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**Running Title: Diagnosis and Chemoprevention of Canine Vector-Borne Pathogens in the Asia-Pacific**

# Title: Advanced Approaches for the Diagnosis and Chemoprevention of Canine Vector-Borne Pathogens and Parasites – Implications for the Asia-Pacific Region and Beyond

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**Key words:** vector-borne disease; dog; next-generation sequencing; metabarcoding; diagnostics; chemoprevention; ectoparasiticide; canine pathogen; Asia-Pacific; Southeast Asia.

## Contents

## Abstract

Vector-borne pathogens (VBPs) of canines are a diverse range of infectious agents, including viruses, bacteria, protozoa and multicellular parasites, that are pernicious and potentially lethal to their hosts. Dogs across the globe are afflicted by canine VBPs, but the range of different ectoparasites and the VBPs that they transmit predominate in tropical regions. Countries within the Asia-Pacific have had limited prior research dedicated to exploring the epidemiology of canine VBPs, whilst the few studies that have been conducted show VBP prevalence to be high, with significant impacts on dog health. Moreover, such impacts are not restricted to dogs, as some canine VBPs are zoonotic. We reviewed the status of canine VBPs in the Asia-Pacific, with particular focus on nations in the tropics, whilst also investigating the history of VBP diagnosis and examining recent progress in the field, including advanced molecular methods, such as next-generation sequencing (NGS). These tools are rapidly changing the way parasites are detected and discovered, demonstrating a sensitivity equal to, or exceeding that of, conventional molecular diagnostics. We also provide a background to the armoury of chemopreventive products available for protecting dogs from VBP. Here, field-based research within high VBP pressure environments has underscored the importance of ectoparasiticide mode of action on their overall efficacy. The future of canine VBP diagnosis and prevention at a global level is also explored, highlighting how evolving portable sequencing technologies may permit diagnosis at point-of-care, whilst further research into chemopreventives will be essential if VBP transmission is to be effectively controlled.

# 1. Introduction

Vector-borne pathogens (VBPs) of canines are a diverse range of agents transmitted by arthropods that encompass viruses, bacteria, protists, and metazoan parasites (Irwin, 2014; Irwin and Jefferies, 2004). Together, they cause a spectrum of disorders in dogs ranging from relatively benign infections to diseases that can be fatal, whilst some are also zoonotic and, therefore, represent a concurrent risk to humans (Irwin, 2014; Otranto et al., 2009a). Although VBPs of canines have been extensively studied in Europe and North America, there is substantially less relevant information on these pathogens in the tropics, particularly in the Asia-Pacific (Colella et al., 2020; Irwin and Jefferies, 2004; Nguyen et al., 2021). The potential for rare or novel VBPs to emerge 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 COVID-19 (Ferri and Lloyd-Evans, 2021; Grange et al., 2021; Johnson and Fooks, 2014; Traub et al., 2015). This chapter will review the state of knowledge of canine VBPs in the Asia-Pacific, the diagnostic tools historically used to detect them as well as exploring emerging next-generation sequencing (NGS) based technologies and how these have already proven their ability to revolutionise the field of diagnostic parasitology. Moreover, we review the arsenal of tools used to protect dogs from ectoparasite infestation and VBPs, including contemporary chemopreventive products and assess whether such products can remain effective even in contexts of substantial infection pressure by parasites, such as within tropical environments (Colella et al., 2020; Nguyen et al., 2020).

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, i.e., approximately 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, several common pathogenic families and genera can be found worldwide (Beugnet and Marié, 2009; Pfäffle et al., 2013).

Some of the most widespread are alphaproteobacteria 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 bacteria can cause some of the most damaging VBP infections in dogs, 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 canines, 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).

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 transmitted via ingestion of the vector and are often only associated with severe disease when found in coinfections (Baneth et al., 2003; Ivanov and Tsachev, 2008). 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).

In contrast, filarial nematode parasites are predominantly transmitted by highly motile mosquito vectors (Megat Abd Rani et al., 2010). The filaroid *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 by causing fatal cardiocirculatory disease (Baneth et al., 2016; Dantas-Torres and Otranto, 2013; Frangipane di Regalbono et al., 2016; Genchi and Kramer, 2017).

Some of the most prevalent and damaging pathogens of dogs 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, in Southern Europe, the Middle East and India (Parola et al., 2013) with some of these *Rickettsia* exhibiting fatality rates as high as 32% in people (Mencke, 2013). Today, there is also growing recognition that *Rickettsia felis* is 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 uses dogs as a reservoir host, whilst in humans it produces the multi-systemic disease Flea-Borne Spotted Fever (FBSF) producing flu-like symptoms, myalgia, and malaise (Levin et al., 2012; Ng-Nguyen et al., 2020; Reif and Macaluso, 2009; Teoh et al., 2016; Williams et al., 2011).

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 zoonotic species *Ehrlichia chaffeensis*

the aetiological agent of 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 and can even be fatal (Kolo et al., 2020; Lee et al., 2020).

Dogs are also known to be key reservoirs of potentially zoonotic filarial worms (Genchi and Kramer, 2017). The filaroids *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).

The main aims of this review are: (i) to provide a background on the key canine ectoparasites and VBPs of the Asia-Pacific with a particular focus on those occurring in tropical regions; (ii) to detail the principal diagnostic methods used to detect and characterise VBP with emphasis on novel molecular techniques, such as next-generation sequencing; (iii) to appraise the available chemopreventive tools used for the protection of dogs against ectoparasite infestation and VBP infections; and (iv) to review recent developments and discoveries in the areas of diagnostics and chemoprevention in geographic areas with elevated VBP infection pressures, such as Southeast Asia, taking lessons particularly from novel research conducted in Cambodia. Finally, this article looks to the future to provide recommendations for the protection against VBPs of canines in the tropics, whilst also highlighting the remaining challenges and emerging opportunities to improve the diagnosis and prevention of diseases caused by these pathogens.

## 2. Important canine ectoparasites of the Asia-Pacific

Arthropods show extensive levels of diversity, with some estimates attributing 80% of all known species to the phylum Arthropoda (Wall and Shearer, 1997). A considerable portion of this phylum have adopted an ectoparasitic and 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 a plethora of bacteria and protozoa and transmit some of the most lethal 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 $CO_2$, 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 VBPs of canines, 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. 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 is quickly established (Wall and Shearer, 1997). Not only can flea infestation cause Flea Allergy Dermatitis (FAD) in dogs but they can also transmit and maintain parasites, 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 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 Australian (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 principal vectors of filarial VBPs and are dependent on static waterbodies for reproduction, thereby strongly tying the effect of local climate on the prevalence of filarial diseases (Carrade et al., 2009; Smout et al., 2016).

## 3. Key canine VBPs of the Asia-Pacific

### 3.1. *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 in multiple studies at prevalences of 27% - 36% of community dogs in Indigenous communities in Queensland and the Northern (Hii et al., 2012; Shapiro et al., 2017). In Southeast Asian studies (Table 1), molecular detection has found *A. platys* at a prevalence of between 4.4% - 25% in Thailand (Huggins et al., 2019a; Liu et al., 2016) and 32% in

Cambodia (Huggins et al., 2021a), 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).

### 3.2. *Babesia* spp.

*Babesia* is a genus of protozoan parasite in the order Piroplasmida, that is phylogenetically placed 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 coinfected 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 (Table 1), 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 (Table 1), *Babesia* has also been found at high prevalence via molecular methods, for example *B. vogeli* in 14% - 32.7% of Cambodian dogs (Huggins et al., 2021a; Inpankaew et al., 2016), but also at lower levels, such as 8% - 13% of stray dogs in Thailand (Do et al., 2021; Huggins et al., 2019b). 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 – 7 mg/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).

### 3.3. *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, in Thailand molecular detection found 1% of dogs to be infected with *Bartonella* spp. (Huggins et al., 2019a), whilst in a separate study 38% of dogs tested were serologically positive (Suksawat et al., 2001). In Cambodia 3% of canines were found positive for *Bartonella clarridgeiae* via molecular means, although prevalence appeared localised around a few urban centres (Huggins et al., 2021a; cf. Table 1). 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 is very limited data, 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).

### 3.4. *Dirofilaria immitis*

*D. immitis* is a filaroid 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 ingested 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 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; cf. Table 1). Microfilaremia can be effectively

treated in dogs using a single dose of a macrocyclic lactones, such as moxidectin. Nonetheless, 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).

### 3.5. *Ehrlichia canis*

*E. canis* is an alphaproteobacterial VBP in the family Anaplasmataceae; it uses ticks as a vector and as an important reservoir for bacteria, which can undergo 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 including pallor, epistaxis, petechiae, haematuria, lameness, ventral oedema, neurological manifestations and secondary infections; this phase of the disease is often fatal (Castro et al., 2022; 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 (Table 1); for example, it was detected via molecular methods in the blood of 9.8% of 81 anaemic dogs tested (Kaewmongkol et al., 2017) and 40% of community dogs in Thailand (Huggins et al., 2019a) as well as 20% - 21.8% of dogs in Cambodia (Huggins et al., 2021a; 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, 2020a; Neave et al., 2022).

