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Author/s:
Huggins, LG;Colella, V;Koehler, AV;Schunack, B;Traub, RJ

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

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1 **Title: A multipronged next-generation sequencing metabarcoding approach unearths hyper-**
2 **diverse and abundant dog pathogen communities in Cambodia**

3 **Running Title: NGS characterises vector-borne pathogens in Cambodian dogs**

4 **Authors:** Lucas G. Huggins[1*], Vito Colella[1], Anson V. Koehler[1], Bettina Schunack[2] & Rebecca J.
5 Traub[1]

6 [1]Faculty of Veterinary and Agricultural Sciences, University of Melbourne, Parkville, Victoria 3050,
7 Australia

8 [2]Elanco GmbH, Heinz-Lohmann-Str. 4, 27472 Cuxhaven, Germany

9 *Correspondence: lghuggins@student.unimelb.edu.au

10 **Summary**

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

32 *Keywords:* next-generation sequencing, vector-borne pathogens, Cambodia, zoonosis,
33 metabarcoding, dogs

34 **Introduction**

35 Vector-borne pathogens (VBP) or those predominantly transmitted by blood-feeding arthropods,
36 inflict a significant toll with regard to morbidity and mortality on dogs, and nowhere is this more

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

12 Some of the most common vector-borne diseases of dogs in the tropics are caused by members
13 of the bacterial Anaplasmataceae family, including *Anaplasma platys* and *Ehrlichia canis* (Little,
14 2010; Ng-Nguyen et al., 2020). *Ehrlichia canis* causes canine monocytic ehrlichiosis (CME) a
15 frequently lethal multisystemic disease. In contrast, *A. platys* is less pathogenic, although clinical
16 signs can be exacerbated when dogs are coinfected with other VBPs (Hii et al., 2015; Inpankaew
17 et al., 2016; Huggins et al., 2019a). Apicomplexan VBPs, such as *Babesia* spp. and *Hepatozoon*
18 *canis* are also endemic to Southeast Asia (Irwin and Jefferies, 2004; Inpankaew et al., 2016; Liu
19 et al., 2016; Huggins et al., 2019b) and generate disease that varies in pathogenicity from
20 relatively mild in the case of hepatozoonosis, to severe, for example in the case of acute
21 haemolytic anaemia caused by babesiosis, in particular when *Babesia gibsoni* is implicated
22 (Ivanov and Tsachev, 2008; Kirk et al., 2017). With the exception of *B. gibsoni,* the
23 aforementioned VBP are all vectored by the brown-dog tick, *Rhipicephalus sanguineus* that is
24 ubiquitous in tropical Southeast Asia (Ng-Nguyen et al., 2020). However, unlike many VBPs, *H.*
25 *canis* is transmitted via ingestion of the infected brown dog tick, as opposed to blood-feeding
26 (Baneth et al., 2003).

27 Additionally, kinetoplastid protozoans such as *Leishmania infantum* also pose a risk to dogs and
28 humans (Alvar et al., 2012; Medkour et al., 2019). In Southeast Asia, *Trypanosoma evansi*, the
29 cause of 'surra' in ruminants, also infects dogs through biting flies or ingestion of infected raw
30 meat to produce severe clinical signs and death (Barameechaithanun et al., 2009; Rjeibi et al.,
31 2015; Bui et al., 2021).

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

1 Next-generation sequencing (NGS)-based methodologies permit surveillance with no *a priori*
2 knowledge of VBPs as they can be designed to target whole communities of organisms via
3 sequencing of conserved genes such as the small ribosomal RNA subunit (16S rRNA) gene in
4 bacteria or 18S rRNA gene in eukaryotes (Huggins et al., 2020; Flaherty et al., 2021).
5 Metabarcoding or the collective amplification and sequencing of all taxonomically informative
6 genetic regions from a group of interest can be used as a powerful tool for characterising
7 pathogen communities in both vector and mammalian hosts (Flaherty et al., 2018; Greay et al.,
8 2018; Chaudhry et al., 2019; Huggins et al., 2019b). These methods are being increasingly utilised
9 to detect known, as well novel bacterial and protozoan haemoparasites from domestic animals
10 and a range of other mammalian species (Huggins et al., 2019a, 2019b; Takhampunya et al., 2019;
11 Ghafar et al., 2020, 2021; Glidden et al., 2020; Mohamed et al., 2021).

