivity was tested using genomic DNA from animal blood or culture for the species *Anaplasma phagocytophilum*, *Bartonella clarridgeiae*, *Bartonella henselae*, *Borrelia burgdorferi*, *Coxiella burnetii*, *Orientia* spp., *Rickettsia australis*, *Rickettsia felis*, *Rickettsia typhi*, *Rickettsia honei*, *Leishmania infantum*, *Trypanosoma evansi* and *Dirofilaria immitis*. Both quadruplex assays were able to detect and amplify genomic DNA from their respective target organisms, with the haemotropic *Mycoplasma* probe specifically confirmed to detect genomic DNA from *M. haemocanis*, *Candidatus Mycoplasma haematoparvum* and *Candidatus Mycoplasma turicensis*. The possibility of cross-reactivity against pathogens targeted by the opposing quadruplex was also assessed, i.e., that the apicomplexans detected by the apicomplexan quadruplex were not detected by the bacterial quadruplex assay and vice versa.

The ability of our multiplex qPCRs to correctly classify an individual as being positive or negative for a particular pathogen, i.e., their diagnostic sensitivity and specificity (Saah and Hoover, 1997), was ascertained using 100 samples previously characterised for canine VBP using both next-generation sequencing (NGS) metabarcoding and conventional PCR (cPCR). The results of these samples previously reported by Huggins et al. (2019a and 2019b) were compared to those ascertained by the newly developed quadruplex assays.

For diagnostic sensitivity calculations the merged results of cPCR and NGS metabarcoding analysis were taken as a gold standard, i.e., samples positive by either cPCR and NGS for any given pathogen were considered true positives, whilst samples negative by both cPCR and NGS for any given pathogen were considered true negatives. Diagnostic sensitivity was calculated as the number of qPCR positives divided by the number of true positives (NGS and/or cPCR), whilst diagnostic specificity was taken as the number of qPCR negatives divided by the number of true negatives (NGS and cPCR).

6.3.3. Samples & DNA extraction

Canine blood samples were collected from geographic locations known to be highly endemic for the canine VBPs targeted by our assay. One hundred samples collected as part of an ongoing research project with Kasetsart University, Thailand, and previously tested for a range of bacterial and apicomplexan pathogens using NGS metabarcoding and conventional PCR (cPCR) were used as a test cohort with which to compare diagnostic parameters of our multiplex qPCRs (Huggins et al., 2019a, 2019b). These samples were collected from stray dogs inhabiting pagoda communities across 35 different field sites, with a qualified veterinarian conducting blood sample collection through a cephalic or jugular puncture. A total of 1ml of blood was collected into EDTA tubes and stored at -20°C until DNA extraction could be completed according to manufacturer's instructions using a E.Z.N.A.[®] Blood DNA Mini Kit (Omega Biotek Inc., Norcross, GA, USA), with DNA eluted in a total of 100 μl elution buffer. A further twelve samples were collected from stray dogs in Nanning, China, previously tested and confirmed positive for the pathogen *B. gibsoni*. Sample collection was only done after informed consent was acquired from dog owners, and the study was carried out under ethics permit OACKU-00758 provided by Kasetsart University.

6.3.4. Methodological comparison and statistical analysis

Kappa statistics (k) used to compare agreement between the NGS and cPCR results vs the qPCR multiplex assays, calculated in IBM SPSS Statistics 27 (SPSS, Chicago, IL, USA). Agreement between tests was considered poor if $k \leq 0.20$, fair if $0.21 \leq k \leq 0.40$, moderate if $0.41 \leq k \leq 0.60$, substantial if $0.61 \leq k \leq 0.80$ and high if $k > 0.81$. Endpoint PCRs were used as another reference point with which to compare our new qPCRs with the exact cPCRs used being a piroplasmid specific (*B. gibsoni* and *B. vogeli* inclusive) 18S targeting nested PCR (Jefferies et al., 2007a), a *H. canis* specific 18S targeting PCR (Inokuma et al., 2002b) and separate *A. platys* (Inokuma et al., 2002a), *E. canis* (Gal et al., 2008) and *Mycoplasma* genus (Criado-Fornelio et al., 2003) specific 16S targeting PCRs.

6.4. Results

6.4.1. Optimisation of multiplex qPCRs for canine VBPs

Via iterative testing of primer and probe concentrations, optimal concentrations were identified (**Table 6.2**) that produced the highest qPCR efficiencies in both a singleplex and multiplex format. Efficiencies ranged from 93% (*B. vogeli*) to 100% (mammalian mtDNA) with an R^2 from between 0.992 to 0.999 and slope from -3.314 to -3.502 (**Figure 6.2** and **Figure 6.3**). When singleplex reactions were added into a quadruplex format no changes were observed in reaction efficiencies, intra-reaction reproducibility and analytical sensitivity (**Figure 6.4** and **Figure 6.5**). No cross-reactivity was observed on gBlock synthetic DNA fragments for any of the target pathogens in either of the two quadruplex reactions.

6.4.2. Sensitivity & specificity of multiplex qPCRs for canine VBPs

Analytical sensitivity of our multiplex qPCRs was assessed by identifying the lowest possible gBlock DNA concentration that was detectable, along with the corresponding Cq value for that concentration, for each pathogen targeted. Mean Cq values associated with minimal detection limits were 36.83 (S.D. ± 0.2) for *B. vogeli*, 37.32 (S.D. ± 0.3) for *B. gibsoni*, 38.68 for *H. canis*, 35.86 (S.D. ± 0.7) for *A. platys*, 36.42 (S.D. ± 0.6) for *E. canis*, 37.06 (S.D. ± 0.8) for haemotropic *Mycoplasma* spp. and 36.85 (S.D. ± 0.4) for mammalian mtDNA corresponding to 1.2×10^{-4} fg of target gBlock DNA for *B. gibsoni*, 1.08×10^{-5} fg for *B. vogeli*, 2.9×10^{-4} fg for *H. canis*, 2.8×10^{-4} fg for *A. platys*, 7.8×10^{-4} fg for *E. canis*, 5.2×10^{-4} fg for haemotropic *Mycoplasma* spp. and 5.2×10^{-3} fg for mammalian mtDNA.

High analytical specificity was confirmed via the absence of any cross-reactivity on off-target genomic DNA samples. Diagnostic sensitivity of our novel qPCRs, when using the merged cPCR and NGS metabarcoding results as a gold standard, was 95.5% (84/88; 95% CI 88.9–98.2), whilst diagnostic specificity was 91.7% (11/12; 95% CI 64.6–98.5).

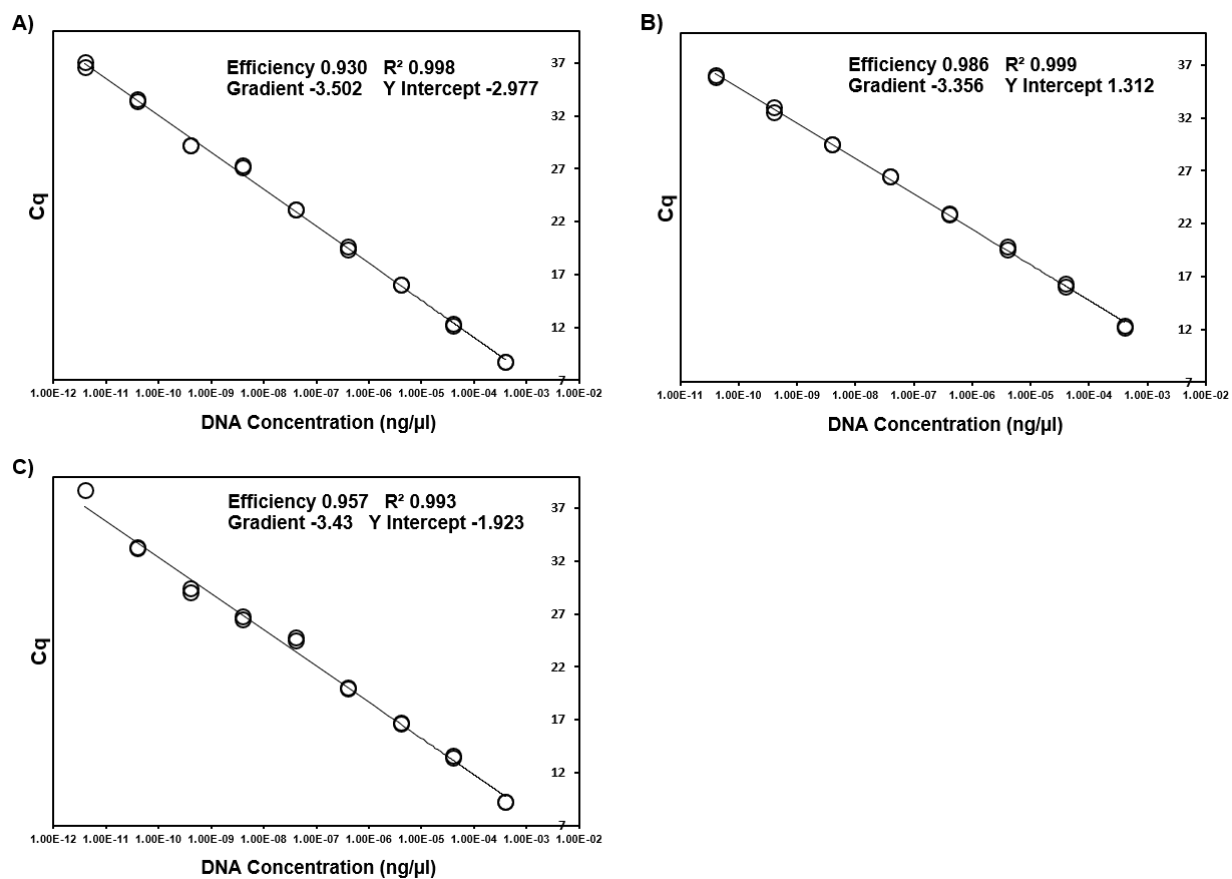


Figure 6.2. Standard curves generated from 12-fold serial dilutions of target gBlock Gene Fragments from the apicomplexans; *B. vogeli* (A), *B. gibsoni* (B) and *H. canis* (C).

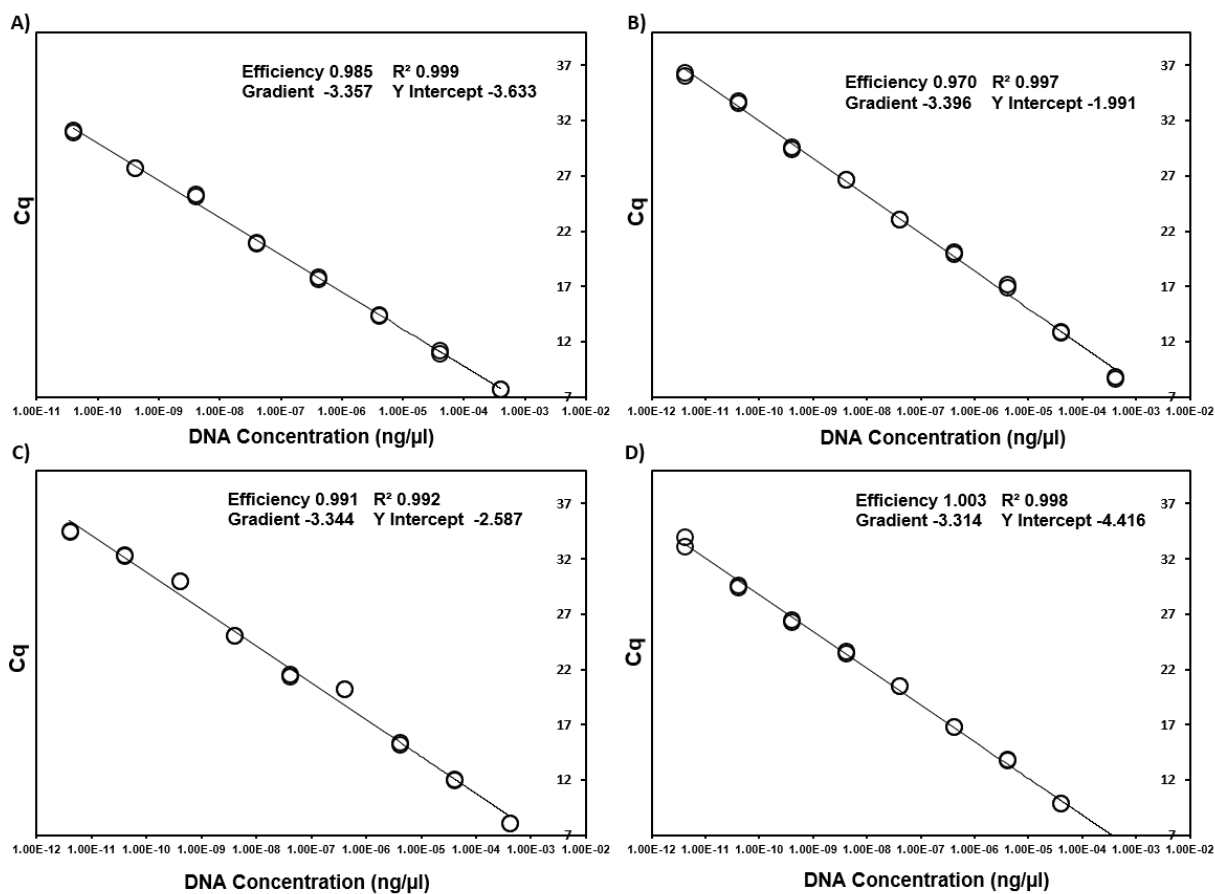


Figure 6.3. Standard curves generated from 12-fold serial dilutions of target gBlock Gene Fragments for *A. platys* (A), *E. canis* (B), *Mycoplasma* spp. (C) and mammalian mtDNA (D).