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).

### 3.6. 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% - 13% for *M. haemocanis* and 2.9% for *Candidatus M. haematoparvum* as well as the presence of a novel canine-infecting *Mycoplasma* in four individuals (Huggins et al., 2021a; Inpankaew et al., 2016). Additionally, studies in Thailand have found haemotropic *Mycoplasma* spp. to be one of the most prevalent VBP of stray dogs at between 19.9% - 39% prevalence (Huggins et al., 2019b; Liu et al., 2016; cf. Table 1). Numerous haemotropic *Mycoplasma* spp. have also been found in Australia, including an undescribed species (Barker et al., 2012; Hii et al., 2015b; Shapiro et al., 2017).

### 3.7. *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* species causing infection in people (Dantas-Torres and Otranto, 2016).

*H. canis* has been reported in dogs across the Asia-Pacific (Table 1), including in Cambodia at a prevalence of 10.9% - 18% (Huggins et al., 2021a; Inpankaew et al., 2016) and in Thailand at a prevalence of 18.8% - 38% (Huggins et al., 2019b; 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).

### 3.8. *Leishmania* spp.

*Leishmania* spp. are one of the most important zoonotic VBPs 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 *Leishmania* species that infects both canines and is zoonotic. *L. infantum* uses dogs as a reservoir host 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 elimination 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 (Table 1). 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).

### 3.9. *Rickettsia felis* and related species

The Rickettsiaceae is a large family of obligate parasites that are 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* species 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 to be conclusively identified (Hii et al., 2013, 2011a). 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 (Table 1) 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) has 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.

### 3.10. *Trypanosoma evansi*

*Trypanosoma* spp. are flagellated protozoans of the Class Kinetoplastida, 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* infection across the Asia-Pacific, including in Vietnam (Bui et al., 2021), Malaysia (Aregawi et al., 2019), Cambodia (Huggins et al., 2021a) and Thailand (Aregawi et al., 2019; Schneeberger et al., 2016) 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; cf. Table 1). 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 (off-label) by intramuscular diminazene aceturate at a low dosage of 3.5mg/kg over five days (Panigrahi et al., 2015), whilst suramins (off-label) have also been shown to lead to a 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).

## 4. Conventional and advanced techniques for diagnosis

Until recently diagnosis of bacterial diseases or infections had been primarily conducted via *in vitro* cultivation on different growth media, followed by phenetic identification/characterisation 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 long lag times until results are obtained. They may 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 require 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, microfilaria or egg life cycle stage is being observed (Tanaka et al., 2014).

Advancing from microscopy-based diagnosis, serological techniques have also been widely used for VBP 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; Pottumarthy 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).

### 4.1. Molecular diagnostic techniques

With the advent of molecular technologies, like the polymerase chain reaction (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; Huggins et al., 2017). 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 prevents 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 predominant pathogen in a sample (Zepeda Mendoza et al., 2015). For diagnosis of VBPs this may mean that mixed infections, which are common, are missed as only the most prevalent parasite sequences are detected. Alternatively, when Sanger sequencing samples with coinfections mixed sequencing chromatograms may be generated which can be challenging to accurately analyse (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 sizes of amplicon fragments that can be run on a gel and used to assess for the presence of multiple pathogens coinfecting a host (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 qPCR, 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; Huggins et al., 2021b; 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 or the product of non-specific amplification (Huggins et al., 2021b; 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 identify novel species (Takhampunya et al., 2019).

One of the principal challenges to using molecular diagnostic methods in the field or in resource-limited settings is the large amount of bulky equipment required that typically needs a constant power supply, for example PCR thermocyclers (García-Bernalt Diego et al., 2021). To preclude such difficulties, numerous isothermal nucleic acid amplification technologies (INAATs) have been developed that can amplify DNA at a single temperature, i.e., via use of a battery-powered heat block, with minimal sample handling (García-Bernalt Diego et al., 2021; Li et al., 2018; Upadhyay et al., 2021). INAAT methods, such as loop-mediated isothermal amplification (LAMP) have been developed for numerous VBPs, including *Babesia* spp., *E. canis*, *H. canis*, *Leishmania* spp., *Plasmodium* spp. and *Rickettsia* spp., and been shown to have

comparable or better sensitivity than other molecular methods like cPCR (Adams et al., 2010; Hayashida et al., 2017; Ikadai et al., 2004; Müller et al., 2010; Noden et al., 2018; Ruang-areerate et al., 2021; Upadhyay et al., 2021). LAMP-based diagnosis for numerous parasites has now been conducted at point-of-care (POC), including in remote field locations, whilst many LAMP assays have also demonstrated less susceptibility to PCR inhibitors, meaning samples can be prepared quicker, cheaper and with less manipulation (García-Bernalt Diego et al., 2021; Hayashida et al., 2017; Noden et al., 2018; Qin et al., 2018; Upadhyay et al., 2021). More recently, the simplicity of INAAT methods has been improved further still with the use of recombinase polymerase amplification (RPA) techniques. RPA can be conducted at lower temperatures and exceed the speed at which a diagnosis can be obtained down to as little as 5 – 30 mins when compared to other INAAT methods like LAMP (Cordray and Richards-Kortum, 2015; Li et al., 2018; Yin et al., 2017). Due to these facets, RPA has exhibited rapid uptake within the scientific community since its development and within the context of VBP research has already been used for the diagnosis of canine babesiosis, equine piroplasmosis, leishmaniasis, malaria and theileriosis (Cordray and Richards-Kortum, 2015; Cui et al., 2018; Khan et al., 2021; Lei et al., 2020; Li et al., 2018; Yin et al., 2017).

### 4.2. Next-generation sequencing methods

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 methods are typically classified into one of two categories; short-read technologies able to sequence reads between 150 to 700 base pairs (bp) in length or long-read

technologies that can reach read lengths as large as 200,000 bp (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 bases present and their positions (Goodwin et al., 2016). Alternatively, sequencing by synthesis, e.g., on the 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 NGS 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 (ONT) 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). ONT's 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, 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 containing all the COX1 DNA barcodes in a sample, known as a metabarcode, that can in turn be used to elucidate all the species that were originally present (Zepeda Mendoza et al., 2015). Analysis of a samples 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 organisms' antibiotic resistance status (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 the numerous benefits NGS brings in terms of quantity and depth of information generated it is also encumbered with various drawbacks. NGS 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 NGS 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). Since the arrival of NGS

technologies their uptake for diagnosis of disease has been much greater in the context of human medicine, whilst their employment for veterinary research has been substantially lagging until only recently, as will be explored in section **6.1**.

## 5. Prevention is better than cure: The role of ectoparasiticides in protecting dogs from VBPs

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 can be very difficult, with some pathogens 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 nations, 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).

### 5.1. 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 it's 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 chemical classes of ectoparasiticide 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; cf. Table 2). 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., 2022).

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; cf. Table 2). Macrocyclic lactones are another important class of ectoparasiticide, that include the chemicals selamectin and moxidectin. Macrocyclic lactones were developed from fermentation chemicals found in various *Streptomyces* species and are broad-spectrum antibiotics that work 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., 2022). These target GABA-gated and glutamate-gated ion channels in many ectoparasite species, disrupting neurotransmission in the invertebrate central nervous system and causing paralysis and death (Shoop et al., 2014; Zhou et al., 2022; cf. Table 2).

Whilst the particular ectoparasiticide given to an animal 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). On the other hand, 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 is easier, as reapplication is less frequent and thus there are fewer chances for infestation to occur if an owner forgets to reapply the product (Brianti et al., 2013; Stanneck et al., 2012a).

### 5.2. Protection from VBP in the tropics: safeguarding dog health where parasite infection pressure reaches its peak

Whilst the risk VBDs pose to canines is global, nowhere is this threat more pronounced than in developing countries in the tropics (Traub et al., 2015). High annual 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 populations of stray dogs 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 or contaminated soil (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 near 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 their life cycles 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 chemopreventives 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., 2013a; 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, a 10% imidacloprid and 4.5% flumethrin chemopreventive collar (Seresto®) was demonstrated as being 100% effective at blocking *B. vogeli* transmission to canines in field conditions in Southern Italy (Dantas-Torres et al., 2013a). Initial prevalence of infection was demonstrated as being between 4.9% to 6% for *Babesia* spp. (Cassini et al., 2009; Dantas-Torres et al., 2013a). 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. Hence, whether the same product could afford a comparable level of protection under these conditions of elevated pathogen intensity is not something that can be presumed.

Recognising that tropical settings present unique challenges regarding 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).