12 Cambodia, like other countries in Asia, harbours a large population of free-roaming or poorly-
13 cared for semi-domesticated dogs, at an estimated rate of one dog per every three people,
14 equating to approximately 5 million dogs (Sor et al., 2018). These dogs are often infected by
15 multiple canine VBPs due to limited veterinary service availability (Inpankaew et al., 2016;
16 Huggins et al., 2020). In light of such data, we used recently developed NGS-based metabarcoding
17 assays (Huggins et al., 2019b, 2019a, 2020) to holistically detect i.e. detect DNA sequences of all
18 relevant bacterial and protozoan haemoparasites, in an unbiased manner from dogs across
19 Cambodia.

20 **Methods:**

21 *Study sites and sample collection*

22 Dog blood samples were collected from five different study areas across Cambodia each
23 containing between three and six study sites. Samples were collected in the months of April, May
24 and September 2019, thereby covering Cambodia's drier, warmer northeast monsoon season
25 and the wetter southwest monsoon season (Brook et al., 2008). Study areas included
26 metropolitan Phnom Penh, the Cambodian capital (11°31'N, 104°55'E), Kampong Chhnang town
27 (12°15'N, 104°39'E), Siem Reap city (13°26'N, 103°45'E), Battambang city (13°25'N, 103°73'E)
28 and Tbong Khmum district (12°01'N, 105°30'E) as displayed in **Figure 1**. Study sites were selected
29 based on trying to achieve a cross-section of densely populated and lightly populated urban
30 areas, including rural towns as well as urban areas surrounded by both farmland and rainforest.
31 Climate across all study sites is similar. Study site selection was also influenced by the existence
32 of local rabies eradication programs, whereby opportunistic canine sampling could be conducted
33 at the same time as administration of rabies vaccinations. Dogs sampled were from a mixture of
34 locally owned animals and pagoda community dogs cared for by monks. Most sampled dogs from
35 all field areas lacked access to adequate veterinary care, including anti-parasiticidal treatments,
36 desexing and vaccination. There were no exclusion criteria for dog sampling apart from whether
37 the individual was too aggressive to capture and restrain.

A total of 467 dogs were sampled across the study's entirety; Phnom Penh (PP), n = 135, Kampong Chhnang (KC), n = 48, Siem Reap (SR), n = 92, Battambang (BB), n = 92 and Tbong Khmum (TK), n = 100. Dogs were humanely restrained, and blood was only taken following informed consent from the relevant monk in the case of pagoda dogs or the owner for semi-domesticated community and pet dogs. Two ml whole blood was collected by a veterinarian via cephalic or jugular puncture into two anti-coagulation EDTA tubes and temporarily kept on ice. Blood samples subjected to NGS assays were stored at -20°C until further use, whilst the second EDTA tube was refrigerated at 4°C and processed within 48 hours for packed cell volume (PCV), total protein and subjected to the commercial SNAP 4Dx Plus Test (IDEXX, Westbrook, ME, USA) which detects antibodies to *Anaplasma platys/phagocytophilum*, *Ehrlichia canis/ewingii*, *Borrelia burgdorferi* sensu lato (all serotypes) and antigens of *Dirofilaria immitis*.

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

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

DNA extraction

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

Bacterial 16S rRNA gene metabarcoding

The 467 blood samples collected were analysed using our previously developed bacterial 16S rRNA gene targeting metabarcoding protocol (Huggins et al., 2019a, 2020). In brief, our methodology involved first-step amplification of 100 blood extracted DNA samples plus 12 controls, per batch. This required use of 1 µl of DNA template, 10 µL of OneTaq® 2X Master Mix with Standard Buffer (New England Biolabs, Ipswich, MA, USA), 0.4 µl of primer WehiNGS_Adp_F and WehiNGS_Adp_R at 10 µM, alongside 1.2 µl of the canine mitochondrial DNA blocking primer; Canis-mito-blk (Huggins et al., 2020) with the reaction made up to 20 µL with Ambion Nuclease-Free Water (Life Technologies, Carlsbad, CA, USA). All sample batches were also run with four positive controls, four no DNA template PCR controls and four blood-omitted sample extraction controls i.e. DNA extracted from extraction reagents only at the same time as blood DNA extractions. Positive controls were uniquely identifiable synthetic DNA constructs as defined