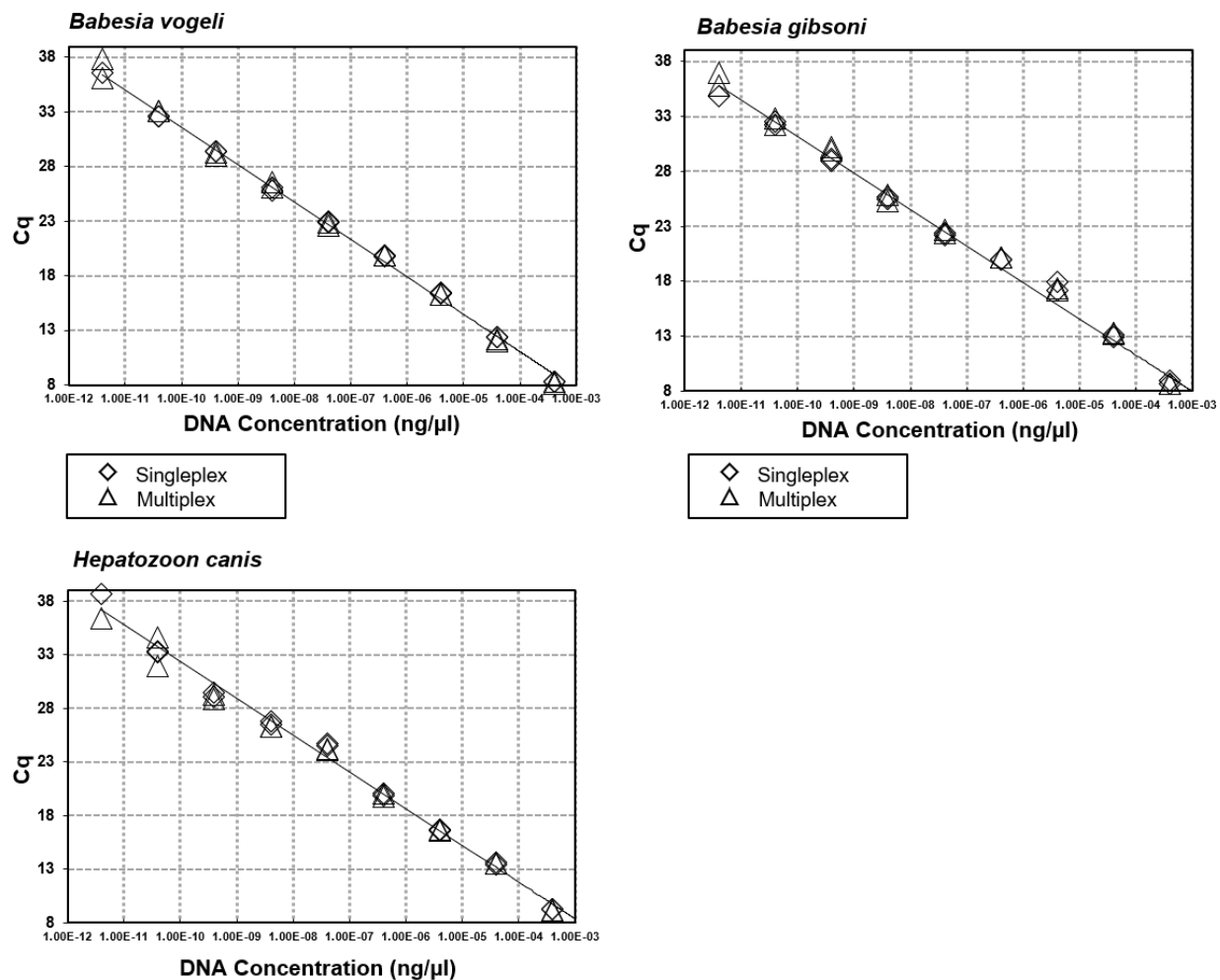


Figure 6.4. Singleplex and multiplex qPCR efficiencies. Optimisation and comparison of the sensitivity and efficiency of each singleplex and multiplex qPCR using gBlock Gene Fragment controls for *B. vogeli*, *B. gibsoni* and *H. canis*.

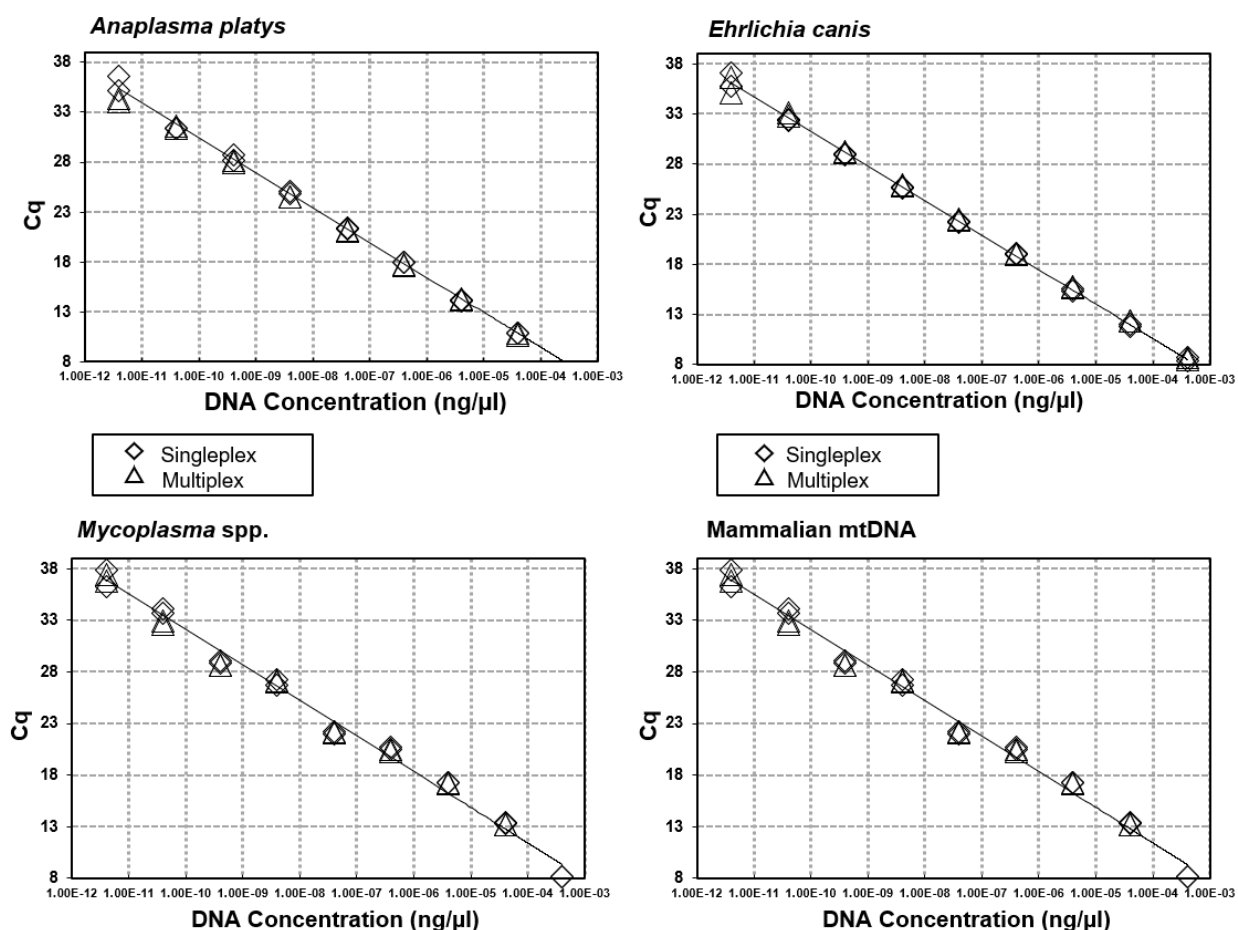


Figure 6.5. Singleplex and multiplex qPCR efficiencies. Optimisation and comparison of the sensitivity and efficiency of each singleplex and multiplex qPCR using gBlock Gene Fragment controls for *A. platys*, *E. canis*, *Mycoplasma* spp. and mammalian mtDNA.

6.4.3. Performance of multiplex qPCRs on field samples

The ability of our newly developed multiplex qPCRs to accurately and sensitively detect the targeted canine VBPs from 100 field samples was assessed using a set of blood-extracted DNA from stray dogs in Thailand, previously characterised by cPCR and NGS metabarcoding (Huggins et al., 2019a, 2019b). The results of these two tests provided an accurate benchmark with which to compare the field sensitivity and specificity of our novel qPCR protocol. For all sample batches tested via qPCR, mammalian DNA, EHV and gBlock positive controls amplified within the expected Cq values, demonstrating successful DNA extraction and the absence of qPCR inhibition. Negative control samples

were always absent of any amplification (with no Cq values), demonstrating that no reaction cross-contamination had occurred.

Comparisons of cPCR vs qPCR performance for these 100 Thai field samples are shown in **Table 6.3**, whilst comparisons of NGS metabarcoding results against those acquired by our novel qPCR are displayed in **Table 6.4**. Overall our multiplex qPCR detected more VBPs in the Thai canine cohort than the cPCR assays for all pathogens apart from haemotropic *Mycoplasma* spp. with total agreement ranging from 72% for *H. canis* to 98% for haemotropic *Mycoplasma* spp. Kappa agreement for VBP detection was high for both *B. vogeli* and haemotropic *Mycoplasma* spp., substantial for *E. canis*, moderate for *A. platys* and fair for *H. canis* with lower levels of agreement observed for these latter three pathogens due to the qPCR detecting numerous VBP positive samples that the cPCR assays had missed. For example, with respect to *H. canis* the multiplex qPCR outperformed the cPCR detecting 24% more infections, whilst in contrast, agreement for *H. canis* detection was substantial between the NGS and qPCR methodologies (**Table 6.4**).

Agreement between a previously developed NGS metabarcoding assay and the multiplex qPCR assays is shown in **Table 6.4**. Methodological agreement was high for the detection of *A. platys*, haemotropic *Mycoplasma* spp. and *B. vogeli*, whilst substantial agreement was identified for *E. canis* and *H. canis* with the qPCR assay detecting a greater number of infections for these two pathogens than the NGS protocol. Agreement between the NGS and qPCR assays was higher for the detection of *A. platys*, *E. canis* and *H. canis* when compared to the agreement for cPCR vs qPCR, whereas for *B. vogeli* and haemotropic *Mycoplasma* spp. detection agreement was similar to that achieved by cPCR vs qPCR.

A kappa statistic diagnostic comparison for *B. gibsoni* could not be made given the absence of this species in the Thai field samples. Nonetheless, a batch of twelve samples from stray dogs in China that were confirmed for the presence of *B. gibsoni* via cPCR and Sanger sequencing (100% query cover and nucleotide identity to accession #: MN134517.1) were also tested using the relevant apicomplexan quadruplex qPCR, with Cq values ranging from 26 to 30.

Table 6.3. cPCR vs multiplex qPCR agreement statistics for five VBPs. POS = positive, NEG = negative, SE = standard error. Kappa agreement level defined as poor if coefficient (k) is ≤ 0.20 , fair agreement if $0.21 \leq k \leq 0.40$, moderate agreement if $0.41 \leq k \leq 0.60$, substantial agreement if $0.61 \leq k \leq 0.80$ and high agreement if $k > 0.81$.

VBP	cPCR	qPCR		Total Agreement (%)	Kappa (Agreement)	Kappa SE
		NEG	POS			
<i>A. platys</i>	NEG	75	13	87	0.581 (moderate)	0.098
	POS	0	12			
<i>E. canis</i>	NEG	53	14	85	0.692 (substantial)	0.071
	POS	1	32			
<i>Mycoplasma</i> spp.	NEG	61	0	98	0.958 (high)	0.03
	POS	2	37			
<i>B. vogeli</i>	NEG	86	1	99	0.957 (high)	0.043
	POS	0	13			
<i>H. canis</i>	NEG	57	26	72	0.365 (fair)	0.084
	POS	2	15			

Table 6.4. NGS vs multiplex qPCR agreement statistics for five VBPs. POS = positive, NEG = negative, SE = standard error. Kappa agreement level defined as poor if coefficient (k) is ≤ 0.20 , fair agreement if $0.21 \leq k \leq 0.40$, moderate agreement if $0.41 \leq k \leq 0.60$, substantial agreement if $0.61 \leq k \leq 0.80$ and high agreement if $k > 0.81$.

VBP	NGS	qPCR		Total Agreement (%)	Kappa (Agreement)	Kappa SE
		NEG	POS			
<i>A. platys</i>	NEG	73	2	96	0.893 (high)	0.052
	POS	2	23			
<i>E. canis</i>	NEG	51	8	89	0.777 (substantial)	0.063
	POS	3	38			
<i>Mycoplasma</i> spp.	NEG	60	2	95	0.893 (high)	0.046
	POS	3	35			
<i>B. vogeli</i>	NEG	85	2	97	0.872 (high)	0.073
	POS	1	12			
<i>H. canis</i>	NEG	55	7	89	0.770 (substantial)	0.065
	POS	4	34			

6.5. Discussion

In the present study, we have demonstrated the development, optimisation and validation of two novel quadruplex qPCR assays for the simultaneous detection of six different canine VBPs. Such diagnostic tools will significantly aid researchers in the accurate diagnosis of common canine VBPs in the Asia-Pacific, permitting epidemiological surveillance of these pathogens in a manner that can easily be scaled up into a high-throughput format.

Clinical signs presented by dogs infected with common VBPs are typically shared by numerous different aetiological agents, whilst successful treatment may vary greatly depending on the species implicated (Kaur et al., 2020; Kidd, 2019; Little, 2010). Specific and sensitive diagnosis of the exact pathogen responsible is critical, with our newly developed multiplex qPCR being capable of meeting such requirements, whilst also providing relatively rapid diagnosis compared with the time and labour needed to conduct microscopy and cPCR. Additionally, our new qPCR protocol obviates many of the challenges associated with microscopy-based analysis such as low sensitivity and the high-levels of expertise required by the microscopist to accurately identify VBPs, particularly in individuals that are coinfecting with species that may be morphologically similar (Megat Abd Rani et al., 2010).

Real-time PCR assays for the detection of *A. platys*, *E. canis*, haemotropic *Mycoplasma* spp., *B. vogeli*, *B. gibsoni* and *H. canis* have been developed previously, although none of these methods have targeted all these VBP agents simultaneously and only a proportion have utilised highly specific, Taq-Man probe-based chemistry (Barker et al., 2010; Buddhachat et al., 2020; Costa-Júnior et al., 2012; Eddlestone et al., 2007; Gal et al., 2008; Hatta et al., 2013; Hulme-Moir et al., 2010; Kaur et al., 2020; Peleg et al., 2010; Qurollo et al., 2017; Shen et al., 2018; Sirigireddy and Ganta, 2005; Troskie et al., 2019; Wengi et al., 2008; Xu et al., 2015).

Many of the aforementioned VBPs are also common across the Asia-Pacific, with some species demonstrating changing distributions that mean accurate surveillance is crucial to assess changing risks to local dog populations. For example, *E. canis* has been identified molecularly in 25.5% of dogs in Malaysia (Koh et al., 2016), 21.8% in Cambodia

(Inpankaew et al., 2016) and 3.9–40% in Thailand (Huggins et al., 2019b; Liu et al., 2016) with further serological evidence of this pathogen from canines in China, Indonesia, the Philippines, Singapore, Taiwan and Vietnam (Colella et al., 2020). In addition, this pathogen has now been found in Australia, a country which was previously considered *E. canis* free but that is now grappling with growing outbreaks in the north, west and centre of the country (Australian Government, 2020b; Mason et al., 2001; Shapiro et al., 2017).

Anaplasma platys, haemotropic *Mycoplasma* spp., *H. canis* and *Babesia* spp. have also been found throughout the Asia-Pacific with apicomplexan species such as *B. vogeli* found in dogs at a prevalence as high as 32.7% in Cambodia (Inpankaew et al., 2016) and 9.4% in Thailand (Liu et al., 2016), whilst the more pathogenic *B. gibsoni* has been identified less commonly in countries such as China, Singapore (Colella et al., 2020) and Australia (Jefferies et al., 2007b; Muhlnickel et al., 2002). *Hepatozoon canis* has also been found parasitising large numbers of canines across the region (Huggins et al., 2019a; Inpankaew et al., 2016; Liu et al., 2016) and again there has been recent evidence of introduction of this species into Australia where previously it was not considered endemic (Greay et al., 2018). Such cases emphasise the critical need for effective pathogen diagnostics alongside strong biosecurity policies to prevent the introduction of novel VBPs to a previously non-endemic country and thereby mitigate the risk to naïve local dog populations that may be particularly susceptible.

Analytical sensitivity demonstrated our qPCRs as capable of detecting DNA quantities as low as 1.08×10^{-5} fg (*B. vogeli*), whilst singleplex reaction efficiencies were high, ranging from 93% to 100% with little difference observed when used in a multiplex format. Analytical specificity of our qPCRs was evaluated through testing on a range of common canine VBP positive blood DNA samples (previously confirmed using cPCR and Sanger sequencing). No off-target amplification was observed for any of these positive control samples, likely aided by the inclusion of LNA bases, as well as thorough *in silico* testing of qPCR primers and probes to ensure any chance of off-target binding was kept to a minimum (You et al., 2006).