### 5.3. Adapting to the threats posed by VBP under a changing climate

The founding of advisory research councils to inform the veterinary community has come at a time of great need, helping to find solutions to problems generated by a rapidly changing planet with transforming zoonotic threats (Beugnet and Marié, 2009). The last 20 years have seen abrupt changes in the epidemiology of VBPs, 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 VBDs 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 and mosquito-borne *D. repens* infection, across Europe (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 pathogen vectors, like mosquitoes (Ramasamy and Surendran, 2011; Smout et al., 2016). Furthermore, increased travel of humans and their pets can rapidly translocate VBPs 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 quarantine measures for pets travelling into the UK from the EU but at the same time led to the importation of non-endemic *Babesia* spp., *Ehrlichia* spp. and *Leishmania* spp. parasites, (Beugnet and Marié, 2009; Hii et al., 2012). Similar outcomes can emerge due to natural disasters as well. For example, after Hurricane Katrina, significant 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, particularly regarding mosquito-borne disease, when compared to their counterparts in temperate regions (Rizzoli et al., 2019). The interaction between environmental modification caused by humans 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.

## 6. Recent advances in the diagnosis and chemoprevention of VBPs of dogs: a focus on the Asia-Pacific

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 to 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 (Colella et al., 2020; Irwin and Jefferies, 2004). Despite this, within the region there are significant knowledge gaps regarding canine VBP research and the impact they have on dog populations. Therefore, to address this, recent work in our laboratory has set-out to improve our understanding of the epidemiology and control of VBP within

the Asia-Pacific region. This has been achieved by: (i) the development of an NGS diagnostic capable of holistically characterising pathogen communities from canine blood; (ii) the utilisation of this diagnostic on a country-wide scale to assess the prevalence, diversity and risk factors associated with canine VBPs; and (iii) the employment of such epidemiological data to develop targeted, economical and high-throughput diagnostics such as multiplex qPCRs. Finally, for the first time, through the execution of a 12-month field efficacy study it has been shown that (iv) topical ectoparasiticides can be highly effective products at preventing the transmission of VBPs in canine populations within the tropics. Therefore, the following sections detail how contemporary research in the field of NGS-based diagnostics and VBP chemoprevention, from our own lab and others, is beginning to grow our understanding of the epidemiology and control of VBP within the Asia-Pacific and across the globe.

### 6.1. Applications of next-generation sequencing

Ever since its inception, NGS 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 pathogens in situations where conventional microscopy and PCR techniques have failed. 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 to conventional diagnosis of visceral leishmaniasis, an important finding given that this zoonosis accounts for roughly 20,000 – 40,000 deaths annually. All these cases powerfully underscore NGS-methodologies' role as an important addition to the global armoury of available diagnostics (Chen et al., 2020; Jerome et al., 2019; Rozo et al., 2020).

Next-generation sequencing has been directly applied to the field of VBP diagnosis. 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., 2015; 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., 2015), 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 (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).

Additionally, large recent expansions in the number of NGS-based tick microbiome studies has led to a greater understanding on the roles of bacterial

endosymbionts in ticks, the effects of coinfection with different VBP as well as the impacts of environment, tick age, sex and organ sampled on the tick microbiome (Chauhan et al., 2020; Gofton et al., 2015b; Greay et al., 2018b; Guizzo et al., 2020; 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 microbiome 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), *Rickettsia* in Thai rodents (Chaorattanakawee et al., 2021), and *Anaplasma* spp. from Sudanese camels (Mohamed et al., 2021). As these methods are further improved, 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 investigated (Huggins et al., 2020; Oney et al., 2021).

The specific employment of NGS to detect bacterial VBP in dogs has been pioneered by our laboratory (Huggins et al., 2021a, 2020, 2019b; Figure 1). Testing, optimisation, and benchmarking of NGS metabarcoding methods on canine blood DNA has identified such methods as being more sensitive for the detection of VBP than conventional PCR and better able to detect rare, novel, and unexpected

pathogens (Huggins et al., 2021a, 2019a). These NGS protocols are robust and sufficiently high-throughput to be utilisable for country-wide epidemiological surveys of canine VBP, as has now been completed in Cambodia (Huggins et al., 2021a). Furthermore, issues of low bacterial biomass from blood affecting diagnostic sensitivity of NGS methods has been resolved by the development of a canine DNA blocking primer (Huggins et al., 2020). This has reduced the amount of extraneous amplification and sequencing of host DNA that can occur during NGS protocols, and can increase the amount of pathogen DNA detected, thereby improving the sensitivity of these methods (Huggins et al., 2020). Nonetheless, due to the high sensitivity that many NGS-based diagnostic methods possess, the use of precautions and techniques to limit contaminant DNA from environmental or kit-derived bacteria is essential. Huggins et al. (2020) compared a variety of different blood DNA extraction kits, revealing striking differences in the amount of endogenous bacterial contaminants such products have, which in turn can greatly affect their suitability for NGS diagnostics.

[Insert Figure 1 here]

**Figure 1. Workflow for conducting next-generation sequencing (NGS) based metabarcoding diagnostics from canine blood.** (1) Approximately 200 µl of whole blood is collected from the subject using aseptic technique, directly into a Vacutainer® collection tube with ethylenediaminetetraacetic acid (EDTA). (2) Whole blood contains bloodstream circulating pathogens and can be processed immediately or stored at -20°C until required. (3) DNA is extracted using a DNA extraction kit that is confirmed sterile or has been tested for use in NGS applications. (4) Polymerase chain reaction (PCR) amplification is conducted using NGS adapted primers that target taxonomically informative barcoding regions of the pathogen group of interest. (5) Amplicons are generated that have all the genetic barcodes from the pathogen

group of interest plus the NGS adapter regions. Amplicons are cleaned at this stage. (6) A second PCR is conducted whereby a unique molecular identifier (UMI) is added to each amplicon from the first PCR, thereby permitting multiple amplicons to be multiplexed, i.e., mixed, onto the same NGS run. (7) Amplicons with incorporated UMIs are cleaned again and pooled, with final quantification and processing steps conducted before the NGS run. (8) The pooled amplicon library is run on an NGS platform, for example Illumina's MiSeq deep-sequencer. (9) NGS data is downloaded from the sequencing platform and bioinformatically processed, undergoing stringent quality control and filtering steps. (10) Demultiplexing is carried out so that all sequences with a specific UMI, belonging to one sample's amplicon can be separated, generating the sample's metabarcode. (11) Each metabarcode undergoes taxonomic classification so that all the pathogens from the original sample can be identified. Created with BioRender.com.

Further advances in NGS methodologies, such as ONT' MinION™ platform, have allowed diagnostic metabarcoding and whole genome data to be accrued from samples in as little as 6 - 24 hours, using highly portable equipment (Quick et al., 2016, 2015). The latest nanopore technology has improved raw base-calling accuracy up to 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). Such technology, spearheaded by Quick et al. (2016) in the context of human disease outbreaks, used the USB-sized MinION™ sequencer along with portable laboratory equipment that could fit into a suitcase to monitor the evolution of the 2014 West African Ebola Outbreak. Now, such

technology has also been used for the diagnosis of vector-borne bacteria in canines as well. Huggins et al. (2022a) found that metabarcoding of the full-length 16S rRNA gene directly from dog blood, could be used to accurately detect and characterise a large range of blood-borne bacteria with a diagnostic sensitivity and specificity comparable to that of qPCR. Using such a compact sequencing platform with bioinformatic pipelines that are not reliant on an internet connection signifies an important next step in our ability to holistically diagnose VBPs of canines rapidly, and in the field (Huggins et al., 2022a).

### 6.2. Next-generation sequencing for the characterisation of protozoan communities

Alike to the success of microbiome studies exploring bacterial microbiomes, more and more research has now been published on parasitic assemblages of protozoa, or the 'haemoprotobiome', via use of the eukaryotic 18S rRNA barcoding gene (Chaudhry et al., 2019). Recent studies have explored this in wildlife and livestock in Zambia (Squarre et al., 2020b), 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.

This 'haemoprotobiome' has also begun to be explored in canines as well (Huggins et al., 2021a, 2019b). NGS-based methods capable of detecting the entire parasitic community of apicomplexans and kinetoplastids from dog blood have been

developed (Figure 1) and shown to have improved diagnostic sensitivity to traditional molecular methods, such as cPCR (Huggins et al., 2019b). When such methods were used alongside bacterial metabarcoding in a nation-wide epidemiological study of stray dogs from Cambodia they found the country to be hyperendemic for a diverse range of VBPs of dogs (Huggins et al., 2021a). This epidemiological data alongside dog metadata were critically important for the identification of significant risk indicators for VBP exposure; information that will be key for veterinarians working in the region that may have limited resources to diagnose dogs with molecular methods (Huggins et al., 2021a). In addition, the strengths of using NGS-based technology were aptly shown through its ability to detect rare VBPs in Cambodian dogs such as *T. evansi* and novel pathogens, including an uncharacterised haemotropic *Mycoplasma* species from four individuals (Huggins et al., 2021a). Moreover, the DNA of numerous putative pathogens was also detected from dog blood encompassing the bacterial groups *Coxiella*, *Neisseria* and Rickettsiaceae, as well as the protozoan genera *Colpodella*, *Parabodo*, and *Bodo* (Huggins et al., 2021a).