in Huggins et al. 2020, diluted to 1 x 10^{-7} ng/µl synthesised by Integrated DNA Technologies (Coralville, IA, USA), see supporting information for sequences. Thermocycling conditions were 95 °C for 3 min followed by 20 cycles of 95 °C for 45 s, 56 °C for 60 s and 72 °C for 90 s with a final elongation at 72 °C for 10 min conducted on a MiniAmp™ Thermal Cycler (Thermo Fisher Scientific, Waltham, MA, USA). PCR product was then cleaned using 1X Ampure Beads (Beckman Coulter, Brea, CA, USA) and checked for quality on an Agilent 2200 Tape Station (Agilent Technologies, Santa Clara, CA, USA) before proceeding. Finally, the second-step PCR addition of library indexes, followed by deep sequencing was conducted according to Huggins et al. (2019a) using an Illumina MiSeq (Illumina, San Diego, CA, USA) with 600-cycle v3 chemistry at the Walter & Eliza Hall Institute Proteomics Facility, Parkville, Australia.

Apicomplexan and kinetoplastid 18S rRNA gene metabarcoding

The same subsample used for the bacterial 16S rRNA gene metabarcoding was also analysed using our previously developed 18S rRNA gene protocol designed to target parasitic apicomplexan and kinetoplastid organisms (Huggins et al., 2019b). In brief, batches of 104 samples were amplified using 2 µl of DNA template and the previously reported ApicomplexF and ApicomplexR primers modified to include indexing overhangs (Huggins et al., 2019b) alongside two no DNA template PCR controls, two DNA extraction controls and two synthetic DNA construct positive controls (Supporting information). For kinetoplastid detection the same samples were also separately amplified using the KinetoF and KinetoR primers, with indexing overhangs plus four negative and two positive controls (Supporting information). For apicomplexan PCR amplification thermocycling conditions were 94 °C for 5 min, followed by 25 cycles of 94 °C for 30 s, 52 °C annealing for 30 s and 72 °C for 30 s with a final elongation at 72 °C for 5 min, this was the same for kinetoplastid PCR except for that annealing was conducted at 56 °C instead. PCR product was then processed in the same manner as that for bacterial 16S rRNA gene metabarcoding apart from that the second step indexing PCR used just 20 cycles of amplification to add on multiplexing indexes as opposed to the 24 cycles originally reported in Huggins et al. 2019b. After addition of indexes, batches of 104 apicomplexan targeting PCR products were pooled with 104 kinetoplastid targeting PCR products, producing multiplexed pools of 208 samples achievable via 16 unique forward indexes and 13 unique reverse indexes. Finally, pools were run on an Illumina MiSeq using 300-cycle v2 chemistry (2 x 150 bp paired-end reads).

Bioinformatics

Raw NGS data was processed according to Huggins et al. 2020 with all bioinformatic steps conducted in QIIME2 version 2020.11. DADA2 truncation parameters were chosen via inspection of read quality plots and subsequent exploration of parameters to find those that generated the most post-filter sequences for each batch of samples (Callahan et al., 2016). Amplicon sequence variants (ASVs) were again chosen over operational taxonomic units (OTUs) so as to minimise data loss at each processing step (Langille et al., 2018; Knight et al., 2018) and sequences were classified using sci-kit learn built classifiers working from the latest available SILVA reference

database (version 138.1) (Pedregosa et al., 2011). Additionally, ASVs were taxonomically identified using the BLASTn program in GenBank (NCBI) to corroborate classifications and in some cases classify down to a lower taxonomic level than possible using QIIME2 classifiers. Alpha rarefaction plots were also generated, using MAFFT and FastTree 2 to confirm that ASV diversity plateaued and hence a sufficient sequencing depth had been achieved (Price et al., 2010; Katoh and Standley, 2013). All NGS data produced in the present study are available from the NCBI BioProject database, BioProjectID: PRJNA601241, BioSampleIDs: SAMN13870739 to SAMN13870796 and SAMN18323289 to SAMN18323754. Raw NGS read data for bacteria, apicomplexans and kinetoplastids is available from the NCBI sequence read archive (SRA) under the aforementioned BioSample accession numbers.