Assessment of our qPCRs' diagnostic sensitivity and specificity against a set of samples from dogs in Thailand previously characterised via NGS and cPCR methods,

demonstrated a high sensitivity at 95.5% in conjunction with good diagnostic specificity at 91.7%. Diagnostic sensitivity and specificity were calculated using merged cPCR and NGS metabarcoding results as a gold standard, given the high concordance between these methods and that NGS analysis has previously been demonstrated to be highly sensitive for detection of some VBPs, particularly when compared to microscopy (Chen et al., 2020; Huggins et al., 2019b, 2019a).

Our new qPCR assays detected more VBP infections than nearly all the cPCR assays used and found similar numbers to those identified by NGS metabarcoding. Kappa agreement between cPCR and qPCR was fair for *H. canis*, moderate for *A. platys* and substantial for *E. canis* with qPCR identifying more infections than cPCR for all pathogens except haemotropic *Mycoplasma* spp. Superior sensitivity of qPCR over cPCR assays, alike to the data found in our study, has been demonstrated previously, including direct demonstration of lower limits of detection for qPCR over cPCR (Bogema et al., 2015; Hii et al., 2018; Massetti et al., 2020). In the present study, differences in sensitivity between cPCR and qPCR for each pathogen are likely due to the method of cPCR visualisation, e.g., gel electrophoresis used in the present study has poorer sensitivity than capillary electrophoresis as well as the particular cPCR assays used, each of which makes use of different reaction conditions and gene target regions (Gupta et al., 2010). Such differences in diagnostic sensitivity are non-trivial and may have large implications for the results of epidemiological surveys. For example, prior studies have found 25.5% of Malaysian dogs (Koh et al., 2016), 21.8% of Cambodian dogs (Inpankaew et al., 2016) and 3.9% of Thai dogs (Liu et al., 2016) positive for *E. canis* via cPCR, whilst in this study qPCR methods found many more dogs positive, at 46%. Hence, whether such disparity represents a true difference in prevalence or is an artefact of differences in diagnostic sensitivity is impossible to discern.

Concordance between the NGS metabarcoding method and our novel qPCRs was higher overall than the comparison against cPCR. Agreement was high for the VBPs *A. platys*, *B. vogeli* and haemotropic *Mycoplasma* spp. with substantial agreement for detection of *E. canis* and *H. canis*. For the pathogens *E. canis*, *H. canis* and *B. vogeli* our qPCRs identified slightly more infections than the NGS method, although differences were

negligible. Detection by qPCR of particular pathogens has been shown to be more sensitive than NGS methods in certain circumstances, whilst also being dependent on the number of samples multiplexed within a given NGS run (Anis et al., 2018; Thorburn et al., 2015).

There was also a small proportion of samples found positive by NGS and negative by qPCR for all pathogens targeted. As many samples are multiplexed and analysed together during NGS protocols there can be occasional Illumina index cross-talk, misreading or sequence hybridisation errors during amplification and deep-sequencing (Huggins et al., 2020; Kim et al., 2017). These facets of Illumina sequencing can lead to reads that were present in one sample appearing to be present in another, thus providing a potential source of false positives that may have generated the discordance between our NGS and qPCR methods (Kim et al., 2017).

Despite the high sensitivity and specificity demonstrated by our novel qPCR assays, there are some inherent limitations with molecular-based diagnostic testing that users should be cognizant of. Any molecular assay requires the circulation of pathogen DNA in the body compartment to be tested to be able to identify an active infection (Espy et al., 2006; Wong et al., 2011). This may not always be the case in asymptomatic or chronic infections where the presence of circulating pathogens in the bloodstream may be cyclical or low if the host has mounted a successful immune response (Portillo et al., 2017; Wong et al., 2011). Additionally, our qPCRs' diagnostic specificity of 91.7% indicates that it may be prone to occasional false positive results, a factor that might be alleviated by the use of three or four replicates per sample that could improve qPCR precision (Svec et al., 2015). Therefore, taking both factors together, we would advise that in a clinical setting additional serology-based tests that detect circulating pathogen-specific antibody, may also need to be conducted to ensure an accurate diagnosis is performed.

Our novel assays detailed herein may not only be a useful diagnostic method for use in a veterinary context but also for pulling apart canine VBP transmission dynamics in other relevant lifecycle hosts, such as arthropod vectors or non-domesticated wildlife. For example, our qPCRs could be used to test samples from wild canids that are known to maintain reservoirs of some VBPs (Millán et al., 2019, 2018), whilst a modification to the

assay's DNA extraction control to detect invertebrate mtDNA could allow the qPCRs to surveil for canine VBP prevalence in collected tick and flea vectors as well.

6.6. Conclusions

Our two newly developed quadruplex qPCR assays for the detection of six common canine VBPs found across the Asia-Pacific demonstrated higher diagnostic sensitivity when compared to cPCR methods and were able to detect coinfections that the individual cPCRs were not able to do. Furthermore, our novel qPCRs demonstrated comparable, if not superior, diagnostic sensitivity when compared to NGS metabarcoding methodologies, being able to achieve similar results at a fraction of the cost and labour. Overall, we envision that these new qPCRs will greatly assist researchers in conducting future epidemiological surveillance of canine VBPs over a geographic region stretching from China, through Southeast Asia to Australia where the target pathogens may be found. Our methods may provide tools to predict and uncover emerging VBP threats to previously non-endemic areas at a time when global climate disruption, increasing movement and globalisation, rapidly change patterns of worldwide disease prevalence (Traub et al., 2015).

6.7. References

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Chapter 7 - Field trial investigating the efficacy of a long-acting imidacloprid 10%/flumethrin 4.5% polymer matrix collar (Seresto[®], Elanco) to monthly topical fipronil for the chemoprevention of canine tick-borne pathogens in Cambodia

7.1. Abstract

The brown dog tick, *Rhipicephalus sanguineus* commonly infests canines in the tropics and is an important vector for disease-causing and sometimes lethal pathogens including *Babesia* spp., *Ehrlichia canis*, *Hepatozoon canis*, *Anaplasma platys* and haemotropic mycoplasmas. In tropical climates ticks and their pathogens exert an extremely high infection pressure on unprotected dogs. To protect canines in such regions, effective acaricidal products possessing a speed of kill that blocks pathogen transmission is paramount. We conducted a twelve-month community trial, of which the first 6-months of follow-up data were available, to compare the chemoprophylactic efficacy of two topical commercial acaricidal formulations; an imidacloprid 10% and flumethrin 5.5%, eight-month acting collar (Seresto®) against a monthly spot-on containing [12% w/v] fipronil (DeTick, Thailand). In a separate analysis, we used baseline data collected at the start of the trial to quantify tick-borne pathogen (TBP) infection status in dogs with a prior history of being administered a systemically-acting (isoxazoline) versus a topically-acting ectoparasiticide. Both topical products in the community trial demonstrated high efficacy at protecting dogs from ticks and tick-transmitted pathogens with just 1% of dogs on Seresto® and 2% of dogs on DeTick acquiring a new TBP. At baseline, dogs treated with commercial systemic isoxazoline acaricides prior to the trial's commencement were more likely to be infected with a TBP (10%, 95% CI 5% to 20%) compared to those treated with topical formulations (3%, 95% CI 1% to 9%), highlighting such isoxazoline products as being less effective than topical products at preventing canine TBP acquisition in a tropical setting.

7.2. Introduction

Ticks are important blood-feeding arthropods that represent a large threat to dog populations worldwide due to their capacity to vector a range of viruses, bacteria, and protozoa, collectively known as tick-borne pathogens (TBPs) (Brianti et al., 2013; Chomel, 2011; Fourie et al., 2013a). The impacts of TBPs are particularly pernicious, given tick species such as the brown dog tick, *Rhipicephalus sanguineus*, are one-host ticks, meaning that local ectoparasite numbers can rapidly increase and facilitate the easy spread of TBPs (Gray et al., 2013). Ticks including *R. sanguineus* and, in the tropics, *Rhipicephalus linnaei*, transmit diseases that can be lethal to dogs, for example *Ehrlichia canis* the aetiological agent of canine monocytic ehrlichiosis (CME). At the same time, protozoan diseases such as *Babesia vogeli* and *Hepatozoon canis* can also inflict significant morbidity (Chomel, 2011; Koh et al., 2016; Little, 2010; Šlapeta et al., 2021).

In the tropics relatively high levels of humidity and mean annual temperatures facilitate the spread of TBPs as local vectors do not experience strong population fluctuations that occur in parts of the world with distinct seasonal cycles (Galay et al., 2018; Snellgrove et al., 2020). Additionally, in low-to-middle income countries where large numbers of semi-domesticated or free-roaming dogs have limited access to veterinary care, conditions are often further conducive for TBP propagation (Inpankaew et al., 2016; Koh et al., 2016).

Given the severe health impacts ectoparasites and the TBPs they transmit generate in dogs, effective chemopreventive medications and products that block TBP transmission are critically important. Topical products that act from the animal's hair and dermis to repel ectoparasites and rapidly kill them are particularly beneficial as they prevent vector haematophagy and thus do not provide a time period over which TBPs can be transmitted (Dantas-Torres et al., 2013; Schorderet-Weber et al., 2017). Such benefits are further highlighted when their effectiveness is compared to ectoparasiticides which rely on systemic uptake of the active ingredient into the host's tissues, e.g. isoxazoline compounds. Systemic ectoparasiticides are ingested at the point of vector feeding, subsequently killing the ectoparasite whilst at the same time also providing a timeframe over which some TBPs can be transmitted (Jongejan et al., 2016; Ohmes et al., 2015). The TBP blocking ability of topical products that rely on chemicals present on the skin of

the dog, thereby repelling and/or quickly killing ectoparasites, has been experimentally demonstrated via challenge infections to be superior to products that rely on systemic chemical uptake for the prevention of TBP transmission, e.g. for *E. canis* (Jongejan et al., 2016; Schorderet-Weber et al., 2017).

An imidacloprid 10% and flumethrin 5.5% vinyl collar (Seresto®, Bayer) designed to provide long-term protection from ticks and fleas for up to eight months has been tested for its ability to repel and kill ectoparasites as well as to prevent the transmission of some TBPs (Krüdewagen et al., 2015; Stanneck et al., 2012c). The two active chemicals in Seresto® collars act synergistically to augment the neurotoxic effects of either alone, on insect and arachnid nervous systems (Stanneck et al., 2012a). A further advantage of these collars is their purported eight-month efficacy, which results in improved owner compliance when compared to products which offer shorter-term protection and therefore need regular administration (Dantas-Torres et al., 2013; Ohmes et al., 2015; Stanneck et al., 2012c).

Seresto® collars have been shown to prevent flea and tick infestations with high efficacy (90% to 100%) for up to eight months, even in regions with high ectoparasite pressure or within canine populations with regular exposure to rain and other environmental factors that might negatively impact on product effectiveness (Beugnet et al., 2018; Brianti et al., 2013; Dantas-Torres et al., 2013; Halos et al., 2014; Stanneck et al., 2012b, 2012c). Moreover, Seresto® collars have been shown to be efficacious at preventing the transmission of key canine TBPs, including *E. canis* (Stanneck and Fourie, 2013), *Babesia canis* (Fourie et al., 2013b), *Borrelia burgdorferi* sensu lato and *Anaplasma phagocytophilum* (Krämer et al., 2020) the latter two of which are important zoonoses for which canines act as a reservoir.

Less contemporary topical ectoparasiticides, such as fipronil, a phenyl pyrazole that has been used on canines since the early 1990s has also been extensively tested to assess its ability to prevent infestation and block TBP transmission. Prior field studies have found fipronil in commercial topical formulations such as Frontline™ (Boehringer Ingelheim) to demonstrate efficacies as high as 94% to 97% for preventing flea infestations and 94% to 100% for ticks, concluding that it is a highly effective topical ectoparasiticide (Kužner

et al., 2013; Rohdich et al., 2014). Moreover, such infestation prevention also translates to an ability to block TBP contraction, with fipronil having been shown to prevent transmission of *E. canis* by *R. sanguineus* in areas of endemicity and high pathogen pressure in Djibouti (Davoust et al., 2003).

Field efficacy studies have also been conducted on Seresto®, predominantly in Europe, with research focusing on testing efficacy at repelling the relevant vectors and blocking the associated TBPs found in these regions (Brianti et al., 2013, 2014; Dantas-Torres et al., 2013; Stanneck et al., 2012c). Nonetheless, in tropical parts of the Asia-Pacific pressure from ectoparasites and the prevalence of TBPs in local dog populations can greatly exceed those found in temperate regions. For example, prior studies in Italy have shown Seresto® collars to be efficacious at blocking *B. vogeli* transmission with an estimated local prevalence of 5% to 6% for this pathogen (Cassini et al., 2009; Dantas-Torres et al., 2013). In contrast, *B. vogeli* has been found in as many as 33% of free-roaming dogs in Cambodia (Inpankaew et al., 2016), 23% of dogs in Cambodia's capital Phnom Penh as ascertained in Chapter 5, 9% to 13% of dogs in Thailand (Huggins et al., 2019; Liu et al., 2016) and 44% of dogs in remote communities throughout parts of tropical and arid regions of Australia (Barker et al., 2012). Such high pathogen pressure environments provide an ideal setting within which to test Seresto® collars to elucidate whether a high level of ectoparasite repellence and TBP-blocking efficacy is still maintained. Comparing Seresto®'s efficacy to fipronil, a topical ectoparasiticide that has been demonstrated effective at preventing tick infestation and is readily available throughout Southeast Asia, provided a benchmark from which to assess the potential TBP blocking efficacy that Seresto® confers.

With this background, we report the methodology and findings from a two-part study designed to document the risks of TBP infection in dogs with a history of using either topical or systemic ectoparasiticides and investigate the efficacy of two topical ectoparasiticides on blocking canine TBP transmission in Cambodia. Firstly, we conducted a baseline assessment of dogs to determine the prevalence of TBP infection in those being treated with topical and systemic ectoparasiticides as well as those with no prior ectoparasiticide treatment. Secondly, we carried out a 12-month prospective

superiority community trial on dogs enrolled into the baseline study to compare the incidence of newly acquired TBPs in dogs treated with two different topical commercial ectoparasiticides; Seresto® collars and fipronil [12% w/v] (DeTick, Thailand), the latter being a topical ectoparasiticide commonly used in Southeast Asia.

The data presented in this chapter is the analysis and synthesis of data accrued up until the six-month time point of this trial which was all that was available at the time of writing. The trial will continue for a further six months and will be reported in subsequent publications.

7.3. Methods

7.3.1. Study design and study area

We conducted a community trial where our primary aim was to compare the chemoprophylactic efficacy of two topical commercial acaricidal formulations; an imidacloprid 10% and flumethrin 5.5%, eight-month acting collar (Seresto®) against a monthly spot-on containing [12% w/v] fipronil (DeTick, Thailand), administered according to labelled instructions.

Two communities of dogs were selected for investigation in Cambodia (Jan - March 2020). The first comprised dogs (pets) that were owned by clients of the Animal Mama Veterinary Hospital in Phnom Penh (11°31'N, 104°55'E) and dogs from the Home of Heroes animal shelter in Siem Reap (13°26'N, 103°45'E). The second community comprised dogs that worked for and were cared for by the Cambodian Mine Action Centre (CMAC). This group included dogs that were kenneled and used for breeding as well as individuals regularly taken into the field to assist with landmine decontamination programs at three locations across the country; Phnom Penh city (11°31'N, 104°55'E) an urban environment, Kampong Chhnang (12°15'N, 104°39'E) a rural town and Battambang (13°25'N, 103°73'E) a medium sized city. Dogs in the first community, owned by clients of Animal Mama Veterinary Hospital, were recruited by email or over the phone in January 2020. A written description of the study together with contact details of investigators and expectations of each dog owner were provided in English and/or Khmer along with a

consent form for signing. Official permission was obtained by the Director General of CMAC in January 2019 to enroll dogs from the second community into this study. This study was approved by the Office of Research Integrity and Ethics, University of Melbourne under ethics permit 1814620.