Not only do such studies provision critical baseline data on the epidemiology of VBPs in countries with limited prior data but they also facilitate the development of novel diagnostics based on cheaper and more high-throughput technologies (Huggins et al., 2021b). For example, information gleaned through NGS-based surveys of Cambodian dogs (Huggins et al., 2021a) and prior epidemiological studies permitted the identification of the most prevalent VBPs of dogs in Cambodia and the Asia-Pacific (Barker et al., 2012; Carslake et al., 2017; Colella et al., 2020; Huggins et al., 2019b, 2019a; Irwin and Jefferies, 2004; Muhlnickel et al., 2002). This data aided in the design and validation of a novel Taq-Man based multiplex qPCR to rapidly and economically

test for six key VBPs from canine blood samples in a platform highly amenable to upscaling for in-depth VBP surveillance in dog populations (Huggins et al., 2021b).

### 6.3. Chemoprevention is central to blocking the transmission of VBPs in the tropics

The elucidation by NGS diagnostics that dog populations in countries of the Asia-Pacific, like Cambodia and Thailand, have high prevalence of VBPs, highlights the importance of finding effective products that can block their transmission (Huggins et al., 2021a, 2019b, 2019a).

Historically, 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 stop transmission of VBPs (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 infect a new host with a VBP. Therefore, research is now focussed on feeding prevention, as well as investigation of how long it takes different VBPs to be transmitted during haematophagy (Jongejan et al., 2016).

Topical chemopreventives that work from the animal's fur and have a rapid ectoparasite knock-down or repellent effect are of particular importance as they stop a vector taking a blood-meal and infecting the host with a VBP (Kužner et al., 2013; Rohdich et al., 2014). Phenyl pyrazole chemicals, such as fipronil, have already been extensively tested and used on canines, continuously demonstrating efficacies of over 94% for the prevention of ectoparasite infestation, whilst also effectively blocking VBP transmission (Davoust et al., 2003; Kužner et al., 2013; Rohdich et al., 2014).

Moreover, in the Asia-Pacific, other, more contemporary topical products have recently become available and shown great promise at blocking VBP transmission, for example, Seresto® (Brianti et al., 2013; Dantas-Torres et al., 2013a). This product, consisting of a 10% imidacloprid and 4.5% flumethrin chemopreventive collar, uses imidacloprid to increase arthropod sensitivity to flumethrin, thereby producing a potent ectoparasiticidal effect that is greater than the sum of either chemical alone (Stanneck et al., 2012a, 2012b).

Seresto® has 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., 2013a; Fourie et al., 2013b; Stanneck et al., 2012b; Stanneck and Fourie, 2013). Laboratory experiments have found Seresto® reduced *C. felis* infestations by 99.8-100% by the second day of collar application whilst its effectiveness at preventing re-infestation by ticks was 96% seven days after collaring. A field study by Brianti et al. (2013) used Seresto® on shelter dogs; a population that prior to collaring had 96.3% of individuals infested with ticks at levels as high as 434 ticks per dog. However, seven days post-collaring, tick infestation was reduced to 15%, with no ectoparasites found at the 14-day sampling point, and this high level of deterrence maintained for three months (Brianti et al., 2013).

The capacity of Seresto® to stop the transmission of VBPs has also been investigated. Within the context of an Italian dog shelter the efficacy of Seresto® collaring on acquisition of new VBPs versus an uncollared control group, found that it controlled new *A. platys* infection by 91.1%, *B. vogeli* by 100% and *H. canis* by 43.4% (Dantas-Torres et al., 2013a). The authors postulated that the collars lower efficacy at reducing *H. canis* infection was due to this protozoan being transmitted via tick ingestion, highlighting the differences between effective control of distinct pathogen

species (Dantas-Torres et al., 2013a). Moreover, Krämer et al. (2020) found Seresto® collars 100% efficacious at blocking the transmission of *A. phagocytophilum* and *Borrelia burgdorferi* sensu lato, whilst Fourie at al. (2013) found similar results regarding blocking *B. canis* infection. Such transmission blocking effects have been demonstrated to persist across the collar's entire eight-month duration of action (Fourie et al., 2019a; Fourie et al., 2013b; Krämer et al., 2020).

Importantly for tropical regions, where there is a greatly elevated pressure of ectoparasite infestation and infection by VBPs, topical chemopreventives that act from the dog's skin, have continued to demonstrate high efficacy at protecting canines (Huggins et al., 2022b). A recent 12-month field efficacy study found that both Seresto® collars and fipronil [12% w/v], as a monthly spot-on, effectively protected local pet and mine-detection dogs that were regularly exposed to stray dogs with high VBP prevalence (Huggins et al., 2022b, 2021a). Over the entire duration of this study all 164 dogs on topical products were not found to have acquired an ectoparasite infestation, whilst new infections with VBP were as low as 1% for the dogs on Seresto® and 2% for those on fipronil. These results are of critical importance for demonstrating the value of topical ectoparasiticides at preventing contraction of VBPs of dogs in tropical regions of the Asia-Pacific. This benefit is especially pronounced in countries such as Cambodia, where prevalence of VBP can be as high as 62% for untreated community dogs (Huggins et al., 2022b, 2021a).

Irrespective of the chemopreventive 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 tenet is highlighted when products that do not prevent vector haematophagy are studied and compared. For example, a growing class of systemic ectoparasiticides that are taken up and stored

within the subject's tissues, typically after ingestion of the product, have recently become popular for canine ectoparasite control (Schorderet-Weber et al., 2017; Zhou et al., 2022). Isoxazoline compounds, such as afoxolaner, fluralaner and sarolaner are the most notable of these and work by killing the ectoparasite only after it has begun feeding from the host animal, thereby providing a window over which VBPs can be transmitted (Jongejan et al., 2016; Schorderet-Weber et al., 2017; Zhou et al., 2022).

The benefits of topical over systemic ectoparasiticides for canine protection was first demonstrated by Jongejan et al. (2016) via testing of three different products against sustained challenges of *R. sanguineus* ticks infected with *E. canis*. Here, blocking efficacies between 33.3% to 66.6% were found for the systemic products compared to 100% for those on a topical chemopreventive (Jongejan et al., 2016). Comparable results have also been obtained in 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 systemic products that rely on vector feeding to work (Fourie et al., 2013a; Jongejan et al., 2015; Witchey-Lakshmanan, 1999).

Recent comparison of VBP prevalence in Cambodian dogs on systemic and topical products has also shown the enhanced protection conferred by topicals within a tropical, high ectoparasite pressure environment (Huggins et al., 2022b). Of the 186 dogs compared, those on a systemic isoxazoline product were 2.7 times more likely to be positive for a VBP than those on a topical product, irrespective of their environment. Additionally, dogs on no chemopreventive were approximately seven times more likely to be positive for a VBP than those on a topical product (Huggins et al., 2022b). Such data is of great significance for veterinarians working in the Asia-Pacific, given the region's high VBP transmission pressure, popular use of systemic

ectoparasiticides and the insidious health impacts endemic VBPs have on canine there (Huggins et al., 2022b, 2021a).

Given that systemic ectoparasiticides permit vector feeding, research into VBP transmission times after vector attachment has also become increasingly important. Such research has shown that the speed at which transmission occurs is highly dependent on the pathogen in question, the species of vector, and a myriad of other factors (Jongejan et al., 2016). For example, *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, there has also been evidence recorded of *E. canis* being inoculated into a new host after just three hours post-attachment of a tick (Fourie et al., 2013). More 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 (Fourie et al., 2019b).

This picture is further complicated by an understanding that other factors, such as whether the tick vector has recently fed, also 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 by systemic ectoparasiticides that may take as long as 24 hours after tick attachment to generate a killing effect (Fourie et al., 2013b; Jongejan et al., 2016; Zhou et al., 2022). This again emphasises the benefits of topical chemopreventives for interruption of VBP transmission and canine protection.

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 fly-borne pathogens, like *D. immitis* (Brianti et al., 2013; Frangipane di Regalbono et al., 2016; Genchi and Kramer, 2017). In the Asia-Pacific, *D. immitis* is one of the most important mosquito-borne VBPs of canines with a substantial body of research into its chemoprevention. Advantage Multi®, 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 highly effective at blocking transmission of both species (Frangipane di Regalbono 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). Nonetheless, there has been limited testing of such products comparative efficacy at preventing *D. immitis* within the Asia-Pacific; an unexploited area of research that is due greater attention.

### 6.4. Preventing VBP transmission in animal populations also benefits humans

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,* 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 important One Health principles 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, clinicians, 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 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). A definitive animal host for SARS-CoV-2 has not yet 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 epidemics caused by SARS and MERS both had their animal 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 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 global 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).