Sequences of taxa found in no-reagent DNA extraction negative controls and no-template PCR negative controls were removed from the overall dataset using the QIIME2 taxonomy-based filtering command which can be used to exclude negative control taxa from further downstream analysis. These taxa were excluded as they likely represent organism's DNA introduced via DNA extraction and PCR amplification materials as well as from the environment. High read counts of these taxa in non-control samples may have been indicative of their endogenous presence in blood samples, however none of such taxa were from pathogenic or putatively pathogenic genera and hence further analysis was not deemed necessary.

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

Conventional PCR and Sanger sequencing

For some samples with reads that could not be assigned to species level classification, endpoint PCR assays were run to amplify larger gene regions or more informative genes for better characterisation (Table 1). These cPCRs were conducted using HotStarTaq DNA Polymerase (Qiagen) according to the relevant author's methods (Table 1). Positive amplicons were then cleaned using ExoSAP-IT™ PCR Product Cleanup Reagent (Thermo Fisher Scientific) and sent to

1 Macrogen (Seoul, South Korea) for Sanger sequencing. Results were then run through BLASTn, to
2 compare them to existing GenBank reference sequences and corroborate with NGS findings, i.e.
3 to ascertain whether cPCR Sanger sequences returned the same taxonomic classification in
4 GenBank as NGS reads classified using the SILVA database.

5 **Table 1.** Corroboratory qPCR and cPCR assays used, only those that were successful at cross-validating
6 NGS results are shown.

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

7

8 *Statistical & risk indicator analyses*

9 The 95% confidence intervals (CIs) were calculated using the Wilson score interval via the open
10 source software Epitools (https://epitools.ausvet.com.au). Chi-square (χ^2) statistical methods
11 calculated using IBM SPSS Statistics 27 (SPSS, Chicago, IL, USA) were used to compare measured
12 categorical variables against NGS positivity for VBPs. A multiple logistic regression model was
13 then used to assess dog risk factors to VBP exposure in GraphPad Prism 9 (GraphPad Software,
14 San Diego, CA, USA). VBP exposure was here defined as dogs found positive to a pathogen via
15 NGS or a SNAP 4Dx Plus Test (IDEXX). Variables that were tested were sampling seasons (wet or
16 dry), dog age, sex, husbandry, temperature, presence of ectoparasites, haircoat condition, body
17 score, dog demeanour, PCV and total protein (as a continuous variable). Additional variables
18 tested were for the presence of enlarged lymph nodes, abdominal palpation condition e.g.
19 hepatosplenomegaly, respiration rate for example panting as well as mucous membrane
20 condition i.e. normal or abnormal with abnormal considered as signs of pallor, jaundice or
21 presence of petechiae. Variables with a significance of < 0.2 found via univariate analysis were
22 included in the regression model. PCV was converted to the binary variable anaemic i.e. PCV <
23 35% or non-anaemic PCV > 35% for this model.

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

1 Kappa statistics were used to compare agreement of NGS positivity for *A. platys* and *E. canis* vs
2 results from SNAP 4Dx Plus (IDEXX) in SPSS Statistics 27. We used the formula defined in McHugh
3 (2012) with the agreement between tests considered poor if $k \leq 0.20$, fair if $0.21 \leq k \leq 0.40$,
4 moderate if $0.41 \leq k \leq 0.60$, substantial if $0.61 \leq k \leq 0.80$ and high if $k > 0.81$ (McHugh, 2012).