Animals were deemed eligible for enrolment if they were over eight weeks of age on 1 March 2020, if they were walked outdoors a minimum of three times per week, were clinically normal on physical examination and were expected to continue living in Cambodia for the next 12 months. Dogs that were previously administered ectoparasiticide products were only enrolled in the study after the window of labelled efficacy for the applied or administered product had ceased.

The trial was conducted between January 2020 and July 2021 for dogs recruited through Animal Mama Veterinary Hospital and March 2020 to March 2021 for the CMAC dogs. Dogs in the first community (clients of the Animal Mama Veterinary Hospital in Phnom Penh and clients from the Home of Heroes animal shelter in Siem Reap) received Seresto® collars (Bayer) and are referred to as the Investigational Veterinary Product (IVP) group in the remainder of this chapter. Dogs in the second community (dogs that worked for and were cared for by the CMAC) received monthly treatment with fipronil (DeTick, Thailand) and are referred to as the Control Product (CP) group. Both of these products act topically with the active compound working from the subject's skin or hair.

On the prospective community trial's date of commencement, a Seresto® collar was fitted to each of the dogs in the IVP group. Dogs in the CP group received the first of their monthly fipronil (DeTick) treatment topically between the shoulder blades as per the manufacturer's instructions. For the IVP group the Seresto® collars were replaced after 8 months, or earlier if the collar detached and was lost (within a 24-hour period).

A community trial study design was necessary because ectoparasiticide treatment could not be randomly allocated to the pool of eligible dogs. This was because over this study period, the CMAC dogs had to continue their regular demining activities and had a strict ectoparasite and endoparasite prevention and treatment protocol that was not permitted to be altered by CMAC for the sake of this study.

Dogs enrolled into the prospective community trial were followed by investigators for a period of 12 months. A 6-month and 12-month follow-up period was used to assess treatment efficacy. **Figure 7.1** provides a diagrammatic representation of the sequence of treatment and testing events carried out as part of the baseline assessment and prospective community trial.

Throughout the follow-up period dogs in the CP group (i.e., CMAC dogs) were kept at centres comprised of large areas of open land, fields and trees. They had access to these sites during the day for training activities. During evenings and nights these dogs were kept in raised cages off the ground. The CMAC centres and grounds were not closed environments and wildlife and occasionally community dogs were able to enter such areas resulting in opportunity for ectoparasite exposure. Dogs in the IVP group were continued to be cared for by their respective owners who were encouraged to continue walking their dogs outside of their premises in areas frequented by community dogs, as they had been doing prior to the study's commencement.

7.3.2. Sampling and diagnostic methods

At enrolment, dogs were subject to a complete physical examination by the study's veterinarians. Each animal's age, sex, breed, neutering status, whether imported, prior ectoparasite and endoparasite control history as well as other ongoing medication details were recorded. Previous ectoparasite and endoparasite control product used with particular focus on whether a topical, systemic or no ectoparasiticide had been administered prior to the study's commencement was key information collected at this baseline assessment point. Furthermore, endo- and ectoparasiticide product compliancy was noted, with compliancy defined as either poor, intermittent or high depending on the adherence to manufacturer recommendations. Compliancy was defined as poor if the owner only applied parasiticides when taking their dog to occasional veterinary check-ups, compliancy was intermittent if a product was used but with gaps over the product's labelled window of efficacy, compliancy was high if the parasiticide was used as per the product's labelled instructions.

Dogs were then subjected to a detailed physical exam by the study veterinarians. Two millilitres of samples were drawn via cephalic or jugular venipuncture from each dog into two EDTA tubes. Blood was subjected to a complete blood count (CBC), examination for TBPs using stained whole and buffy coat smears and SNAP 4Dx Plus (IDEXX, Westbrook, ME, USA) to test for the presence of antibodies to anaplasmosis, ehrlichiosis, Lyme disease and antigens of *Dirofilaria immitis*. The second tube of whole blood was frozen at -20 °C and transported on ice to the Melbourne Veterinary School at the University of Melbourne. At the Melbourne Veterinary School DNA extraction was conducted using a DNeasy Blood & Tissue Kits (Qiagen, Hilden, Germany) and subject to our previously developed multiplex real-time (qPCR) assays for canine vector-borne pathogens, capable of detecting *Anaplasma platys*, *Babesia gibsoni*, *Babesia vogeli*, *Ehrlichia canis*, *Hepatozoon canis* and haemotropic *Mycoplasma* spp. (Huggins et al., 2021). Collection of blood for qPCR analysis was also conducted at the six and 12-month examination time points.

7.3.3. Clinical and safety observations

Throughout the follow-up period dogs were examined at monthly intervals for clinical signs consistent with TBP infection as well as potential adverse effects to their chemoprophylaxis treatment (e.g., dermatological changes at the site of application). Dogs were also checked for the presence of ticks at common feeding sites e.g., head, ears, inter-digital regions, with those found collected and preserved in 80% ethanol. At this time, the integrity of the Seresto® collars were also confirmed and CP dogs administered fipronil (DeTick, Thailand). Owners were also questioned to ensure compliance with the study protocol as per eligibility criteria at enrolment.

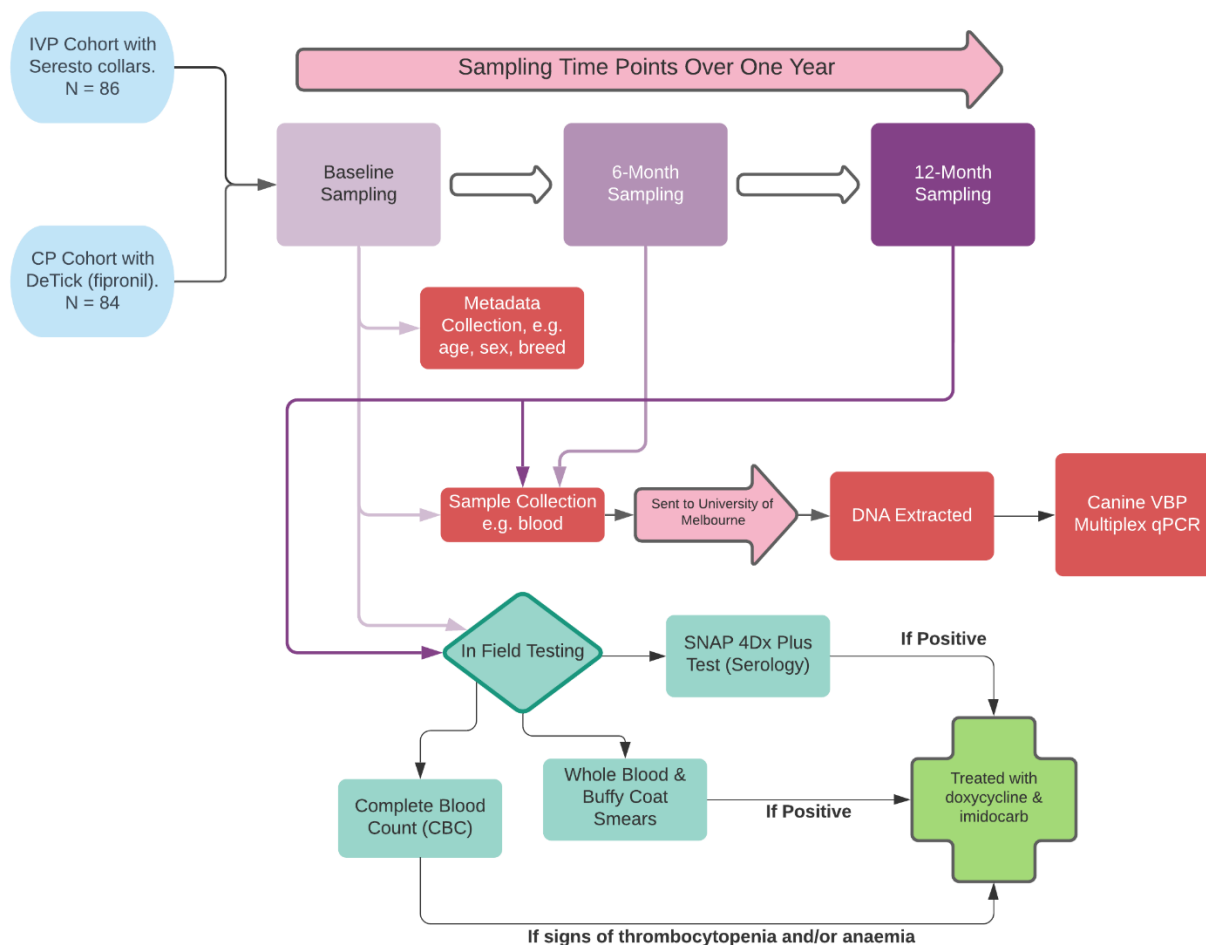


Figure 7.1. Experimental design for (1) baseline assessment where metadata collected was used to compare rate of vector-borne pathogen (VBP) positivity in canines treated with systemic, topical and no ectoparasiticides prior to the study's commencement and (2) prospective community trial comparing two topical ectoparasiticides (Seresto® and DeTick) for prevention of TBP contraction.

7.3.4. Treatment and rescue treatment

Dogs that were found to have subclinical anaemia or thrombocytopenia at the time of enrolment, and/or those found exposed to a TBP via SNAP 4Dx Plus testing or positive via blood smears were put on a course of doxycycline 10 mg/kg SID. The course of doxycycline was for 14 days if positive to *A. platys*, whilst a 28-day course was used for *E. canis*. At baseline, dogs found to be anaemic and/ or thrombocytopenic, were

administered imidocarb dipropionate 5 – 7 mg/kg IM over a two-week interval, to clear any potential concurrent *B. vogeli* infection.

Rescue treatment was also initiated using the treatment protocols described for enrolled dogs found to be vector-borne pathogen positive by smear or qPCR at any of the follow-up examinations (six and twelve-months). Additionally, dogs found positive to an apicomplexan were treated with imidocarb dipropionate 5 – 7 mg/kg IM twice, two weeks apart to ensure TBP negativity (Sainz et al., 2015). Dogs found positive for haematropic mycoplasmas were given a 14-day course of doxycycline at 10 mg/kg PO SID.

7.3.5. Data handling and statistical analysis

Sample size calculations conducted for the prospective community trial were carried out to determine the number of dogs that needed to be enrolled into the study to provide sufficient power to demonstrate that the confidence limit for the difference between the two treatments excluded zero. This takes the assumption that the TBP prevalence for the IVP group was less than the TBP prevalence for the CP group by an amount called the smallest clinically important difference, delta. Given 80% power, a 95% confidence at the 5% level of significance, an estimated TBP prevalence of 10% and 30% in the IVP and CP group, respectively, and a smallest clinically important difference between treatment groups of 5%, the total sample size of dogs needing to be enrolled and complete the study was 166. In addition to this, a study drop-out rate of 15% was estimated based on prior data and conversations with CMAC and Animal Mama regarding regular adoption of CMAC canines or movement of clients and dogs to other regions of the country, as well as dog mortality. Hence, minimum sample size required was increased to 195 dogs. Sample size calculations were carried out using the 'sample size for a cohort study' function in EpiTools (<https://epitools.ausvet.com.au>). The sample size required for the baseline assessment was calculated using the 'Sample size to detect a significant difference between two proportions' function in EpiTools and was found to be less than that required for the prospective community trial hence the community trial's sample size was assessed to be suitable for both parts of this investigation (Sergeant, 2018).

The prevalence of TBP infection as determined by qPCR results and TBP exposure as determined by serology, was calculated using the `epi.directadj` function in the contributed `epiR` package (Breslow and Day, 1987; Stevenson et al., 2021) in R (Core Team, 2021). These calculations were used to compare dogs on systemic, topical or no ectoparasiticide products prior to the study's commencement, directly adjusting for the confounding effect of the environment in which dogs were kept (urban, rural). This provided TBP prevalence estimates that were comparable, given that the environment in which a dog is kept can give rise to markedly different TBP infection pressures (Farkas et al., 2009; Gracia et al., 2008; Smith et al., 2011). Dogs that were on a treatment that had been administered with poor compliance, see 'Sampling and diagnostic methods', were included in the no ectoparasiticide category for this analysis, whilst those administered their ectoparasiticide with moderate or high compliance were included in their respective product group. Baseline assessment TBP crude (i.e. unadjusted) prevalence estimates for systemic, topical and no ectoparasiticide treated dogs and their 95% confidence intervals were presented as error bar plots, using the data from both the qPCR and serological analyses. In addition, using the qPCR data, calculations were carried out to identify the number of dogs needed to be treated for benefit (NNTB), defined as the inverse of the attributable risk (the directly adjusted probability of a dog on a topical ectoparasiticide being TBP positive minus the directly adjusted probability of a dog on a systemic ectoparasiticide being TBP positive). NNTB estimates were interpreted as the number of dogs that would need to be treated with a topical ectoparasiticide to prevent one additional dog from being TBP positive. Confidence intervals for the NNTB estimates are reported using the approach described by Altman (1998).

For the prospective community trial comparing the topical ectoparasiticides Seresto® and DeTick for the 6-months' qPCR data, the frequency of TBP positivity for the CP and IVP groups were expressed as incidence rate measures, that is the number of incident TBP cases divided by the total number of dog trial-days at risk. This approach allowed for the possibility of owners withdrawing their dogs from the study prior to the study completion date. Again, the `epi.directadj` in `epiR` was used, adjusting for the confounding effect of the environment in which a dogs was kept. Crude TBP incidence rate estimates for IVP (Seresto®) and CP (DeTick) treated dogs and their 95% confidence intervals were

presented as error bar plots. NNTB calculations were also conducted and interpreted as the number of dogs that would need to be treated with the IVP ectoparasiticide (Seresto®) to prevent one additional dog from becoming TBP positive.

Additionally, for the prospective community trial the IVP (Seresto®) collar efficacy $EVBP_{IVP}$ was calculated as follows:

$$EVBP_{IVP} = \left(\frac{P_{CP} - P_{IVP}}{P_{CP}} \right) \times 100 \quad \text{Equation 1}$$

In Equation 1 P_{CP} and P_{IVP} equal the proportion of dogs in the CP group and IVP group that were newly TBP pathogen positive at the end of the follow up period, respectively.

The efficacy of the IVP (Seresto®) collar against ectoparasites EEP_{IVP} was calculated using a similar approach:

$$EEP_{IVP} = \left(\frac{EP_{CP} - EP_{IVP}}{EP_{CP}} \right) \times 100 \quad \text{Equation 2}$$

where EP_{CP} and EP_{IVP} equal the mean number of ectoparasites found attached to dogs in the CP group and IVP group at the end of the follow up period, respectively. The IVP group's Seresto® collars were deemed effective against TBPs and ectoparasites if the calculated efficacy, based on means, was at least 90% (Marchiondo et al., 2013). The chi-squared test was used to assess whether there was a statistically significant difference in the number of dogs that dropped out of the study up to the six-month time point between the CP and IVP groups within the prospective community trial.

7.4. Results

7.4.1. Baseline assessment comparing topical and systemic ectoparasiticides

A total of 164 dogs were comparable within the baseline assessment that had information provided on ectoparasiticide usage prior to the study's commencement ($n = 99$ on topical treatments and $n = 59$ on systemic treatments, $n = 6$ on no ectoparasiticide).

Key information collected at baseline apart from the type of ectoparasiticide used prior to the study's commencement was the compliancy of product usage, as defined in the

'Methods'. No differences were observed in moderate or high owner compliancy categories between dogs being treated with systemic or topical ectoparasiticides, whilst those on poorly compliant regimes were included in the no ectoparasiticide used group for subsequent analysis. The majority of dogs on a systemic product were using an isoxazoline product, such as Bravecto® (MSD Animal Health) and Nexgard® (Boehringer Ingelheim).