# 7. Discussion and conclusions

## 7.1. VBP control in canine populations in the tropics

Despite the relatively limited data available regarding the epidemiology of canine VBP in the Asia-Pacific, what is known is that when investigated, prevalence is consistently high, underscoring the need for effective management of VBP risk as well as control in the region (Colella et al., 2020; Huggins et al., 2021a; Irwin and Jefferies, 2004; Nguyen et al., 2021). Veterinarians must be kept up-to-date of novel findings regarding the prevalence of VBPs, with a particular focus on the identification of potentially zoonotic pathogens, such as *Bartonella* spp., *Rickettsia* spp., *Leptospira* spp. and *Brugia* spp. that have been identified in prior studies (Colella et al., 2020; Huggins et al., 2021a; Inpankaew et al., 2016; Koh et al., 2016; Liu et al., 2016). 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 select 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 effective topical ectoparasiticide products such as spot-on fipronil and Seresto® collar formulations, amongst others. Additionally, dog owners should be warned of the risks of systemic isoxazoline medications that do not prevent vector feeding and thereby permit transmission of VBPs, particularly given the popularity of these products in the Asia-Pacific (Huggins et al. 2022b; Jongejan et al., 2016). Other management strategies that should be communicated to veterinary clients is the use of environmental modification to reduce risk (Figure 2). Simple measures such as cutting grass, removing leaflitter from gardens, checking kennels for the presence of ticks, sealing of cracks and crevices where tick species like *R. sanguineus* s.l. 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., 2013b; Gray et al., 2013; Otranto and Wall, 2008; Šlapeta et al., 2021). In addition, limiting the amount of contact pet dogs have with stray animals and wildlife, as well as reducing the time spent in areas frequented by itinerant dogs, may diminish the chance of infestation by ectoparasites (Otranto and Wall, 2008).

Nonetheless, individual measures alone will not be sufficient to tackle the issue of prevention and control of canine VBPs, 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, as well as education of owners to prevent their animals from wandering (Otranto and Wall, 2008). All such actions will reduce the reservoir of canine VBPs formed by large vagrant dog populations and reduce the

risk to pet dogs (Guven et al., 2017; Jimenez-Coello et al., 2010; Figure 2). These measures can also 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 for local cultural and religious practices, giving communities a clear understanding and voice on the programs being implemented on their lands (Rupprecht et al., 2020).

Finally, the elucidation of a high prevalence and diversity of VBPs of dogs across the Asia-Pacific has relevance to the risk management and biocontrol of other countries around the globe that are non-endemic for these VBPs. Exportation of pets and livestock presents a serious risk of seeding VBPs into previously naïve countries, for example the UK's Pet Travel Scheme which introduced *Babesia* spp., *Ehrlichia* spp. and *Leishmania* spp. to Great Britain for the first time (Beugnet and Marié, 2009; Hii et al., 2012; Johnson and Fooks, 2014; Shapiro et al., 2017). 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 conclusively known (Australian Government, 2020a, 2020b; Neave et al., 2022). Regardless, the importance of strict bio-surveillance and biosecurity measures to restrict human-mediated spread of VBPs has never been more urgent given the elevated rates of animal translocation that occur today (Chomel, 2011; Johnson and Fooks, 2014; Traub et al., 2015). Therefore, given the greater incidence of canine VBP that occurs in many nations of the Asia-Pacific, local and importing governments may need to impose strict screening procedures for dogs destined to leave the country, particularly for those

moved between higher risk countries. At the same time, importing countries will need to test and quarantine arrivals with novel diagnostics to ensure a diverse range of VBPs are not inadvertently introduced, particularly given the ubiquitous presence of *R. sanguineus* s.l. that could vector them if given a chance to establish (Fèvre et al., 2006; Gray et al., 2013; Johnson and Fooks, 2014; Šlapeta et al., 2021).

[Insert Figure 2 here]

**Figure 2. Recommendations for the diagnosis and control of vector-borne pathogens (VBPs) of canines in the Asia-Pacific region.** Further research into diagnostic tools for canine VBP will benefit from harnessing the power of deep-sequencing methodologies that can detect a large range of different pathogenic agents simultaneously, whilst concurrent use of serological tests may highlight latent infections that would otherwise be missed by molecular methods alone. Control of canine VBP is complex and multifaceted, relying on approaches that surveil for VBPs, reduce their reservoirs, and alter the local environment to reduce the likelihood of vector survival. Additionally, the application of topical ectoparasiticides to pet and working dogs will be crucial in reducing the spread of VBPs within these communities. Future research priorities should focus on the development of portable point-of-care tools that can test for VBP rapidly and in environments that may be resource limited, whilst the discovery of novel ectoparasiticides will be essential to keep us abreast of drug resistance within vector populations. Further research into the microbiome of canines from different bodily compartments, such as the blood, skin, and mucosal surfaces, will also be important to permit the accurate identification of commensal, opportunistic and putatively pathogenic bacteria.

### 7.2. Novel diagnostics provide new insights into mammalian microbiomes

The employment of deep-sequencing diagnostics to target, amplify and characterise the 16S rRNA gene from mammalian blood, not only provides us with important information on bacterial pathogens present but also gives us substantial insight into the entire blood microbiome (Huggins et al., 2021a, 2020). When exploring the canine blood microbiome, prior research has found that on average over 100 unique 16S rRNA gene sequences are found from a single host, with as many as 1,532 unique sequences found in the most bacterially diverse samples. Such diversity, coupled with high sequencing depth that can reach averages of 110,944 reads per sample, generates phenomenally varied and complex datasets from which key microbiome data can be elucidated and analysed (Huggins et al., 2021a, 2020).

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). Many bacterial taxonomic hits from the canine blood microbiome have been found to match entries labelled with ‘canine oral microbiome’ identifying that they had first been detected through microbiome experiments on this body site (Huggins et al., 2021a, 2020, 2019a). Insights into other canine microbiomes apart from that in the bloodstream are also 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., due to *Pasteurella multocida* (Razali et al., 2020).

Novel diagnostic methods not only assist researchers in elucidating the dog blood bacterial microbiome but also via sequencing of the 18S rRNA gene the 'haemoprotobiome' i.e., the collection of protozoa found in the bloodstream, can be discovered. Studies exploring haemoprotozoan pathogens have now not only been completed in dogs but also 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; Huggins et al., 2021a, 2019b; Mohamed et al., 2021; Ortiz-Baez et al., 2020; Squarre et al., 2020). Such research has identified taxonomic hits from organisms that are unusual, poorly studied and not typically associated as pathogenic or commensal to dogs, for example the kinetoplastid genera *Parabodo*, *Bodo* and *Neobodo*, as well as the apicomplexans *Colpodella* and *Goussia* (Huggins et al., 2021a). From limited prior research conducted into mammalian 'haemoprotobiomes' other authors have identified *Parabodo*, *Bodo* and *Colpodella* in a spectrum of other hosts too, 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 requires future exploration, using corroboratory diagnostics alongside attempted experimental demonstration of Koch's postulates, to resolve whether they truly represent novel pathogens.

### 7.3. Remaining challenges for the use of novel diagnostic tools

Despite the benefits of NGS technologies regarding high sequencing depth and accuracy, like those afforded by Illumina's platforms, many are also severely restricted with regards to the length of reads they can sequence. For example, one of Illumina's longest read kits (MiSeq v3) is capable of sequencing amplicons at 2 × 300 bp, restricting amplicons to a maximum size of approximately 470 – 500 bp long, if paired end overlap is sufficient and sequence quality kept high (Fadrosh et al., 2014; Ravi et al., 2018). This caps the length of the utilisable barcoding gene, meaning that for some 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 many bacterial primers, hence for these taxa species level identification is not possible using short-read metabarcoding alone (Greay et al., 2018b; Gutiérrez et al., 2017; Portillo et al., 2017; Santibáñez et al., 2013). To achieve species level resolution auxiliary molecular methods such as cPCR targeting different gene may need to be used, which can have their own inherent drawbacks such as reduced sensitivity when compared to NGS (Chen et al., 2020; Greay et al., 2018b; Huggins et al., 2019b, 2019a).

For bioinformatic analysis of NGS metabarcoding results, accurate classification of reads is also highly dependent on good reference databases. Databases such as SILVA used by many scientists in the field of microbiome research confer a degree of sequence classification reliability as they are more curated than NCBI's GenBank, with sequences checked for accuracy at the time of submission (Huggins et al., 2021a, 2019b, 2019a; Nilsson et al., 2016; Pruesse et al., 2007; Quast et al., 2013; Shen et al., 2013). Nevertheless, inaccurate taxonomic classifications of sequence entries in both SILVA, GenBank and other repositories are inevitable when

databases are open source, meaning there will always be some reads that are incorrectly classified in microbiome experiments (Balvočiute and Huson, 2017; Nilsson et al., 2016; Shen et al., 2013).

When analysing microbiomes of animals generated by NGS methods it is not always clear if taxa identified are pathogenic, commensal or contaminant, due to the sensitivity of the technology, meaning that all such categories are detected. Some taxa may be from genera that are typically pathogenic or have pathogenic representatives, although whether the exact species found is a pathogen of the host organism it was collected from is not always known (Huggins et al., 2019b; Kim et al., 2017). When assessing these putative pathogens, the employment of rigorous negative controls in NGS methods is invaluable, given that they can highlight the bacteria introduced at the DNA extraction and DNA amplification stages (Huggins et al., 2020). This is particularly important when conducting NGS diagnostics on blood, which has low microbial biomass, sometimes with quantities of bacterial DNA that can be comparable to those found in 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). Hence, accurate and thorough reporting of negative 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).