5 **Results**

6 *Sample collection*

7 Our study sample of 467 dogs assessed for vector-borne pathogens consisted of 213 (45.6%)
8 females, 246 (52.7%) males and 8 (1.7%) with unreported data (Table 2). Dog ages ranged from
9 one month to seven years and older with 142 (30.4%) dogs in the youngest age category of six
10 months and younger, 96 (20.6%) dogs between six months and a year, 200 (42.8%) dogs between
11 one and seven years old, 18 (3.9%) dogs over seven years of age and 11 (2.3%) with unreported
12 data. Only 12 (2.6%) dogs were pure bred, including an Alsatian, a Golden Retriever, an English
13 Bulldog, a Labrador, Huskies and German Shepherds. Just two dogs (0.4%) were found to be
14 neutered. It was more common for dogs to have at least one ectoparasite present, with 344
15 (73.7%; 95% CI = 69% - 77%) of dogs found infested with ticks, fleas and/or lice (Table 2).

16

17 **Table 2.** Distribution, sex, age, breed and presence of ectoparasites across the 467 canine samples
18 assessed for vector-borne pathogens by our NGS-based methodology (PP = Phnom Penh, KC = Kampong
19 Chhnang, SR = Siem Reap, BB = Battambang, TK = Tbong Khmum). At some study sites category totals do
20 not equal the relevant sample area total due to unreported data.

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

21

22 *Bioinformatic processing*

23 A total of five separate 600-cycle MiSeq runs were used to deep-sequence all 467 samples for
24 bacterial pathogens as well as 60 negative and positive control samples, with the highest raw
25 reads obtained for any run at 14,880,659 and the lowest at 9,636,742 (mean ± SE = 11,446,984 ±
26 907,014) reads. After bioinformatic filtering and quality control steps, the mean total filtered
27 reads per batch was 9,106,146 ± 818,499 (Table 3). This translated to an average of 9,191 ± 1,610
28 ASVs or unique reads per batch and an average median read per sample of 74,640 ± 9,454.

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

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

1 **Table 3.** NGS read data across MiSeq batches pre and post-filtering. Bacterial batches show results from
2 600-cycle MiSeq kits, whilst apicomplexan and kinetoplastid samples were pooled and run on 300-cycle
3 MiSeq runs, hence the lower raw and filtered read values. The median number of reads per sample in
4 each batch and number of amplicon sequence variants (ASVs), i.e. unique reads, per batch are also shown,
5 alongside relevant means and standard errors (S.E.).

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

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1 *NGS-based detection of confirmed canine vector-borne pathogens*

2 Key species and genera of known canine pathogens were identified as *Anaplasma platys*,
3 *Bartonella* spp., *Babesia vogeli*, *Ehrlichia canis*, *Hepatozoon canis*, *Candidatus* Mycoplasma
4 haematoparvum, *Mycoplasma haemocanis* and *Trypanosoma evansi*, which were then extracted
5 from the overall datasets for each batch (Table 4). Across all field sites 62% (95% CI = 57% - 66%)
6 of dogs were found to be infected with at least one of these eight pathogens with the highest
7 apparent prevalence of infection found in Battambang at 75% (95% CI = 65% - 83%) and lowest
8 in Tbong Khmum at 47% (95% CI = 37% - 56%).

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

15 Mixed infections, with between two to four pathogens detected simultaneously, were common
16 with 28% (95% CI = 24% - 32%) of dogs found to be coinfected across all field sites (Table 4). The
17 most common coinfection was *A. platys* and *E. canis* in 4% (95% CI = 3% - 6%), followed by *A.*
18 *platys* and *H. canis* in 3% (95% CI = 2% - 5%) of dogs. The most common coinfection comprising
19 three pathogens was *A. platys*, *B. vogeli* and *H. canis* in 2% (95% CI = 1% - 4%) of dogs. The most
20 common coinfection with four pathogens was *A. platys*, *B. vogeli*, *E. canis* and *H. canis* in 1.2%
21 (95% CI = 1 - 3) of dogs. **Figure 2** shows the relative proportion of infection-free, single infection
22 and coinfected dogs at the five field sites. Battambang had the most coinfected dogs at 42% (95%
23 CI = 32% - 52%), followed by Phnom Penh at 29% (95% CI = 22% - 37%), whilst Tbong Khmum had
24 the least; 15% (95% CI = 9% - 23%).