At baseline, five dogs on systemic ectoparasiticides were identified as ectoparasite positive, including three with ticks, one with fleas and one with an undocumented ectoparasite. In contrast, only one individual on topical ectoparasiticides was identified as ectoparasite positive (a tick). Of the six dogs that were not on an ectoparasiticide, three were found infested with ticks with one of these three also infested with fleas. Using our previously developed multiplex qPCR within the dogs on systemic products six were positive for any TBP, whilst for dogs on topical products three were positive. Of the dogs on no ectoparasiticides two were TBP positive. See **Table 7.1** for details of the TBP species identified. All TBP-positive individuals in both groups were identified using qPCR with no pathogens detected via whole blood or buffy coat smears.

Figure 7.2 shows the crude apparent TBP prevalence estimates for systemic, topically and no ectoparasiticide treated dogs, conditioned by the environment in which the dog was kept (urban, rural). After adjusting for the effect of environment there were 12 (95% CI 3 to 32) TBP-positive systemically treated dogs per 100 dogs at risk, compared with 4 (95% CI 1 to 12) TBP-positive topically treated dogs per 100 dogs at risk. This result can be alternatively expressed as an apparent prevalence risk ratio, i.e. dogs that were systemically treated were 3.0 (95% CI 0.67 to 14) times more likely to be TBP-positive compared with dogs that were topically treated. For dogs on no ectoparasiticide product there were 17 (95% CI 2 to 60) TBP-positive dogs per 100 dogs at risk. After accounting for the confounding effect of environment we estimate that 12 (95% CI 5 to 37) dogs needed to be treated topically to avoid one case of TBP.

Table 7.1. Between group comparison of the number of qPCR positive dogs (+) and apparent pathogen prevalence (%) with 95% CI found for single, mixed and overall infections at study baseline. No dogs were found *B. gibsoni* positive.

Pathogen	Systemic Products (n = 59)		Topical Products (n = 99)		No Ectoparasiticide (n = 6)	
	+	% (CI)	+	% (CI)	+	
Single & mixed infection						
<i>Anaplasma platys</i>	1	2 (0 - 9)	2	2 (1 - 7)	2	33 (10 – 70)
<i>Babesia vogeli</i>	2	3 (1 - 12)	0	0 (0 - 4)	1	17 (3 – 56)
<i>Ehrlichia canis</i>	0	0 (0 - 6)	1	1 (0 - 6)	0	0 (0 – 39)
<i>Hepatozoon canis</i>	1	1 (0 - 6)	0	0 (0 - 4)	0	0 (0 – 39)
Haemotropic <i>Mycoplasma</i> spp.	2	3 (1 - 12)	1	0 (0 - 4)	0	0 (0 – 39)
Mixed infection						
<i>A. platys</i> + <i>E. canis</i>	0	0 (0 - 6)	1	1 (0 - 6)	0	0 (0 – 39)
<i>A. platys</i> + <i>B. vogeli</i>	0	0 (0 - 6)	0	0 (0 - 4)	1	17 (3 – 56)
Total positive	6	10 (5 - 20)	3	3 (1 - 9)	2	33 (10 – 70)
Total negative	53	90 (80 - 95)	96	97 (91 - 99)	4	67 (30 – 90)

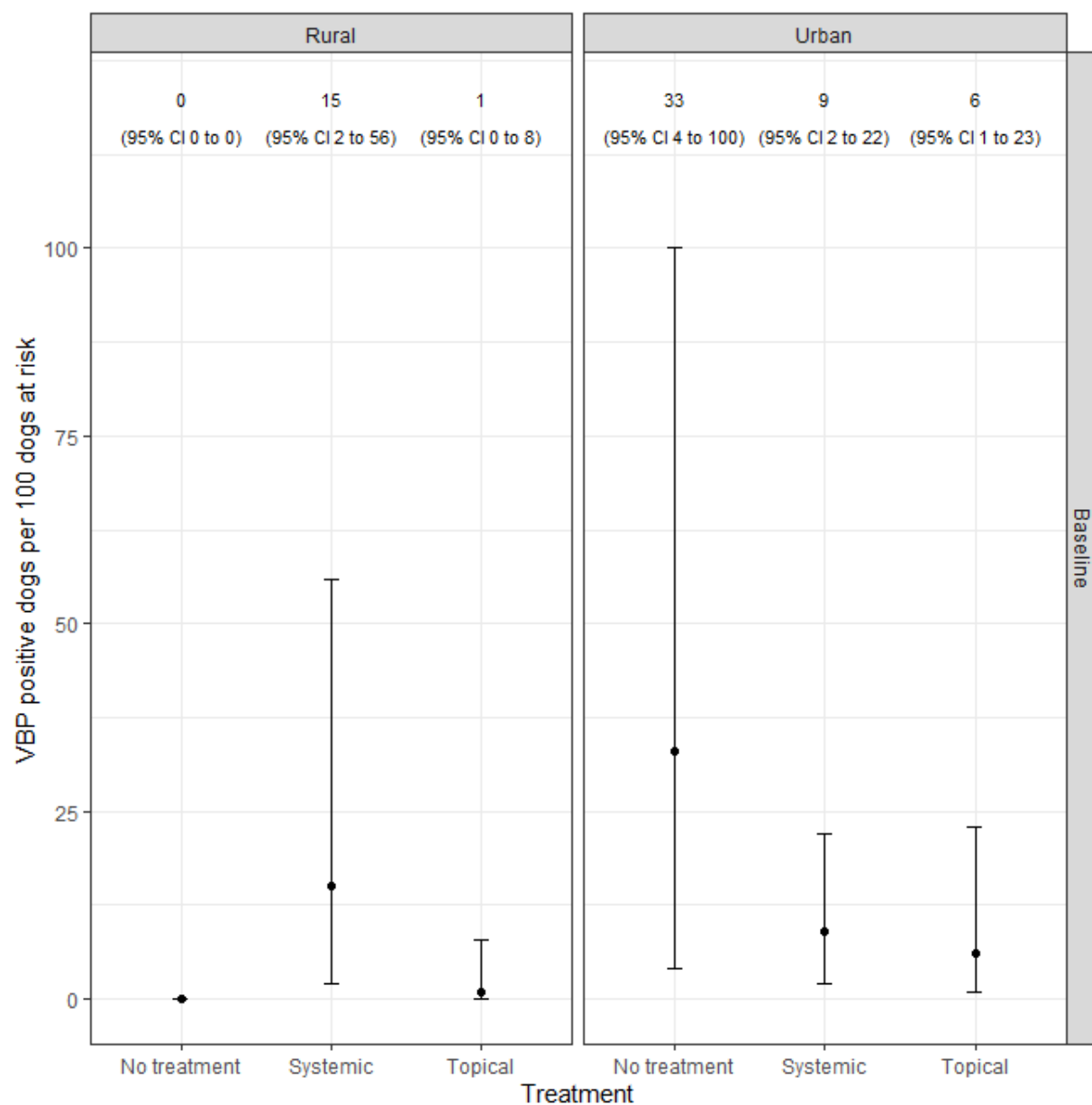


Figure 7.2. Error bar plot showing crude (unadjusted) vector-borne pathogen (VBP) apparent prevalence estimates as assessed by qPCR for dogs treated with systemic, topical and no ectoparasiticide products, conditioned by environment (urban, rural). VBP prevalence is expressed as the number of VBP positive dogs per 100 dogs at risk. The error bars show the lower and upper bounds of the 95% confidence interval for the VBP prevalence estimates.

More dogs were serologically tested than those that underwent qPCR analysis and hence a total of 180 dogs were comparable at the baseline assessment for SNAP 4Dx testing ($n = 115$ on topical treatments and $n = 59$ on systemic treatments and $n = 6$ on no ectoparasiticides). Serology identified 22 (37%, 95% CI 26% to 50%) TBP exposed dogs on systemic products and 16 (14%, 95% CI 9% to 21%) on topical products (**Table 7.2**). Dogs that were on no ectoparasiticides had higher relative levels of TBP exposure with four of six dogs serologically positive. Positivity was only found for the pathogens *A. platys* and *E. canis* across all the dogs tested, see **Table 7.2** for details of the apparent prevalence of different TBPs identified.

Figure 7.3 shows the crude apparent TBP serological exposure prevalence estimates for systemic, topical and no ectoparasiticide treated dogs, conditioned by the environment in which the dog was kept (urban, rural). After adjusting for the effect of environment the apparent prevalence of TBP-exposure was 24 (95% CI 15 to 42) per 100 dogs at risk for those systemically treated, compared with an apparent prevalence of 16 (95% CI 8 to 28) per 100 dogs at risk for those topically treated. For dogs on no ectoparasiticide product the apparent prevalence of TBP exposure was 33 (95% CI 9 to 85) per 100 dogs at risk.

Table 7.2. Between group comparison of the number of serologically positive dogs (+) and apparent pathogen prevalence (%) with 95% CI found for single, mixed and overall infections, as detected by SNAP 4Dx Plus Test at study baseline. No dogs were *B. burgdorferi* or *D. immitis* positive. For dogs in the topical group more dogs were serologically tested than analysed by qPCR, hence the difference in sample size from **Table 7.1.**

Pathogen	Systemic Products (n = 59)		Topical Products (n = 115)		No Ectoparasiticide (n = 6)	
	+	% (CI)	+	% (CI)	+	% (CI)
Single & mixed infection						
<i>Anaplasma</i> spp.	17	29 (19 – 41)	11	10 (5 – 16)	2	33 (10 – 70)
<i>Ehrlichia</i> spp.	14	24 (15 – 36)	8	7 (4 – 13)	3	50 (19 – 81)
Mixed infection						
<i>Anaplasma</i> + <i>Ehrlichia</i>	9	15 (8 – 27)	3	3 (1 – 7)	1	17 (3 – 56)
Total positive	22	37 (26 – 50)	16	14 (9 – 21)	4	67 (30 – 90)
Total negative	37	63 (50 – 74)	99	86 (79 – 91)	2	33 (10 – 70)

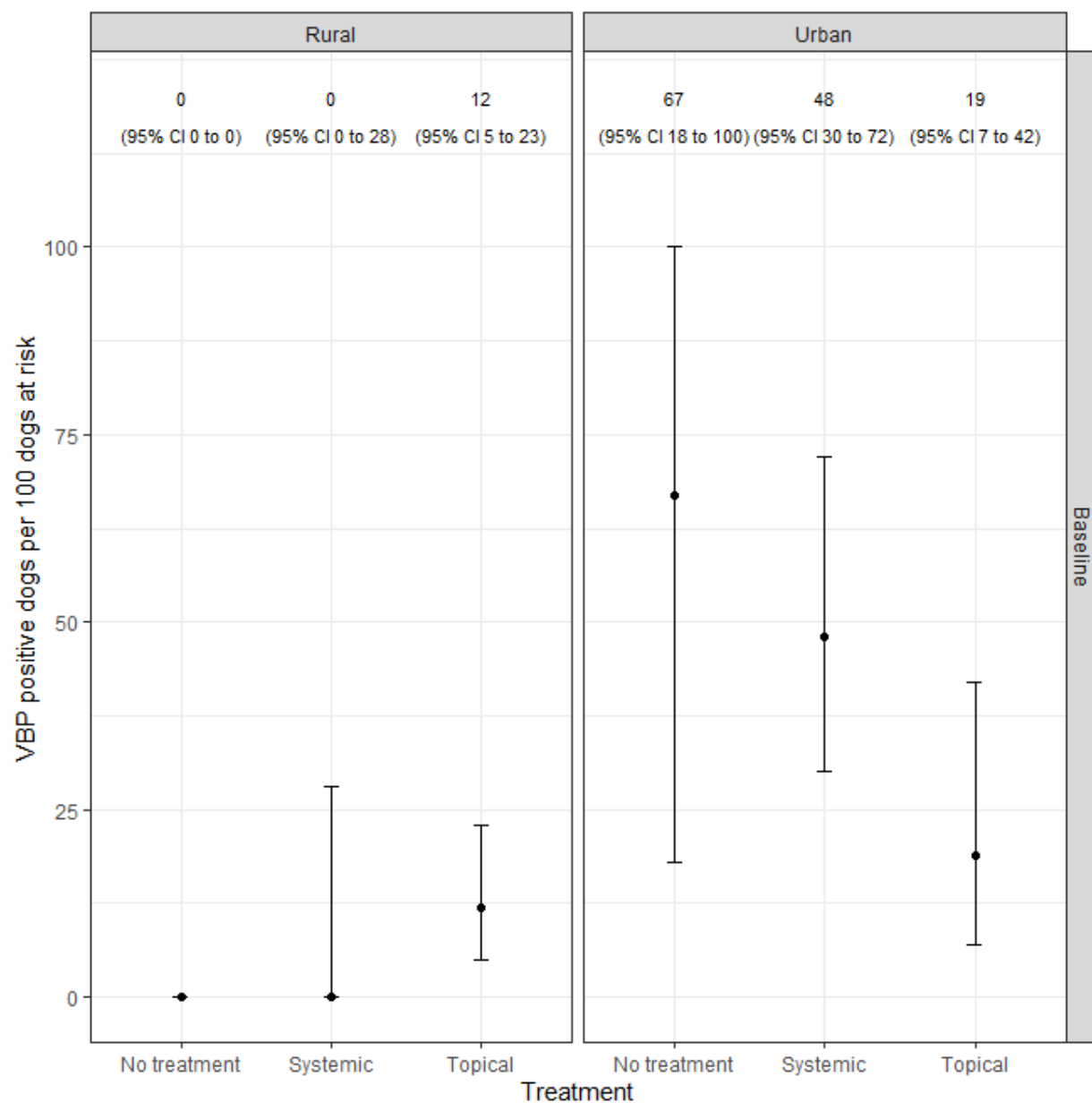


Figure 7.3. Error bar plot showing crude (unadjusted) apparent vector-borne pathogen (VBP) exposure prevalence estimates as assessed by serology for dogs treated with systemic, topical and no ectoparasiticide products, conditioned by environment (urban, rural). VBP prevalence is expressed as the number of VBP exposed dogs per 100 dogs at risk. The error bars show the lower and upper bounds of the 95% confidence interval for the VBP prevalence estimates.

7.4.2. Prospective community trial comparing two topical ectoparasiticides: Results at 6-months

A total of 197 dogs met the eligibility criteria and were enrolled into the prospective community trial which was greater than the minimum required ($n = 166$) to accommodate drop-out of study subjects. The number of dogs that completed the prospective community trial to the six-month time point was 169 ($n = 85$ in the CP group and $n = 84$ in the IVP group) i.e. 28 dogs dropped out due to sickness unrelated to the trial, mortality unrelated to the trial or clients having left the study locations with their animals. There was no statistically significant difference in the number of dogs that dropped out of the community trial in the IVP and CP groups at the $P < 0.05$ level as determined by a chi-squared test (χ^2 test statistic 0.144, n 197 df 1; $P = 0.704$).

The composition of dogs enrolled into the prospective community trial by age and sex was similar for both treatment groups (**Table 7.3**) while there were more purebred dogs in the CP group compared with the IVP group due to CMAC favoring the use of Belgian Malinois to conduct landmine decontamination. In the IVP group dog breeds were more varied including German Shepherds, Chihuahuas, Golden Retrievers, Bichon Frise, Poodles, Dachshunds, Cocker Spaniels, Labradors and Beagles. There were greater numbers of neutered dogs in the IVP group compared with the CP group (**Table 7.3**). Dogs in the IVP group were from Phnom Penh (PP, 81%) and Siem Reap (SR, 19%) while dogs in the CP group were from Kampong Chhnang (KC, 80%), Battambang (BB, 15%) and PP (5%).