The challenges of correctly identifying contaminant sequences from those truly in the biological sample are further exacerbated by a dearth in prior research exploring microbiomes of dogs (Figure 2). 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., 2018b; 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, to date there has still been just a handful of studies examining the blood microbiome of dogs, highlighting the need for further scientific attention to progress this nascent field of research (Huggins et al., 2021a, 2020, 2019b, 2019a; Scarsella et al., 2020).

Whilst some taxa elucidated by 16S rRNA metabarcoding experiments cannot easily be allotted into pathogen, commensal or contaminant categories other taxa can be clearly shown to be artefacts of DNA extraction procedures or non-sterile kits (Huggins et al., 2020; Kim et al., 2017). Huggins et al. (2020) 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 microbiome due to it not being sterile (Hornung et al., 2019; Kim et al., 2017). DNA extraction kit-derived reads have been shown to be as many as 80% – 86% of total reads in some cases, an amount high enough to limit the amount of meaningful read data obtainable from the biological samples themselves (Huggins et al., 2020). Data on the 'kitomes' of different extraction kits is therefore crucial to guide microbiome researchers to the best kit for their needs and to maximise data accrued when conducting costly NGS experiments.

The use of positive controls throughout metabarcoding research is also crucial as they can mitigate common challenges associated with NGS experiments, such as primer amplification biases or sequencing errors. The use of commercial microbial

DNA standards can confirm that primers chosen for metabarcoding experiments do not introduce amplification bias, or, if they do, that these are accommodated for accordingly (Hornung et al., 2019; Huggins et al., 2020). Additionally, uniquely identifiable DNA positive controls, such as the synthetic ones used by Huggins et al. (2020), allow for researchers to identify and control for indexing cross-talk, DNA hybridisation, misreading, and cross-contamination during NGS experiments (Huggins et al., 2021a, 2020; Kim et al., 2017).

### 7.4. VBPs of dogs: future opportunities for their diagnosis and chemoprevention

Much of the research herein discussed presents scientists with a spectrum of future possibilities for harnessing the power of novel diagnostics and chemopreventives for the protection and accurate diagnosis of domesticated animals, wildlife and even humans. Many NGS-based methodologies by their very nature show broad utility for the diagnosis of any bacterial or protozoan pathogen, meaning they could be used for the characterisation of VBPs from human patients where infection is suspected but clinical diagnosis has not been possible (Huggins et al., 2021a; Takhampunya et al., 2019). Moreover, such tools may be used on different vertebrate hosts from an ecosystem, to tease apart transmission dynamics and uncover reservoir hosts that may be facilitating the persistence of a given pathogen (Takhampunya et al., 2019).

In fact, prior research in countries of the Asia-Pacific like Cambodia and Thailand, has already used NGS to attempt to explain undiagnosed human sepsis and febrile illness that may be aetiologically linked to zoonoses circulating in nearby animals (Rozo et al., 2020; Takhampunya et al., 2019). Here, the power of NGS to identify pathogens missed by conventional diagnostics has been excellently

demonstrated, 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 emphasise numerous future avenues of research importance, e.g., using NGS diagnostics to classify the VBPs of dog ectoparasites and identify if these overlap with those found on the dog itself, whilst also exploring the blood microbiota of humans and wildlife that live nearby. In addition, other pathogen groups should be targeted, such as filarial nematodes and blood-borne viruses, that would build an even more holistic understanding of the 'pathobiome' that infects canines.

To mitigate the inherent limitations of short-read sequencing, future VBP related work should continue to explore the use of ONT' long-read sequencing platforms, as has been done by Huggins et al. (2022a). Nanopore sequencing is already beginning to be employed at an ever-growing rate for clinical disease diagnosis, with the long-read lengths achievable 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; Huggins et al., 2022a; Petersen et al., 2020). Furthermore, ONT' MinION™ devices are highly portable, meaning that sequencing can be quickly conducted in the field (Quick et al., 2016). MinION™-based surveillance of canine VBPs 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 effectiveness of topical chemopreventive products to protect canines from the risks posed by VBP in the Asia-Pacific, and globally, can also not be overstated.

Such products have been unequivocally demonstrated to reduce transmission of VBPs, even within high infection risk environments, by preventing the feeding of vectors and thereby removing opportunities over which pathogens can be transmitted (Davoust et al., 2003; Fourie et al., 2019a; Fourie et al., 2013a; Huggins et al., 2022b; Jongejan et al., 2016, 2015; Krämer et al., 2020; Pereira et al., 2009). Novel delivery methods for such products, particularly those that extend the window of efficacy over which they act is essential, whilst further research to isolate and synthesise novel compounds capable of killing or repelling vectors is also of critical importance (Figure 2). Moreover, attention within the scientific and veterinary communities should continue to be drawn to the disadvantages of systemic chemopreventives, given that there is mounting evidence these products fail to protect canines from contracting VBPs in settings where ectoparasite pressure is elevated (Huggins et al., 2022b; Jongejan et al., 2016).

The development and use of new sequencing technologies and chemopreventives will undoubtedly play a significant role in what is possibly the 21st century's greatest threat to humankind: anthropogenic climate change (Brooks and Hoberg, 2007; Caminade et al., 2019; Hoberg and Brooks, 2015). Because arthropod vectors are ectothermic, with many depending on water for the completion of life cycle stages, these vectors have an intrinsic connection to climactic 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 including 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). This, in turn, has a large impact on VBP prevalence and transmission as has already been observed with 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, alongside effective topical chemopreventives to protect pets and livestock from infection, will keep us abreast of evolving threats, safeguarding both animal and human health well into the future (Brooks et al., 2014; Zinsstag et al., 2018).

## 8. Concluding remarks

This review reveals how recent advances in novel NGS techniques for pathogen identification and characterisation as well as the development and field validation of ectoparasiticidal products are significantly enhancing our understanding of the VBPs of canines. The use of metabarcoding methods will continue to discover rare, re-emerging and novel pathogens of both dogs and other animals, particularly in regions of the world that are neglected with respect to scientific investigation. This article has highlighted the benefits conferred by diagnosis through deep-sequencing, but also the challenges, including the current need for substantial technical expertise regarding experimental design and bioinformatic analyses when working with NGS datasets. Furthermore, this article has appraised existing options for chemoprevention of canine VBPs, with a particular focus on regions with high infection pressure in the Asia-Pacific. Current research into topical ectoparasiticides repeatedly demonstrates their ability to prevent vector feeding and stop VBP transmission in a way that is not mirrored by systemic products that rely on ectoparasite haematophagy to take effect. Such information should be a clarion call for veterinarians working in the Asia-Pacific to inform and recommend appropriate chemopreventive products, whilst the lessons

learnt from the tropics also have critically important implications for the betterment of canine health across the globe as well.

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# Tables

**Table 1. Vector-borne pathogens detected in dogs from the Asia-Pacific.** Diagnostic methods include next-generation sequencing (NGS) metabarcoding, conventional PCR (cPCR), quantitative PCR (qPCR) and serology. Percentage of positive dogs detected, total dogs sampled and 95% confidence intervals for each study are shown.

| Vector-borne pathogen | Country | Diagnostic method | % Test-positive (proportion; 95% confidence interval) | Reference |
|---|---|---|---|---|
| **Bacteria** | | | | |
| *Anaplasma platys* | Australia | cPCR | 21 (49/230; 17 - 27) | Hii et al. (2012) |
| | Cambodia | NGS metabarcoding | 32 (150/467; 28 - 37) | Huggins et al. (2021a) |
| | China | Serology | 0.2 (1/481; 0 - 1) | Colella et al. (2020) |
| | Indonesia | cPCR | 12 (6/51; 6 - 23) | Disna Faizal et al. (2019) |
| | Japan | cPCR | Single case report | Unver et al. (2003) |
| | Malaysia | cPCR | 13 (4/30; 5 - 30) | Mokhtar et al. (2013) |
| | Myanmar | cPCR | 0.25 (1/400; 0 - 1) | Hmoon et al. (2021) |
| | Philippines | cPCR | 40 (10/25; 23 - 59) | Ybañez et al. (2016) |
| | Samoa | cPCR and serology | 8 (20/237; 6 - 13) | Carslake et al. (2017) |
| | Singapore | Serology | 2.6 (3/116; 1 - 7) | Colella et al. (2020) |
| | Taiwan | Serology | 3 (4/132; 1 - 8) | Colella et al. (2020) |
| | Thailand | NGS metabarcoding | 25 (25/100; 18 - 34) | Huggins et al. (2019a) |
| | Vietnam | cPCR | 4 (1/25; 1 - 20) | Chien et al. (2019) |
| *Bartonella clarridgeiae* | Cambodia | NGS metabarcoding | 3 (12/467; 1 - 4) | Huggins et al. (2021a) |
| | Thailand | NGS metabarcoding | 1 (1/100; 0.2 - 5) | Huggins et al. (2019a) |
| *Bartonella elizabethae* | Thailand | cPCR | 0.5 (1/192; 0 - 3) | Bai et al. (2010) |
| *Bartonella henselae* | Australia | cPCR | Single case report | Shapiro et al. (2017) |
| | Japan | Serology | 8 (4/52; 3 - 18) | Tsukahara et al. (1998) |
| | Philippines | cPCR | 11 (13/116; 7 - 18) | Singer et al. (2020) |
| | Taiwan | Serology | 4 (65/1630; 3 - 5) | Yi Ting et al. (2009) |
| *Bartonella quintana* | New Zealand | cPCR | Single case report | Kelly et al. (2006) |