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1 **Table 4.** Number of positive dogs (+) and apparent pathogen prevalence (%) with 95% confidence intervals
2 (CI) found for both single and mixed infections, as detected by our bacterial, apicomplexan and
3 kinetoplastid targeting NGS-method at five different study areas in Cambodia (PP = Phnom Penh, KC =
4 Kampong Chhnang, SR = Siem Reap, BB = Battambang, TK = Tbong Khmum). Table shows results from only
5 known canine-pathogenic genera and species.

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

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

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1 *Serology*

2 Of 467 dog blood samples subjected to NGS metabarcoding, 457 samples were also subjected to
3 the SNAP 4Dx Plus Test (IDEXX). Across all field sites 42% (95% CI = 37% - 46%) of dogs were found
4 exposed to *Anaplasma* spp. and 32% (95% CI = 27% - 36%) to *Ehrlichia* spp., whilst 23% (95% CI =
5 20% - 27%) had been exposed to both (Table 5). One dog from Tbong Khmum was serologically
6 positive to *B. burgdorferi* sensu lato (and *Ehrlichia* spp.), whilst no dogs were found positive for
7 *D. immitis*.

8 *Statistical and risk indicator analyses*

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

17 Our multiple logistic regression model identified presence of ectoparasites, abnormal mucous
18 membrane condition, increase in total protein, and PCV (as an indicator of anaemia) as significant
19 predictors of VBP exposure in dogs (Table 6). Via our model, we estimate that the odds ratio of
20 VBP exposure for dogs with ectoparasites to be between 1.33 and 3.59, whilst holding PCV, total
21 protein and mucous membrane variables constant. The odds ratio for VBP exposure in dogs with
22 abnormal mucous membrane condition ranged between 1.29 and 4.92, when the other variables
23 were kept constant. We also estimate that as total protein increases by one unit the odds of VBP
24 infection increases by 1.35 to 1.92, holding other variables constant. Moreover, the odds ratio of
25 VBP exposure for dogs with a PCV indicative of anaemia (below 35%) was between 1.01 and
26 3.085, if total protein, ectoparasite presence and mucous membrane condition variables were
27 kept constant.

28 Kappa statistics, used to compare agreement between two diagnostic tests, assessed the level of
29 concordance between detection of antibodies using the SNAP 4Dx Plus Test and detection of
30 pathogen DNA using our NGS method (Table 7). Agreement for detection of *E. canis* was 0.517
31 (SE 0.044, $P < 0.05$) indicating moderate agreement, with 81.2% total agreement, whilst for *A.*
32 *platys* the kappa value was 0.162 (SE 0.046, $P < 0.05$) indicating poor agreement, with 60.6% total
33 agreement (Table 7).

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1 **Table 5.** Number of serologically positive dogs (+) and pathogen prevalence (%) with 95% confidence
2 intervals (CI) found for mixed and overall infections, as detected by SNAP 4Dx Plus Test at five different
3 study areas in Cambodia (PP = Phnom Penh, KC = Kampong Chhnang, SR = Siem Reap, BB = Battambang,
4 TK = Tbong Khmum). No dogs were *D. immitis* antigen positive and 10 tests had a failed internal control
5 and were therefore excluded from the overall results.

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

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7 **Table 6.** Clinical parameter estimates and odds ratios (95% profile likelihood) for exposure to vector-borne
8 pathogens in 467 dogs from Cambodia.

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

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Table 7. NGS vs SNAP 4Dx Plus test agreement statistics for the canine VBPs *E. canis* and *A. platys*. POS = positive, NEG = negative, SE = standard error. Kappa agreement level defined as poor if coefficient (k) is ≤ 0.20, fair agreement if 0.21 ≤ k ≤ 0.40, moderate agreement if 0.41 ≤ k ≤ 0.60, substantial agreement if 0.61 ≤ k ≤ 0.80 and high agreement if k > 0.81.

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

Confirmatory Sanger sequencing and suspected pathogen detection

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

Using a confirmatory cPCR assay (Criado-Fornelio et al., 2003) all of the four haemotropic *Mycoplasma* positive samples found via NGS that could not be identified to species level (1% of sampled population; 95% CI = 0% - 2%) had a 575bp long amplicon match with 100% query cover and identity to an uncharacterised *Mycoplasma* species (accession #: HE577612.1), previously identified in dogs from Indigenous communities in Australia (Barker et al., 2012).