At the 6-month time point two dogs in the IVP group were found with ticks on them (2%; 95% CI 0.6% to 8%), whilst one dog in the CP group was found with a tick (1%; 95% CI 0.2% to 6%). However, none of these ticks were found attached or engorged.

At the 6-month follow-up one IVP group dog was found to have acquired a new TBP infection with *A. platys* by qPCR (1%; 95% CI 0.2% to 6%), whilst two CP dogs were found newly TBP positive (2%; 95% CI 0.6% to 8%), of these one was positive to *A. platys* and one to *E. canis* (both 1%; 95% CI 0.2% to 6%). All dogs initially found TBP positive or anaemic at baseline that were treated for a bacterial TBP infection were found TBP negative at 6-months, indicating successful treatment with doxycycline. TBP blocking

efficacy of the IVP, as defined by the equation in the 'Methods', was 49% and therefore not deemed substantially more effective at blocking TBPs than the fipronil used in the CP group as defined by Marchiondo et al. (2013). In contrast, the ectoparasite prevention ability was 100% for the IVP, given as no ticks were found attached or engorged in either group.

Figure 7.4 shows the crude TBP incidence rate estimates for IVP and CP treated dogs, conditioned by the environment in which the dog was kept (urban, rural). After adjusting for the effect of environment there were 8 (95% CI 1 to 30) TBP-positive CP dogs per 100 dog-years at risk compared with 1 (95% CI 0 to 25) TBP-positive IVP dogs per 100 dog-years at risk. CP-treated dogs were 6.5 (95% CI 0.6 to 71) times more likely to be TBP-positive compared with IVP-treated dogs. We estimate that 13 (95% CI 11 to 92) dogs needed to be treated with the IVP (Seresto®) to avoid one case of TBP.

Table 7.3. Relative composition of canine sex, age, breed and neutering status in the IVP (Seresto®) group and CP (DeTick) group. Local dog breeds refer to mongrels that do not have obvious recognisable characteristics of a common dog breed.

Sex	IVP Group	CP Group		Neutered	IVP Group	CP Group
Female	47 (56%)	36 (42%)		Yes	30 (36%)	0 (0%)
Male	37 (44%)	45 (53%)		No	19 (23%)	71 (84%)
Unreported	0 (0%)	4 (5%)		Unreported	35 (41%)	14 (16%)
Age	IVP Group	CP Group		Breed	IVP Group	CP Group
< 6 Months	6 (7%)	0 (0%)		Local	37 (44%)	0 (0%)
> 6 Months < 12 Months	16 (19%)	13 (15%)		Cross	13 (15%)	14 (16%)
> 12 Months	52 (62%)	69 (81%)		Pure	33 (40%)	69 (81%)
Unreported	10 (12%)	3 (4%)		Unreported	1 (1%)	2 (2%)

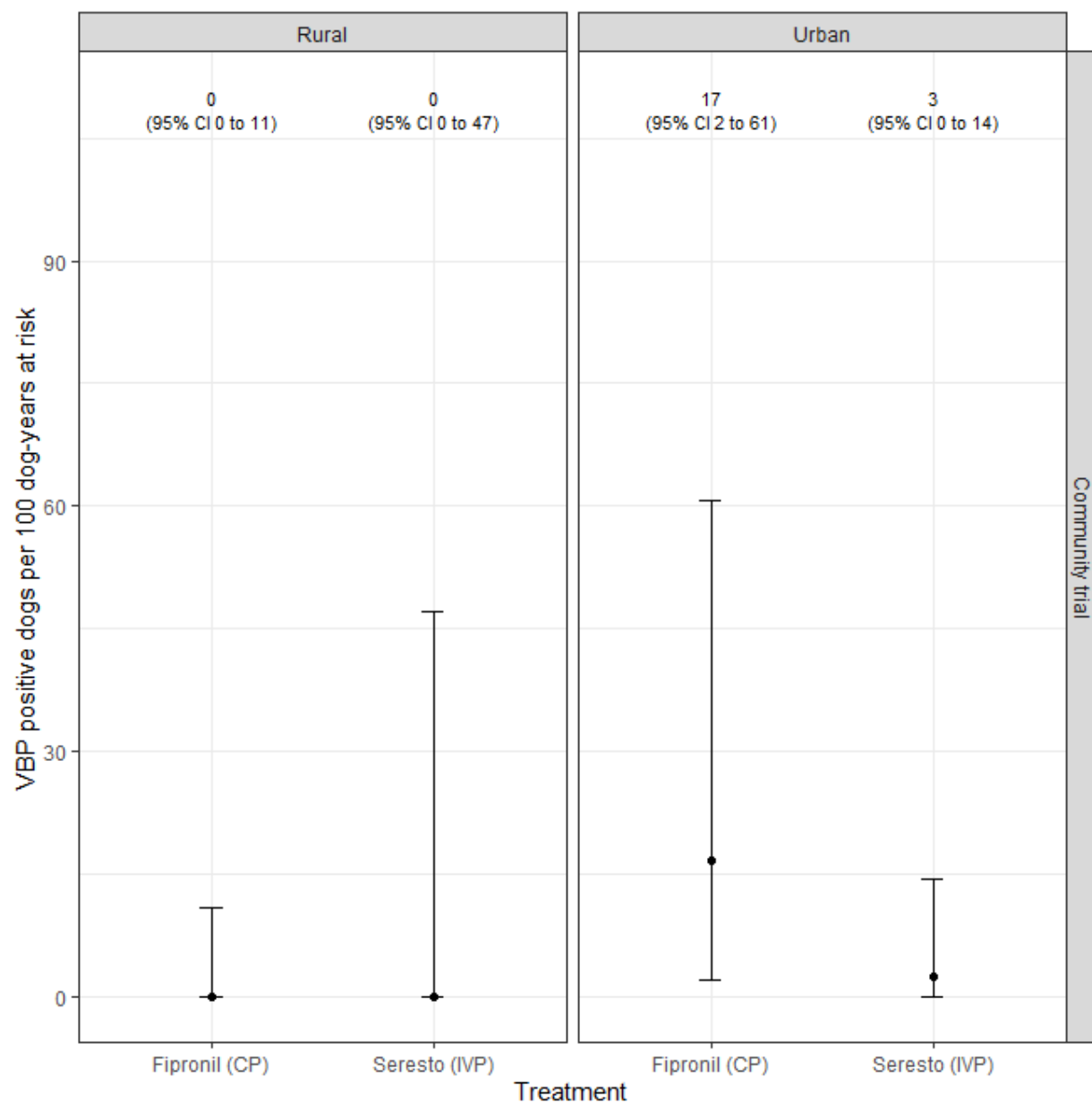


Figure 7.4. Error bar plot showing the crude (unadjusted) vector-borne pathogen (VBP) incidence rate estimates, as assessed by qPCR, for dogs treated with CP (fipronil) and IVP (Seresto® collars), conditioned by environment (urban, rural). VBP incidence rate is expressed as the number of VBP positive dogs per 100 dog-years at risk. The error bars show the lower and upper bounds of the 95% confidence interval for the VBP incidence rate estimates.

7.5. Discussion

7.5.1. Baseline assessment comparing topical and systemic ectoparasiticides

The collection of canine blood samples and relevant metadata at enrolment for this study provided us with the data necessary to complete a baseline assessment of apparent TBP prevalence in dogs using either a topical or systemic ectoparasiticide prior to the study's commencement. At enrolment, 115 dogs (64%) were on a topical ectoparasiticide, which acts from the dog's dermis and hair to prevent ectoparasite feeding, whilst 59 dogs (33%) were on a systemic ectoparasiticide which kills ectoparasites only after they have begun a bloodmeal and six dogs (3%) were on no ectoparasiticide.

We used three separate analyses to compare the effect of topical, systemic and no ectoparasiticide product used with rates of TBP positivity as determined by qPCR, whilst also adjusting for the potential impact of canine environment. An error bar plot (**Figure 8.2**) showed that pathogen positivity was higher in dogs using systemic products, particularly if they were from a rural environment where TBP infection pressure may be higher. Moreover, dogs on no ectoparasiticide had higher rates of TBP positivity than those on both systemic and topical treatments, demonstrating that any ectoparasiticide is better than none. Nonetheless, substantial overlap in confidence intervals between product types and no product meant that differences in TBP positivity could not be demonstrated as statistically significant at the $P < 0.05$ level. Such results were supported by our calculations of direct adjustment for apparent TBP prevalence between groups that also factored in the impact of canine environment. These identified TBP positivity at 12 dogs per 100 at risk using systemic products compared to 4 dogs per 100 at risk for topically treated dogs and 17 dogs per 100 at risk for those on no ectoparasiticide. Overall, systemically treated dogs were 3.0 (95% CI 0.67 to 14) times more likely to be TBP-positive compared to topically treated ones. Such results demonstrate the TBP transmission blocking impact of topical products that do not permit ectoparasite blood-feeding. Additionally, the NNTB calculations ascertained that after accounting for the effect of environment, 12 dogs would need to be treated with a topical product to avoid one case of TBP contraction, further emphasising the benefits of ectoparasiticides that act from the canine dermis and hair.

The comparison of rates of TBP-exposed dogs between those in the topical, systemic and no ectoparasiticide groups, as determined by serology, mirrored the results gleaned through qPCR analysis of current infections. Dogs on systemic and no ectoparasiticide products in urban environments had higher levels of TBP-exposure than those on topical products, although this same pattern was not reflected in the dogs tested in rural environments (**Figure 7.3**). However, when direct adjusted values were calculated that factor in the potentially confounding impact of dog environment, the same trend in product performance was identified as previously, showing that dogs on systemic products were more likely to be exposed (24 per 100 dogs at risk) than those on topical products (16 per 100 dogs at risk). Once more, dogs on no ectoparasiticide product had the highest levels of TBP exposure (33 per 100 dogs at risk).

Despite these results, the small sample size of dogs for which ectoparasiticide product data was available, in conjunction with the low numbers of TBP positive and exposed dogs, meant that confidence intervals for the direct adjustment values were relatively high with substantial overlap, making unequivocal conclusions hard to draw. A larger study, specifically exploring and comparing prevention of TBP transmission via the performance of topical vs systemic products, alongside those on no ectoparasiticide, would need to be conducted to more conclusively demonstrate a difference between these product types.

Even though our limited data may only be indicative of a difference in TBP blocking impact of topical, systemic and no ectoparasiticide products, these tentative conclusions are supported by existing research. Jongejan et al. (2016) compared the *E. canis* blocking impact of two systemic products (afoxolaner and fluralaner) and one topical product (a spot-on combination of permethrin and imidacloprid) by *R. sanguineus* ticks in a laboratory controlled comparative efficacy study. Whilst the topical product consistently demonstrated a higher speed of kill and anti-attachment effect on ticks, its most valuable impact was its 100% blocking efficacy on *E. canis* transmission, whilst the blocking efficacy for the systemic product afoxolaner was 33.3% and for fluralaner was 66.7%. The authors concluded that speed of kill of the systemic products tested was not rapid enough to prevent *E. canis* transmission, which begins in under eight hours of tick attachment but may be as rapid as just a couple of hours (Fourie et al., 2013c; Jongejan et al., 2016).

Additionally, a study by Varloud et al. (2018a) identified similar results when comparing the blocking efficacy of transmission of *Babesia canis* by *Dermacentor reticulatus* ticks between systemic and topical ectoparasiticides. In this study the orally administered systemic product (fluralaner) offered no protection from *B. canis* transmission whilst dogs on the topical product (a combination of dinotefuran, permethrin, pyriproxyfen) had a five times lower risk of contracting this infection (Varloud et al., 2018a). Nonetheless, similar research investigating the *B. canis* transmission blocking efficacy of afoxolaner and fluralaner have found it to be 100% effective at preventing contraction of this TBP during challenge trials (Beugnet et al., 2014; Taenzler et al., 2015). Differences in results between these studies may be because in the Varloud et al. (2018a) investigation *D. reticulatus* ticks could feed on donor sheep and become engorged prior to infestation on dogs, whilst unengorged ticks were used in the latter two studies. Tick engorgement may speed up TBP transmission with pre-engorged *D. reticulatus* having been demonstrated to transmit *B. canis* within eight hours of feeding commencement (Varloud et al., 2018b). Therefore, despite multiple factors impacting canine TBP transmission times, an ectoparasiticide approach that prevents ectoparasite feeding may always be optimal, as it limits any risk of TBP transmission, unlike systemic products.

7.5.2. Prospective community trial comparing two topical ectoparasiticides

The data from the first six months of our prospective community trial comparing the TBP-blocking efficacy of Seresto® in the IVP group and fipronil in the CP group found both products to be highly effective at preventing the contraction of canine TBPs. Of the 169 dogs from which data was collectable at 6-months, just 2% in the CP group acquired a new TBP, whilst 1% contracted a new TBP in the IVP group. Given the high efficacy of fipronil in the CP group, the relative TBP blocking efficacy of Seresto® was just 49% as defined by Marchiondo et al. (2013). Prior research supports data found in the present study, that fipronil is a highly effective product at preventing ectoparasite infestation, in species such as *R. sanguineus*, *D. reticulatus*, *Ctenocephalides felis* and *Trichodectes canis* as well as blocking the pathogens they transmit, for example *E. canis* in field settings (Davoust et al., 2003; Kužner et al., 2013). Fipronil has also been demonstrated

to cause salivary gland degeneration and prevention of engorgement in some tick species, thereby providing an additional mechanism by which TBP transmission is blocked (Pereira et al., 2009). Regarding prevention of ectoparasite infestation, these two products performed equally well, with no dogs in either group being found with ticks attached nor engorged at the 6-month time point.

Additionally, the direct adjusted values that took into consideration the confounding effect of environment at the six-month time point demonstrated a higher incidence of newly contracted TBPs in the CP group at 8% compared to 1% in the IVP group. However, the large and substantially overlapping confidence intervals ascertained for these direct adjusted values mean that no conclusions can be drawn regarding a superior or inferior TBP-blocking capacity for either product. A larger sample size and further sampling time points as this study progresses into its final six to twelve-month period may develop this initial difference between both groups into a more substantive one.

Within the prospective community trial, the number needed to treat for benefit (NNTB) was 13, meaning these many dogs would need to be treated with the IVP (Seresto®) to see one dog benefit and not contract a TBP relative to the CP group. However, the confidence interval for the NNTB was again high, ranging up to 92. Given the modest price difference of $\$3 \times 8 \text{ months} = \$24 \text{ USD}$ for DeTick vs $\$30 \text{ USD}$ for an 8-month duration Seresto® collar, the NNTB value calculated may mean the small benefit in reduced TBP incidence that Seresto® confers is worth the slightly higher ~\$6 USD price, depending on the economic situation of the purchaser. Nonetheless, the remaining data from the final 6-months of this study is needed to fully assess whether the IVP product's TBP protection is maintained right until the end of its purported eight-month window of efficacy.

Additional key information obtained through our 6-month time point data was evidence of successful treatment of dogs that were *A. platys* and/or *E. canis* positive at baseline. Three dogs were found qPCR positive to *A. platys* at baseline and another was found coinfecting with *A. platys* and *E. canis*, all four of these were treated with doxycycline at 10mg/kg SID for the relevant course duration and were found negative for these pathogens at 6-months. Prior studies have found doxycycline to be highly efficacious at

treating canine *A. platys* infections, whilst the data for *E. canis* treatment is more equivocal (Gaunt et al., 2010; Mylonakis et al., 2019). There is some evidence of doxycycline failing to achieve complete cure, with molecular evidence of maintained *E. canis* DNA presence after a 4-week period of doxycycline treatment in some dogs (Mylonakis et al., 2019). Nevertheless, as this trial progresses, later time point data may shed more light on the efficacy of our 4-week doxycycline treatment regime at achieving a curative *E. canis* outcome.