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| | Thailand | cPCR | 0.5 (1/192; 0 - 3) | Bai et al. (2010) |
| *Bartonella taylorii* | Thailand | cPCR | 0.5 (1/192; 0 - 3) | Bai et al. (2010) |
| *Bartonella vinsonii* | China | cPCR | 3 (2/71; 0.8 - 10) | Li et al. (2006) |
| | Taiwan | Serology | 4 (67/1630; 3 - 5) | Yi Ting et al. (2009) |
| | Thailand | cPCR | 5 (10/192; 3 - 9) | Bai et al. (2010) |
| *Borrelia burgdorferi* (s.l.) | China | Serology | 0.2 (1/600; 0 - 1) | Xia et al. (2012) |
| | Japan | Serology | 10 (32/314; 7 - 14) | Uesaka et al. (2016) |
| | Indonesia | Serology | 1 (1/95; 0.2 - 6) | Colella et al. (2020) |
| | Philippines | Serology | 0.8 (1/120; 0.2 - 5) | Colella et al. (2020) |
| | South Korea | Serology | 3 (12/430; 2 - 5) | Miranda et al. (2022) |
| | Thailand | cPCR | 0.2 (1/402; 0 - 1) | Sthitmatee et al. (2016) |
| *Coxiella burnetii* | Australia | Serology | 4 (43/1223; 3 - 5) | Shapiro et al. (2016) |
| | Japan | Serology | 15 (95/632; 12 - 18) | Htwe et al. (1992) |
| | Malaysia | cPCR | 33 (62/188; 27 - 40) | Tukur et al. (2019) |
| | Taiwan | cPCR | 6 (42/720; 4 - 8) | Chou et al. (2014) |
| *Ehrlichia canis* | Australia | cPCR and serology | Multiple case reports | Neave et al. (2022) |
| | Cambodia | NGS metabarcoding | 20 (93/467; 17 - 24) | Huggins et al. (2021a) |
| | China | Serology | 2 (9/481; 1 - 4) | Colella et al. (2020) |
| | Indonesia | Serology | 36 (34/95; 27 - 46) | Colella et al. (2020) |
| | Japan | cPCR | Single case report | Unver et al. (2003) |
| | Myanmar | cPCR | 0.75 (3/400; 0.2 - 2) | Hmoon et al. (2021) |
| | Malaysia | cPCR | 2 (10/500; 1 - 4) | Nazari et al. (2013) |
| | Philippines | cPCR | 3 (2/70; 1 - 10) | Corales et al. (2014) |
| | Singapore | Serology | 5 (6/116; 2 - 11) | Colella et al. (2020) |
| | South Korea | Serology | 6 (14/229; 4 - 10) | Lim et al. (2010) |
| | Taiwan | Serology | 2 (2/132; 0.4 - 5) | Colella et al. (2020) |
| | Thailand | NGS metabarcoding | 40 (40/100; 31 - 50) | Huggins et al. (2019a) |
| | Vietnam | Serology | 26 (31/120; 19 - 34) | Colella et al. (2020) |
| *Candidatus Mycoplasma haematoparvum* | Australia | cPCR | 1 (3/230; 0.4 - 4) | Hii et al. (2012) |
| | Cambodia | NGS metabarcoding | 0.2 (1/467; 0 - 1) | Huggins et al. (2021a) |
| | South Korea | cPCR | 43 (190/440; 39 - 48) | Suh et al. (2017) |
| | Thailand | NGS metabarcoding | 3 (3/100; 1 - 8) | Huggins et al. (2019a) |
| *Candidatus Mycoplasma haemobos* | Australia | cPCR | 0.4 (1/230; 0 - 2) | Hii et al. (2012) |
| | China | cPCR | 20 (11/55; 12 - 32) | Shi et al. (2022) |

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| *Candidatus Mycoplasma haemominutum* | China | cPCR | 3 (1/40; 0.4 - 13) | Zhuang et al. (2009) |
| | Japan | cPCR | Single case report | Obara et al. (2011) |
| | Thailand | cPCR | Unspecified of 181 total dogs | Liu et al. (2016) |
| *Mycoplasma haemocanis* | Australia | cPCR | 10 (23/230; 7 - 15) | Hii et al. (2012) |
| | Cambodia | NGS metabarcoding | 13 (60/467; 10 - 16) | Huggins et al. (2021a) |
| | China | cPCR | 2 (3/162; 0.6 - 5) | Zheng et al. (2017) |
| | Japan | cPCR | 4 (37/913; 3 - 6) | Sasaki et al. (2008) |
| | Philippines | qPCR | 0.8 (1/120; 0.2 - 5) | Zarea et al. (2022) |
| | South Korea | cPCR | 38 (168/440; 34 - 43) | Suh et al. (2017) |
| | Taiwan | qPCR | 2 (2/132; 0.4 - 5) | Zarea et al. (2022) |
| | Thailand | NGS metabarcoding | 34 (34/100; 25 - 44) | Huggins et al. (2019a) |
| *Candidatus Mycoplasma turicensis* | Thailand | NGS metabarcoding | 2 (2/100; 1 - 7) | Huggins et al. (2019a) |
| *Rickettsia canadensis** | Thailand | Serology | 4 (2/49; 1 - 14) | Suksawat et al. (2001) |
| *Rickettsia conorii* | China | cPCR | 4 (3/78; 1 - 11) | Liang et al. (2012) |
| *Rickettsia felis* | Australia | cPCR | 2 (3/130; 1 - 7) | Hii et al. (2011b) |
| | Cambodia | cPCR | 11 (11/101; 6 - 18) | Inpankaew et al. (2016) |
| | China | cPCR | 0.8 (8/1,059: 0.3 - 1) | Zhang et al. (2014) |
| *Rickettsia japonica* | Japan | Serology | 2 (20/1,207; 1 - 3) | Tabuchi et al. (2007) |
| *Rickettsia prowazekii** | Thailand | Serology | 24 (12/49; 15 - 38) | Suksawat et al. (2001) |
| *Rickettsia raoultii* | China | cPCR | 6 (28/496; 4 - 8) | Shao et al. (2021) |
| *Rickettsia rickettsii** | Thailand | Serology | 12 (6/49; 6 - 24) | Suksawat et al. (2001) |
| *Rickettsia* spp.^ | Thailand | cPCR | 15 (15/100; 9 - 23) | Huggins et al. (2019a) |
| *Candidatus Rickettsia tarasevichiae* | China | cPCR | 0.4 (2/496; 0.1 - 1) | Shao et al. (2021) |
| **Protozoans** | | | | |
| *Babesia gibsoni* | Australia | cPCR | Three case reports | Muhlnickel et al. (2002) |
| | China | cPCR | 2 (11/481; 1 - 4) | Colella et al. (2020) |
| | Japan | cPCR and serology | 30 (35/115; 23 - 39) | Miyama et al. (2005) |
| | Malaysia | cPCR | 3 (1/30; 1 - 17) | Mokhtar et al. (2013) |
| | Singapore | cPCR | 0.9 (1/116; 0.2 - 5) | Colella et al. (2020) |
| | South Korea | cPCR | 25 (29/117; 18 - 33) | Lee et al. (2009) |