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

In addition to the detection of confirmed canine pathogens by our metabarcoding method, the bacterial 16S rRNA gene deep-sequencing identified several samples with reads from bacterial genera that contain canine pathogens or species of zoonotic importance. Nonetheless, from the NGS data alone, reads could only be classified to genus-level due to the conserved nature of the 16S rRNA gene region targeted for some bacterial taxa. Bacterial genera of potential pathogenic

significance found across all dogs sampled included *Leptospira* spp. in 3% (95% CI = 2% - 4%) of dogs with the NGS reads most similar to existing GenBank entries returning a 100% query cover and 98.4% identity with *Leptospira interrogans* (accession #: DQ522190.1).

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

Other genera of pathogenic relevance were *Mycobacterium* spp. found in 7% (95% CI = 5% - 9%) of dogs, reads from members of the Rickettsiaceae family that includes the VBP-relevant genus *Rickettsia* in 3% (95% CI = 2% - 5%) of canines, *Coxiella* spp. in 8% (95% CI = 6% – 10%), *Neisseria* spp. in 2% (95% CI = 1% - 3%), *Rhodococcus* spp. in 5% (95% CI = 3% - 8%), and *Treponema* spp. in 3% (95% CI = 1% - 4%). Moreover, a substantial diversity of reads classified as *Prevotella* spp. were also found in dogs from all field sites, including one dog with a high number of reads identified as *Prevotella colorans,* a species previously characterised from a human wound (Buhl et al., 2016). *Bordetella* spp. reads were also identified from one dog, BLASTn revealed this sequence to share 99.2% identity with *Bordetella bronchiseptica* and *Bordetella petrii*, the former of which is a canine pathogen and one of the agents responsible for canine infectious respiratory disease (CIRD) (Schulz et al., 2014).

The apicomplexan and kinetoplastid 18S rRNA gene metabarcoding also returned reads classified from putative pathogens, which were then identified against the NCBI database using BLASTn, see Table 8.

1 **Table 8.** BLASTn results from putative pathogens detected by the apicomplexan and kinetoplastid-
2 targeting NGS metabarcoding protocol, including prevalence with confidence intervals (CI) of these
3 sequences found across all study sites. Query cover and identity ranges are included as multiple ASVs
4 were identified belonging to the same genus in some cases.

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

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6 **Discussion**

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

16 Apparent prevalence of the most common VBPs identified here is consistent with previous
17 studies in the region. A cPCR-based study conducted in Cambodia's central-northeastern
18 province of Preah Vihear found canine VBPs in 71.3% of dogs, specifically *B. vogeli* in 32.7% of
19 dogs vs 14% in our data*, E. canis* 21.8% vs 20%*, H. canis* 10.9 % vs 18% and *M. haemocanis* 9.9%
20 vs 13% reported here. The only discrepancy between studies was the lack of *A. platys* found by
21 Inpankaew et al. (2016), nonetheless, we found apparent *A. platys* prevalence to be lowest at
22 our Siem Reap and Kampong Chhnang study sites i.e. those which are geographically closest to

Preah Vihear. Our data also mirrors results found in nearby countries, for example *Hepatozoon* spp. (18.8%), *Babesia* spp. (9.4%) and *Mycoplasma* spp. (19.9%) in stray dogs in Thailand (Liu et al., 2016) as well as *E. canis* (25.8%) and *A. platys* (13.3%) serological exposure demonstrated in Vietnam (Colella et al., 2020).