This study has some limitations which may have impacted the 6-month results thus far analysed and potentially reduced the differences observed in performance between the IVP and CP products. Possibly the most significant of these is that numerous canines in the IVP group ripped off or tore their Seresto® collars at some stage in the trial. Within the IVP group 13 dogs (15%) broke their collars before 6-months, with average collar replacement times taking between 1 - 2 days. The IVP group dog found *A. platys* positive at six months was one of these 13 dogs that had torn off their collar. Therefore, whether such breakages may have had an impact on the effectiveness of the Seresto® collars, possibly by reducing the amount of active chemical on the dog's skin for a few days and thereby reducing the collars ectoparasite repellency is difficult to discern. This also highlights the advantage of the topical fipronil used in the CP group, which doesn't have the issue of potential removal by the dog, although reapplication must be much more frequent than for the Seresto® collars at one vs eight months.

Additional factors that may have impacted the efficacy of the Seresto® collars in the IVP group was the local Cambodian climate, that features a distinct wet monsoon season from May to September (Bairagi et al., 2020). Under conditions of regular rain exposure Seresto® collars have been demonstrated to show a significantly reduced efficacy at preventing tick and flea infestation, whilst topical fipronil does not show such a waning of efficacy under the same conditions (Beugnet et al., 2018; Halos et al., 2014). Taking this into consideration, the tropical field conditions our trial subjected the Seresto® collars to may have meant they were not working optimally and therefore reduced their TBP-blocking efficacy when compared to topical fipronil. Nevertheless, such data is critically important as the temperate climactic conditions Seresto® has been substantially tested in

before may provide performance results that do not directly translate to tropical environment contexts (Brianti et al., 2013; Dantas-Torres et al., 2013; Stanneck et al., 2012c).

7.6. Conclusions

We have demonstrated firstly that topical ectoparasiticides demonstrate an advantage over systemic isoxazoline products at reducing the contraction of canine TBPs in Cambodia, within both rural and urban contexts. Such findings are critical to inform local veterinarians in the region that may commonly use systemic products, believing them to be effective at preventing TBP transmission to their clients' dogs. Our data will be directly translated and disseminated to local veterinary practitioners, hopefully with the outcome of changing ectoparasiticide practices in the country and reducing the amount of TBP circulation within the country. These findings are non-trivial, given the substantial pathogenicity of many of the TBPs that systemic products are unable to block, particularly *E. canis* that frequently generates fatal canine disease (Little, 2010; Mylonakis et al., 2019). Secondly, our prospective community trial data to date, does not show any clear benefit of Seresto® collars over monthly topical fipronil (DeTick) at blocking TBP transmission, with both products being highly effective. Given the slight benefit in regard to affordability and availability of DeTick when compared to Seresto® this is important information, as it means that an economical and readily available product in Cambodia can be used by local people to protect and improve canine health, thereby reducing the TBP burden of disease in pet and working mine detection dogs.

7.7. References

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Chapter 8 - General Discussion

8.1. Thesis aims revisited

The Asia-Pacific spans a diverse range of nations with a gamut of different cultures, religions and human interaction types with livestock and wildlife (Erian et al., 2019; Irwin and Jefferies, 2004). Nonetheless, it is unified, particularly in Southeast Asia, by a typically tropical and humid climate providing an environment conducive for a great diversity of ectoparasites and the VBPs they transmit (Irwin and Jefferies, 2004; Traub et al., 2015). Such factors are exacerbated in some countries by large populations of free-roaming dogs and in poorer nations frequently limited access to veterinary care, creating an overarching picture of a region highly endemic for diverse canine VBPs (Colella et al., 2020; Irwin and Jefferies, 2004). Many VBPs here are also zoonotic and hence garnering information on their prevalence and biodiversity is critical from a public health standpoint as well (Colella et al., 2020; Otranto et al., 2009).

Considering these points, in this thesis I set out to foremostly explore and characterise the VBP communities of dogs using novel NGS-based molecular techniques in the Southeast Asian nation of Cambodia, a country and region that has historically received very little attention with regard to canine VBP research. Next, using these data, the most common canine VBPs in the region were assessed, a multiplex qPCR developed to detect them and this tool employed to assess the efficacy of a 10.0% imidacloprid/4.5% flumethrin collar (Seresto®, Bayer) at blocking VBP transmission in a community field trial.

Addressing aim (1) in Chapters 2, 3 and 4 I designed, tested, optimised and validated a novel NGS metabarcoding method capable of holistically characterising the entire bacterial, apicomplexan and kinetoplastid haemoparasite communities from dogs. At the time of Chapter 2 and 3 publication this was the first study to conduct such methods using samples from dogs, whilst today there is still only a handful of studies that have conducted such characterisation in animals, with few benchmarking the sensitivity of such methods against traditional molecular diagnostics. In Chapter 4 I further improved on my initial bacterial metabarcoding protocol by elucidating an optimal DNA extraction kit and developing a host specific mitochondrial DNA (mtDNA) blocking primer, with both processes significantly increasing the diagnostic sensitivity of the assay.

Chapter 5 addressed aim (2), using my optimised NGS method to not only make the first reports of *Anaplasma platys*, *Trypanosoma evansi* and *Bartonella clarridgeiae* infection in dogs from Cambodia but also identify canine VBPs as hyperendemic, with 62% of dogs infected with at least one VBP. Additionally, ectoparasite infestation was very common with 73% of dogs infested with ticks, fleas and lice. Canine VBP prevalence was not uniform across Cambodia, with a higher prevalence of infection in Battambang and a lower prevalence in Tbong Khmum. Overall, my results were similar to the one other molecular canine VBP study done in Cambodia prior which found 71.3% of dogs infected (Inpankaew et al., 2016), whilst my results showed higher VBP prevalence than that found previously in Indonesia, Malaysia, the Philippines, Thailand and Vietnam (Colella et al., 2020; Corales et al., 2014; Kaewmongkol et al., 2017; Mokhtar et al., 2013; Prakash et al., 2018).

Statistical analysis for elucidation of risk indicators to predict the probability of VBP exposure in canines found that variables such as the presence of ectoparasites, low PCV, abnormal mucous membrane condition and elevated total protein were informative. Such information is invaluable for clinicians working in such regions, where using this information may allow them to make quick assessments on the probability that a given dog is VBP exposed. In addition, the use of my unbiased NGS-based characterisation of canine haemoparasites also highlighted a large diversity of putatively pathogenic organisms, presenting a wealth of future avenues to explore with regard to novel and emerging canine disease in the region.

Data accrued through Chapter 5 informed my decision to develop a highly sensitive and specific Taq-Man multiplex qPCR to detect the canine VBPs *A. platys*, *Babesia vogeli*, *Ehrlichia canis*, *Hepatozoon canis* and haemotropic *Mycoplasma* spp., thus addressing aim (3) in Chapter 6. The VBP *Babesia gibsoni* was also included as a qPCR target given its high pathogenicity in dogs and that it has been detected in Australia, China and Singapore before, hence giving my assay greater utility to be used across the Asia-Pacific (Colella et al., 2020; Jefferies et al., 2007; Muhlnickel et al., 2002).

Finally aim (4) was informed by an ongoing community field trial to compare the chemoprophylactic efficacy of Seresto® vs topical fipronil for preventing VBP

transmission, presented in Chapter 7. The results of the first six months of this trial have demonstrated that both products are equally efficacious at preventing the contraction of canine VBPs, with only 1% to 2% of dogs in each treatment group becoming newly infected. Additionally, baseline data gathered during this trial suggests that the use of systemic isoxazoline products confers less protection against the contraction of canine VBPs when compared to topical products, such as fipronil and Seresto®. These data are critically important for veterinarians working in the Asia-Pacific given the region's high VBP transmission pressure, popular use of systemic ectoparasiticides and insidious effects the endemic VBPs here have on canines.

8.2. Recommendations for canine VBP control in the Asia-Pacific

The high levels of canine VBP prevalence identified in Cambodia through this thesis and through the limited studies by other authors in surrounding countries of the Asia-Pacific, highlights the need for effective management of canine VBP risk as well as control in the region. Veterinarians must be kept up to date of novel findings regarding canine VBP prevalence, with a particular focus on the identification of potentially zoonotic pathogens, such as *B. clarridgeiae*, *Rickettsia* and *Leptospira* that were identified in my cross-sectional survey. This information must be passed on from practicing veterinarians to their clients, to warn of the potential dangers to their pets and themselves of circulating VBPs in conjunction with information on measures to reduce the chance of contracting VBPs (Braks et al., 2014; Traub et al., 2015). Veterinarians should be cognizant of the signs and symptoms of canine and zoonotic VBPs, particularly given that they themselves are at a significantly higher risk of contracting zoonoses through their work, as demonstrated by various case studies and higher than average seroprevalence to VBPs, such as *Rickettsia felis* and *Rickettsia typhi* (Breitschwerdt et al., 2008, 2007; Maggi et al., 2013; Teoh et al., 2017).

Clients also need to be informed of the benefits of contemporary and effective topical ectoparasiticide products such as Seresto® collars, herein tested, as well as the risks of systemic isoxazoline medications that do not prevent vector feeding and thereby permit VBP transmission (Jongejan et al., 2016). This is especially pertinent given the high use

of isoxazolines for dogs in the Asia-Pacific. Other management strategies that should be communicated to dog owners is the use of environmental modification to reduce risk. Simple measures such as cutting grass and removing leaf litter from gardens, checking kennels for the presence of ticks and sealing of cracks and crevices where tick species like *Rhipicephalus sanguineus* and *Rhipicephalus linnaei* commonly reside, as well as the use of outdoor ectoparasiticide sprays are all likely to be beneficial in reducing tick exposure (Dantas-Torres et al., 2013; Gray et al., 2013; Otranto and Wall, 2008; Šlapeta et al., 2021). In addition, limiting the amount of contact pet and mine detection dogs have with stray animals and wildlife as well as reducing the time spent in areas frequented by stray dogs may reduce the chance of ectoparasite infestation (Otranto and Wall, 2008).

Nonetheless, individual VBP prevention measures alone will not be sufficient to tackle the issue of canine VBP prevention and control, with national and local government initiatives warranted as well. Key actions need to be taken such as free-roaming dog control, spay-neuter and release programs and educating owners to prevent their animals from wandering (Otranto and Wall, 2008). All of such actions will reduce the canine VBP reservoir formed by large vagrant dog populations and reduce the risk to pet and mine detection dogs (Güven et al., 2017; Jimenez-Coello et al., 2010). These measures could work alongside existing rabies elimination programs that are common in the Asia-Pacific, where mass vaccination and neutering are conducted, hence improving the public health reach of such initiatives (Rupprecht et al., 2020; Sor et al., 2018). To maximise the effectiveness of such programs there also needs to be comprehension and accommodation of local cultural and religious practices, giving communities a clear understanding and voice on the programs being implemented within them (Rupprecht et al., 2020).

Finally, the studies presented in this thesis have relevance to the risk management and prevention of introduction of novel VBPs to non-endemic countries. Exportation of pets and livestock present a serious risk of seeding canine VBPs into previously naïve countries, for example the UK's Pet Travel Scheme introduced *Babesia* spp., *Ehrlichia* spp. and *Leishmania* spp. for the first time to Great Britain via infected dogs (Beugnet and Marié, 2009; Hii et al., 2012; Johnson and Fooks, 2014; Shapiro et al., 2017). In addition,

the recent discovery of *E. canis* in Australia, a country that was previously deemed free of this lethal canine pathogen may be due to sub-optimal biosecurity practices, although at this stage whether *E. canis* was recently introduced from imported dogs is not yet known (Australian Government, 2020a, 2020b). Regardless, the importance of strict bio-surveillance and biosecurity measures to restrict human-mediated spread of VBPs has never been more urgent given the high rates of animal translocation that occur today (Chomel, 2011; Johnson and Fooks, 2014; Traub et al., 2015). Therefore, given the high incidence of canine VBP positivity across Cambodia, local and importing governments may need to impose strict screening procedures for dogs destined to leave the country, particularly those within mine detection dog programs which are frequently moved between high-risk countries. At the same time, receiving countries will need to test and quarantine canine arrivals to ensure no VBPs are inadvertently introduced, particularly given the existence of ubiquitous *R. sanguineus* and *R. linnaei* vectors that could transmit them (Fèvre et al., 2006; Gray et al., 2013; Johnson and Fooks, 2014; Šlapeta et al., 2021).

8.3. The canine blood microbiome

One of the initial aims of my PhD project was to use 16S rRNA-targeting NGS methods to characterise bacterial VBPs from dog blood, however, an unexpected consequence of this work was that employing such methods also gave us substantial insight into the blood microbiome of canines. It became apparent that the huge diversity of bacterial sequences being detected during each MiSeq run was providing us with much more information than VBP presence or absence data alone. The diversity of unique sequences detected, or Amplicon Sequence Variants (ASVs), peaked at 15,139 in one run, with an average of 114 ASVs detected per sample and as many as 1,532 ASVs in the most sequence-diverse sample identified. Such diversity coupled with the large numbers of reads per sample (reaching averages of 110,944 per sample in the batch with the highest output) generated phenomenally varied and complex datasets from which key data relevant to my research question had to be extracted.

Exploration into the microbiomes of blood provides us with valuable information about the microbiomes of other body sites as well, such as the gut, skin and mouth, demonstrating the close and dynamic relationship between microbiota across mammalian bodies (Mohamed et al., 2021; Scarsella et al., 2020). There is mounting evidence of bacterial translocation from the gastrointestinal tract, as well as movement of bacteria from the skin and oral cavity into the bloodstream, either through wounds, ectoparasite bites or other mechanisms (Mohamed et al., 2021; Scarsella et al., 2020). From my own data many ASVs were classified with a bacterial genus in addition to the label ‘canine oral microbiome’ identifying that they had first been detected through microbiome experiments. Insights into other canine microbiomes apart from that in the bloodstream are valuable, for example, the bacterial flora identified from the mouths of canines can be directly linked to zoonotic infections caused by dog bites e.g. by *Pasteurella multocida* (Razali et al., 2020).

Tackling such extensive microbiome datasets involves identifying key taxonomic species and genera from known VBP groups, followed by assessing genera with known pathogenic, or putatively pathogenic representatives. This secondary assessment of putative pathogens was more challenging given that many ASVs were only classified to genus level and that most bacterial genera consist of both parasitic and environmental members (Kim et al., 2017). Therefore, whether the ASV in question pertained to the former or latter category typically needed further experimental corroboration, e.g. via use of cPCR to amplify larger barcoding regions (Takhampunya et al., 2019). When assessing these putative pathogens, the employment of rigorous negative controls proved invaluable, given that they highlighted the bacteria introduced at the DNA extraction and DNA amplification stages. Diversity of sequences found in DNA extraction controls were as high as 100 unique ASVs in some cases, whilst ‘no DNA template’ negative controls had up to 20 ASVs. Reporting of negative and positive control data is essential to permit bioinformatic cross-validation of experimental results and to maintain transparency of data processing and analysis (Kim et al., 2017). In the current research all such control data was made publicly available in the NCBI BioProject database and was explicitly reported in Chapter 4.