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| | Taiwan | Unspecified | 1 (36/3174; 0.8 - 2) | Liu and Su, (2015) |
| | Thailand | cPCR | 6 (11/174; 4 - 11) | Do et al. (2021) |
| *Babesia vogeli* | Australia | cPCR | 11 (24/215; 8 - 16) | Brown et al. (2006) |
| | Cambodia | NGS metabarcoding | 14 (67/467; 11 - 18) | Huggins et al. (2021a) |
| | China | cPCR | 5 (8/162; 3 - 9) | Zheng et al. (2017) |
| | Malaysia | cPCR | 2 (5/240; 1 - 5) | Prakash et al. (2018b) |
| | Philippines | cPCR | 7 (17/248; 4 - 11) | Galay et al. (2018) |
| | Taiwan | qPCR | 0.8 (2/265; 0.2 - 3) | Yang et al. (2022) |
| | Thailand | NGS metabarcoding | 13 (13/100; 8 - 21) | Huggins et al. (2019b) |
| *Hepatozoon canis* | Australia | cPCR | Single case report | Greay et al. (2018a) |
| | Cambodia | NGS metabarcoding | 18 (84/467; 15 - 22) | Huggins et al. (2021a) |
| | China | cPCR | 0.8 (4/481; 0.3 - 2) | Colella et al. (2020) |
| | Japan | cPCR | 41 (81/496; 13 - 20) | El-Dakhly et al. (2013) |
| | Malaysia | cPCR | 3 (8/240; 2 - 6) | Prakash et al. (2018a) |
| | Philippines | cPCR | 2 (6/248; 1 - 5) | Galay et al. (2018) |
| | South Korea | cPCR | Single case report | Kwon et al. (2017) |
| | Taiwan | cPCR | 2 (15/714; 1 - 3) | Huang and Tsang, (2011) |
| | Thailand | NGS metabarcoding | 38 (38/100; 29 - 48) | Huggins et al. (2019b) |
| | Vietnam | cPCR | 0.8 (1/120; 0.2 - 5) | Colella et al. (2020) |
| *Leishmania infantum* | China | Serology | 0.4 (2/481; 0.1 - 2) | Colella et al. (2020) |
| | Philippines | Serology | 0.8 (1/120; 0.2 - 5) | Colella et al. (2020) |
| | South Korea | cPCR | Single case report | Bhang et al. (2013) |
| | Vietnam | Serology | 0.8 (1/120; 0.2 - 5) | Colella et al. (2020) |
| *Trypanosoma evansi* | Cambodia | NGS metabarcoding | 0.2 (1/467; 0 - 1) | Huggins et al. (2021a) |
| | Malaysia | Microscopy | 0.07 (3/4084; 0 - 0.2) | Rajamanickam et al. (1985) |
| | Thailand | NGS metabarcoding | 1 (1/100; 0.2 - 5) | Huggins et al. (2019b) |
| | Vietnam | cPCR | Single case report | Bui et al. (2021) |
| **Filaroids** | | | | |
| *Brugia malayi* | Thailand | cPCR | 7 (4/57; 3 - 17) | Satjawongvanit et al. (2019) |
| | Vietnam | cPCR | 0.8 (1/120; 0.2 - 5) | Colella et al. (2020) |
| *Brugia pahangi* | Malaysia | cPCR | 2 (1/45; 0.4 - 12) | Colella et al. (2020) |
| | Thailand | cPCR | 23 (13/57; 14 - 35) | Satjawongvanit et al. (2019) |
| *Dirofilaria immitis* | Australia | Serology | 2 (13/566; 1 - 4) | Orr et al. (2020) |

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| | Cambodia | cPCR | 16 (16/101; 10 - 24) | Inpankaew et al. (2016) |
| | China | cPCR | 24 (147/886: 14 - 19) | Hou et al. (2011) |
| | Japan | Serology | 23 (23/100; 16 - 32) | Oi et al. (2014) |
| | Indonesia | Microscopy | 15 (22/151; 10 - 21) | Erawan et al. (2017) |
| | Malaysia | cPCR | 7 (3/45; 2 - 18) | Colella et al. (2020) |
| | Philippines | cPCR | 14 (16/120; 8 - 21) | Colella et al. (2020) |
| | Samoa | Serology | 47 (111/237; 41 - 53) | Carslake et al. (2017) |
| | Singapore | Serology | 3 (3/116; 1 - 7) | Colella et al. (2020) |
| | South Korea | Serology | 21 (17/81; 14 - 31) | Song et al. (2010) |
| | Taiwan | cPCR | 5 (6/132; 2 - 10) | Colella et al. (2020) |
| | Thailand | cPCR | 24 (95/394; 20 - 29) | Kamyingkird et al. (2017) |
| | Vietnam | cPCR | 0.8 (1/120; 0.2 - 5) | Colella et al. (2020) |
| *Thelazia callipaeda* | China | cPCR | 2 (8/481; 0.9 - 3) | Colella et al. (2020) |
| | South Korea | Microscopy | 18 (28/154; 13 - 25) | Seo et al. (2002) |
| | Taiwan | Microscopy | Single case report | Yang et al. (2006) |
| | Thailand | Microscopy | Single case report | Bhaibulaya et al. (1970) |

*Ability for serological detection to accurately classify to a species level taxonomy was not confirmed by the authors of these references.

^*Rickettsia* species confirmed as either *Rickettsia asembonensis* or *Rickettsia felis.*

**Table 2. The major ectoparasiticide classes and compounds used in dogs for protection against ectoparasite infestation and vector-borne pathogen (VBP) infection.** Ectoparasiticide method of application, either topical, systemic or both are shown, alongside the target receptor in the arthropod vector, against which the ectoparasiticide acts. GABA refers to gamma-aminobutyric acid chloride channels.

| Drug group | Ecto-parasiticide | Products containing ectoparasiticide: Trade name and company | Method of application | Arthropod target receptor | Reference |
|---|---|---|---|---|---|
| Amidines (Formamidines) | Amitraz | Mitaban® (Zoetis), Preventic® (Virbac), Certifect® (Merial) | Topical | Octopamine (G-protein coupled) | Taylor (2001) |
| Carbamates | Propoxur | Kiltix® (Elanco), Bolfo® (Elanco), Proxurr™ (Veko) | Topical | Acetylcholinesterase | Taylor (2001) |
| Isoxazolines | Afoxolaner | NexGard® (Boehringer Ingelheim), NexGard Spectra® (Boehringer Ingelheim) | Systemic | Chloride channels (GABA & glutamate gated) | Zhou et al. (2021) |
| | Fluralaner | Bravecto® (MSD Animal Health) | Systemic | Chloride channels (GABA & glutamate gated) | Zhou et al. (2021) |
| | Lotilaner | Credelio® (Elanco) | Systemic | Chloride channels (GABA & glutamate gated) | Zhou et al. (2021) |
| | Sarolaner | Simparica® (Zoetis), Simparica Trio® (Zoetis) | Systemic | Chloride channels (GABA & glutamate gated) | Zhou et al. (2021) |
| Macrocyclic Lactones | Ivermectin | Heartgard® (Merial), Exelpet™ (Mars) | Systemic | Chloride channels (GABA & glutamate gated) | Taylor (2001) |
| | Milbemycin oxime | Interceptor™ (Elanco), Interceptor™ (Elanco), Trifexis® (Elanco) | Systemic | Chloride channels (GABA & glutamate gated) | Taylor (2001) |
| | Moxidectin | Advantage Multi® (Elanco), Advocate® (Elanco), ProHeart®12 | Topical & Systemic | Chloride channels (GABA & glutamate gated) | Petry et al. (2015) |

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| | | (Zoetis), Simparica Trio® (Zoetis) | | | |
| | Selamectin | Revolution® (Zoetis), Paradyne®(Zoetis), Stronghold® (Zoetis) | Topical & Systemic | Chloride channels (GABA & glutamate gated) | Taylor (2001) |
| Neonicotinoids | Dinotefuran | Vectra® (Ceva), Vectra 3D® (Ceva) | Topical | Acetylcholine | Franc et al. (2012) |
| | Imidacloprid | Advantage® (Elanco), Seresto® (Elanco), Advantix® (Elanco), Advocate® (Elanco) | Topical | Acetylcholine (Nicotinic) | Stanneck et al. (2012c) |
| | Nitenpyram | Capstar™ (Elanco) | Systemic | Acetylcholine (Nicotinic) | Dobson et al. (2000) |
| Oxadiazines | Indoxacarb | Activyl® (MSD Animal Health), Activyl Tick Plus® (MSD Animal Health) | Topical | Sodium channels | Dryden et al. (2013) |
| Phenypyrazoles | Fipronil | Frontline® (Boehringer Ingelheim), Parastar® (Novartis), Effipro® (Virbac) | Topical | GABA gated | Dryden et al. (2013) |
| Pyrethrins & Synthetic Pyrethroids | Deltamethrin | Scalibor® (MSD Animal Health), Canishield® (Beaphar), Salvo™ (Durvet), Proact™ (Tevrapet) | Topical | Sodium channels | Kazimoto et al., (2018) |
| | Flumethrin | Seresto® (Elanco), Kiltix® (Elanco) | Topical | Sodium channels | Stanneck et al. (2012c) |
| | Permethrin | Advantix® (Elanco), Vectra 3D® (Ceva), Activyl Tick Plus® (MSD Animal Health) | Topical | Sodium channels | Franc et al. (2012) |
| | Pyrethrin | Shampoo (Fido's), Flea & Tick Shampoo (Aristopet Animal Health), Flea & Tick Dip (Bio-Groom) | Topical | Sodium channels | Taylor (2001) |
| Spinosyns | Spinosad | Comfortis™ (Elanco), Comfortis Plus™ | Systemic | Acetylcholine (Nicotinic) | Blagburn et al. (2010) |

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| | | (Elanco), Trifexis® (Elanco) | | | |