Using information on the common bacterial genera found in negative controls was important in aiding decision making as to which putative pathogen sequences should be further investigated. This was particularly important given that blood is a low microbial biomass sample type with quantities of bacterial DNA that can be comparable to those found in DNA extraction negative controls (Eisenhofer et al., 2019; Glassing et al., 2016). Due to this, the impacts of contaminant DNA can have a greater proportional effect, with a high number of contaminant reads relative to true pathogen reads (Eisenhofer et al., 2019; Karstens et al., 2019). Nonetheless, the processing of such negative control data still has to be balanced by the possibility that some contaminant species also share a genus with important pathogens (Karstens et al., 2019). For example, *Haemophilus* spp. can be common environmental contaminants in microbiome experiments but also have canine pathogenic species (Eisenhofer et al., 2019; Razali et al., 2020; Yu et al., 2020). Moreover, simple subtraction of taxa found in negative controls from the overall dataset has been demonstrated to be a crude method that loses relevant biological information (Karstens et al., 2019). Instead, a nuanced approach that bioinformatically processes negative control data to proportionally remove contaminant sequences from the overall dataset may be more appropriate (Karstens et al., 2019).

The challenges of correctly identifying contaminant sequences from those truly in the biological sample was further exacerbated by a dearth in prior research exploring the canine microbiome. Substantial research into the gastrointestinal microbiome of mammals (Barko et al., 2018; Fliegerova et al., 2014; Schneeberger et al., 2016; Velásquez-Mejía et al., 2018), the human circulating blood microbiome (Gosiewski et al., 2017; Gyarmati et al., 2015; Tamburini et al., 2018) and the tick microbiome (Greay et al., 2018; Narasimhan and Fikrig, 2015) has generated a baseline understanding of common commensal and contaminant bacteria in these contexts (Eisenhofer et al., 2019). Such research has also dispelled the previously held understanding that the blood compartment is sterile (Gyarmati et al., 2015). Nonetheless, at the beginning of this project there was no equivalent research examining the canine blood microbiome until Scarsella et al. (2020) investigated the blood microbiota of just 36 dogs, identifying common bacteria across all subjects as well as an impact of canine diet (Scarsella et al.,

2020). This further demonstrates the novelty of the data found in my thesis and its value in progressing this nascent field.

Whilst some sequences elucidated by my 16S metabarcoding experiments could not be easily allotted into pathogen, commensal or contaminant categories other sequences were easily demonstrated as being artefacts of DNA extraction procedures or non-sterile kits (Kim et al., 2017). For example, in Chapter 4 I demonstrated how large numbers of reads from bacteria only found in one DNA extraction kit, but not others, could highlight the phenomenon of a 'kitome' i.e. that a DNA extraction kit can have its own unique bacterial microbiome due to it not being aseptic (Hornung et al., 2019; Kim et al., 2017). As demonstrated in Chapter 4 the quantity of kit-derived reads could be as many as 80% – 86% of total reads, an amount so severe that it limited the amount of meaningful data obtainable by overwhelming bacterial reads that derived from canine blood samples. Data on different DNA extraction kit suppliers' 'kitomes' is therefore crucial to guide microbiome researchers to the best kit for their experimental needs and to maximise data accrued when conducting expensive deep-sequencing runs.

The development and role of positive controls throughout my metabarcoding research also proved invaluable, provisioning ways of mitigating common challenges in NGS-based research. The use of a commercial Microbial Community DNA Standard (ZymoBIOMICS) used within bacterial MiSeq runs, confirmed that my 16S-targeting primers did not introduce amplification bias as my results had the same sequence proportions as that described by the manufacturer (Hornung et al., 2019). On the other hand, my synthetic DNA positive control allowed us to identify and control for indexing cross-talk, DNA hybridisation, misreading as well as cross-contamination during library preparation (Kim et al., 2017). The number of uniquely identifiable synthetic positive control sequences that appeared in non-control samples, provided us with a proxy for such issues and allowed development of bespoke cut-offs for true infections for each sequencing run. For some runs, the distribution of positive control sequences in non-control samples was clustered around a positive control well on a 96-well plate, thus indicating some cross-contamination, however, this is a common finding previously reviewed by Kim et al. (2017).

The novelty of my research's exploration into the dog blood bacterial microbiome is echoed by that of my 18S-based exploration of the 'haemoprotobiome' i.e. the collection of protozoa found in the bloodstream. Numerous other studies have explored haemoprotezoa infecting buffalo, sheep, camels and a range of wild and domestic ungulates and marsupials (Chaudhry et al., 2019; Ghafar et al., 2021; Glidden et al., 2020; Mohamed et al., 2021; Ortiz-Baez et al., 2020; Squarre et al., 2020), however, my initial experiments were the first to do this in dogs. The novelty of this field means that discerning taxa as pathogenic or not is even more challenging as there is even less prior research to be guided by.

These challenges came to prominence in my research through the repeated identification of *Parabodo*, *Bodo* and *Neobodo*, as well as individual dogs that had ASVs classified as *Colpodella* and *Goussia*. Some insight could be provided by the limited existing research conducted into mammalian 'haemoprotobiomes' wherein other authors had identified *Parabodo*, *Bodo* and *Colpodella* in a spectrum of other hosts, including dogs and arthropod vectors (Dario et al., 2017; Medkour et al., 2020; Northover et al., 2019; Szöke et al., 2017; Vandersea et al., 2015). Nevertheless, such corroboration does not necessarily signify that these organisms are parasites or even present in the bloodstream (Dario et al., 2017). Given the sensitivity of NGS-methods these protozoans may have been introduced into the blood from the skin at the point of sample collection venipuncture, or via laboratory contamination (Dario et al., 2017). Therefore, the identification of such organisms require future exploration, using corroboratory diagnostics alongside attempted experimental demonstration of Koch's postulates, to resolve whether they represent putative or novel pathogens. Overall, as the number of 18S-based metabarcoding experiments increases, so too will our understanding of the complexities of this field and the mammal-inhabiting eukaryotic microbiome it uncovers.

8.4. Research limitations

The NGS platform used throughout my research was the Illumina MiSeq, a deep-sequencing technology capable of highly accurate base calling ($\geq$ Q30) alongside substantial sequencing depth; typically 3.3 to 15 Gb of data generated per run (Fadrosh

et al., 2014; Ravi et al., 2018). Nonetheless, using MiSeq v3 kits capable of sequencing amplicons at 2×300 bp, still restricts amplicons to a maximum size of approximately 470 – 500 bp long, if paired end overlap is sufficient and sequence quality kept high. This severely caps the length of the utilisable barcoding gene, meaning that for many organism's taxonomic resolution is limited. Many pathogen genera, particularly bacteria, such as *Rickettsia*, *Coxiella* and *Bartonella* have highly conserved 16S rRNA genes, especially at the fourth hypervariable (V4) region targeted by my bacterial primers, hence for these taxa species level identification could not be conducted using metabarcoding alone (Greay et al., 2018; Gutiérrez et al., 2017; Portillo et al., 2017; Santibáñez et al., 2013). To achieve species level resolution auxiliary molecular methods such as cPCR were used, which have their own inherent limitations such as reduced sensitivity when compared to NGS (Chen et al., 2020; Greay et al., 2018; Huggins et al., 2019b, 2019a). This was reflected by the fact that often a cPCR method could not amplify from an NGS positive sample for certain pathogens e.g. *Rickettsia* in Chapter 5 and therefore the pathogen species remained unknown. This was also compounded by the fact that there were few pre-existing cPCR or qPCR assays for certain organisms I wanted to cross-validate the presence of, for example the *Bodo* and *Parabodo* kinetoplastids, making corroboration even more challenging.

For bioinformatic analysis of NGS metabarcoding results, accurate classification of ASVs is also highly dependent on good reference databases. The SILVA database used by my NGS bioinformatic pipeline confers a degree of sequence classification reliability as it is more curated than NCBI's GenBank with sequences checked for accuracy at the time of submission (Nilsson et al., 2016; Pruesse et al., 2007; Quast et al., 2013; Shen et al., 2013). Nevertheless, the SILVA database is smaller and more restricted than GenBank, potentially explaining a lower resolution of ASV classification when compared to using NCBI's BLAST tool on the same sequence (Balvočiute and Huson, 2017; Pruesse et al., 2007). Furthermore, inaccurate taxonomic classifications in both SILVA and GenBank are inevitable in such open-source databases, meaning there will likely be some ASVs that were incorrectly classified (Balvočiute and Huson, 2017; Nilsson et al., 2016; Shen et al., 2013). I also found some ASVs with low similarity to any entries in either database which could reflect completely novel 16S and 18S rRNA sequences or the generation of next-

generation sequencing artefacts, such as chimeras that may have been missed by bioinformatic quality-control steps (Pollock et al., 2018).

Verification of protozoan NGS results was also attempted by using microscopy of stained blood smears on samples positive for genera such as *Colpodella*, *Parabodo* and *Bodo* to try to capture images of each organisms' morphology. Whilst microscopy is considered the 'gold standard' diagnostic by the WHO in other fields of parasitology e.g. egg burdens for gastrointestinal nematodes, there is no unanimous equivalent when investigating bloodborne VBPs (Kuleš et al., 2017; WHO Expert Committee, 2002). In this field, microscopy is recognised as being notoriously insensitive and requiring great technical expertise to morphologically identify specimens accurately (Kuleš et al., 2017; Megat Abd Rani et al., 2010). Hence, the finding that my NGS positives for unusual protozoa were microscopy negative does not necessarily mean that the NGS assessment is incorrect, as differences in results could be due to microscopy's poorer sensitivity.

Further limitations that were generated by the financial and logistical confines of this project where an inability to collect serum samples, maintain cold chain consistently from field site to the University of Melbourne and delays in sample export, the final two factors of which may have marred sample quality. In addition, funding constraints meant that 208 canine dog blood samples collected could not be extracted and analysed by NGS metabarcoding. In an ideal research context, supplementary funding would have permitted sample collection from a wider range of field sites from Cambodia, including coastal and remote rainforest locations that may have different ecology and could have unearthed a wider range of VBPs than those I was able to sample.

Regarding my community trial to assess the VBP blocking capacity of Seresto® against monthly topical fipronil, a key limitation was the composition of canines in both groups i.e. their ownership and where they resided. Under ideal study conditions, dogs would have been randomly assigned to either group from the total pool of available dogs. However, because the dogs given monthly fipronil were working mine detection dogs, with an ectoparasiticide treatment regime that their owning agency did not permit to be changed, we had to manage having a separate group and therefore demographic of dogs that the Seresto® collars could be given to. This generated some differences in the environment

i.e. rural or urban, that dogs in both groups experienced as well as the amount of weekly exposure to that environment – all factors that may have influenced VBP transmission pressure and therefore my results. Nonetheless, the direct adjustment calculations conducted to analyse these data (Chapter 7) provided a means with which to control for the confounding effect of environment (i.e. rural versus urban dogs) permitting valid comparison of any differences between topical fipronil versus Seresto® collar treatment (Chapter 7).

8.5. Applications of developed methods and future directions

My NGS metabarcoding protocols for the characterisation of the entire bacterial, apicomplexan and kinetoplastid communities from the bloodstream of canines present the scientific community with a valuable diagnostic method with which the blood microbiomes of many other mammals could be explored. The canine mtDNA blocking primers developed that were demonstrated to reduce cross-amplification of host DNA during 16S bacterial metabarcoding, may also work using a range of different mammalian blood samples. Using *in silico* analysis of the blocking primer it was found that they can also bind to the mtDNA sequences of numerous other mammals including humans, reindeer, cattle, horses, rodents, marten, boar, chimpanzees and walrus, amongst others. Such broad utility could allow my protocol to be used for characterisation of VBPs from human patients where VBP infection is suspected but clinical diagnosis has not been possible or be used on different mammalian hosts from an ecosystem, to tease apart transmission dynamics and uncover reservoir hosts that may be facilitating the persistence of a pathogen (Takhampunya et al., 2019).

Through an anthropocentric lens, prior research in Cambodia and Thailand has used NGS methods to attempt to explain previously undiagnosed human sepsis and undifferentiated febrile illness that may be aetiologically linked to zoonoses circulating in nearby domestic animals and wildlife (Rozo et al., 2020; Takhampunya et al., 2019). Here, the power of NGS to identify pathogens missed by conventional diagnostics is best typified, whilst also highlighting the interconnected nature of pathogen transmission and

bringing the relevance of a One Health approach to the fore (Overgaauw et al., 2020; Takhampunya et al., 2019; Traub et al., 2015). Successful bio-surveillance for zoonoses in conjunction with prevention and control cannot look at one host in a parasite's life cycle in isolation (Day, 2011; Overgaauw et al., 2020). These findings underscore numerous paths with which the present research can be carried forward, for example using NGS to classify the VBPs of canine ectoparasites and identifying if these overlap with those found in the dog host, as well as exploring the blood microbiota of humans and wildlife that may have close interaction with dogs in the region. In addition, other pathogen groups could be investigated that were not tested for in the current research, such as filarial nematodes or blood-borne viruses, that would build an even more holistic understanding of the pathobiome infecting canines.

Whilst my NGS methodologies represent a powerful but costly method for mining emerging and novel VBPs the Taq-Man based multiplex qPCR developed in Chapter 6, represents an economical and highly scalable diagnostic that could be used for large epidemiological surveys of the common canine VBPs in the Asia-Pacific. Moreover, the relatively little technical expertise needed to conduct qPCR when compared to NGS, means these methods may be more useful for low-to-middle income countries that are often in most need of them to detect canine VBP threats in the region. Further development of these qPCRs could include other VBPs elucidated through metabarcoding in Cambodia, such as *Bartonella* spp. and *T. evansi*.

To mitigate the inherent limitations of Illumina sequencing, future work could explore the use of third-generation long-read sequencing such as Oxford Nanopore Technologies (ONT) MinION platform, a small USB sized sequencer easily able to sequence reads over 1,000 bp long, with 10,000 to 30,000 bp sequences being common (Amarasinghe et al., 2020; Petersen et al., 2020). ONT technology has already been used for clinical disease diagnosis before, with long read lengths making species or even bacterial strain level taxonomic classification relatively simplistic, as the entire 16S or 18S rRNA gene can be amplified in one run (Hall et al., 2020; Petersen et al., 2020). Furthermore, MinION devices are highly portable, meaning that sequencing can be conducted in the field as has been done for the 2014 Ebola outbreak in West Africa (Quick et al., 2016). Within the

context of my own work, MinION-based VBP surveillance of canines could mean investigation of disease outbreaks in real-time within the country of origin or used in a biosecurity scenario, where diagnosis of canine VBPs could be conducted in dogs being exported to disease-free countries.

The development and use of novel sequencing technologies will undoubtedly play a role in what is possibly the 21st century's greatest threat to humanity: anthropogenic climate change (Brooks and Hoberg, 2007; Caminade et al., 2019; Hoberg and Brooks, 2015). Due to arthropod vectors being ectothermic, with many depending on water for the completion of life cycle stages these vectors have an intrinsic connection to climatic variables such as temperature, rainfall and humidity (Brooks and Hoberg, 2007; Caminade et al., 2019; Ogden, 2017). These variables affect every aspect of a vector's life; biting rates, phenology, speed of development, completion of life cycles and the geographic range the vector occupies (Beugnet and Marié, 2009; Caminade et al., 2019; Ogden, 2017). In turn, there is a large impact on VBP prevalence and transmission that has already been observed in mosquito-borne infections like malaria and arboviruses as well as tick-borne ones, such as Lyme disease and tick-borne encephalitis (Caminade et al., 2019; Forman et al., 2008; Rocklöv and Dubrow, 2020). Therefore, the use of emerging NGS diagnostics to surveil for VBPs and uncover novel ones within people, domestic animals and wildlife, will keep us abreast of evolving threats and further enshrine the importance of a One Health scientific approach to create a better future for humankind and our planet (Brooks et al., 2014; Zinsstag et al., 2018).

8.6. References

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