Vector-borne pathogen coinfection was common with 28% of dogs across all field sites found to have between two and four confirmed VBPs. Coinfection rates peaked in Battambang, followed by Phnom Penh and were lowest in Tbong Khmum (Table 4 & Figure 2). The most common coinfection identified was *A. platys* and *E. canis* in 4% of dogs – this is unsurprising given that the two pathogens share a common vector and have been consistently found as one of the most prevalent coinfections identified in Asian dogs before (Huggins et al., 2019a; Rucksaken et al., 2019; Colella et al., 2020; Díaz-Regañón et al., 2020). Mixed infections comprising three and four pathogens were also detected which is important from both an epidemiological and clinical standpoint, as simultaneous vector-borne pathogen infection is associated with more severe pathology, such as exacerbated immune-mediated hemolytic anemia or lower PCV (Suksawat et al., 2001; Rojas et al., 2014; Rawangchue and Sungpradit, 2020)

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

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

DNA of *Leptospira* spp. was also identified in 3% of dog blood samples via NGS. BLASTn cross-validation of NGS taxonomic classification found one ASV to be similar to *L. interrogans* (accession #: DQ522190.1), sharing 98.4% identity, whilst other ASVs ranged down to 95% identity with the

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

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

Further significant bacterial taxonomic groups identified were Rickettsiaceae (3% of samples) which includes the genus *Rickettsia* of which many species are vector-borne pathogens, including the zoonosis *R. felis* which uses dogs as a natural asymptomatic reservoir (Ng-Nguyen et al., 2020). *Mycobacterium* (7%) and *Coxiella* (8%) DNA was also detected across all study sites, a relevant finding given that serology suggests dogs can harbour the human pathogens *Mycobacterium tuberculosis* and *Coxiella burnetii* (Parsons et al., 2012; Shapiro et al., 2016; Ma et al., 2020). Previous work has molecularly identified *Coxiella*-like endosymbiont DNA of ticks in both the vector and mammalian host, providing an alternative explanation for the *Coxiella* DNA found in this study (Khoo et al., 2016). *Neisseria* species found in 2% of our samples can be part of the normal canine oral microbiome, however dog bite wounds have been implicated in fatal cases of *Neisseria* infection in dogs and people (Cantas et al., 2011; Cobiella et al., 2019). *Rhodococcus* species, such as *Rhodococcus equi* can generate serious pathology in dogs, especially those that are immunocompromised, whilst *Treponema* spp. have been implicated in

canine periodontal disease, the former pathogen being found in 5% and the latter in 3% of dogs in this study (Takai et al., 2003; Bryan et al., 2017; Nises et al., 2018).

Nonetheless, with all classifications that could only be made to family or genus level, pathogenicity could not be confirmed given that all these taxonomic groupings also contain non-pathogenic environmental species. In many cases high 16S rRNA gene homology across the genus prevented species level discrimination, an issue highlighted extensively before with regard to *Rickettsia* classification (Santibáñez et al., 2013; Portillo et al., 2017; Greay et al., 2018).

Rarer ASVs found in a limited number of dog samples included the human-wound isolated *Prevotella colorans* (Buhl et al., 2016) and an ASV sharing 99.2% identity to *Bordetella bronchiseptica* which can cause canine infectious respiratory disease (CIRD). The detection of *Wolbachia* DNA sharing 99.2% to 100% identity from *Wolbachia* found in different species of the parasitic filarial worm genus *Dirofilaria* is also significant as *D. immitis*, the aetiological agent of heartworm, is endemic to northeast Cambodia and Southeast Asia more generally, whilst *D. repens* has not been found in the region before (Inpankaew et al., 2016; Colella et al., 2020). Heartworm in particular, is a commonly lethal canine infection and in this case, the potential detection of its bacterial endosymbiont's DNA could be indicative of an active infection, as has been previously demonstrated (Taylor et al., 2005; Huggins et al., 2019a; Laidoudi et al., 2020).

Our 18S rRNA gene deep-sequencing protocols also elucidated a plethora of sequences from organisms detected in dog blood (Table 8). The apicomplexan method detected an ASV in one dog from *Colpodella* spp. a genus which has typically been associated with free-living species, but that has been increasingly found in the bloodstream and haemolymph of various mammals and arthropod vectors including humans, cattle, *Ixodes* and *Rhipicephalus* ticks (Yuan et al., 2012; Matsimbe et al., 2017; Jiang et al., 2018; Sam-Yellowe et al., 2020; Squarre et al., 2020). In cases of previously identified human infections, pathology was generated including fever, neurological symptoms and haemolytic anaemia (Yuan et al., 2012; Jiang et al., 2018). Here, the detection of *Colpodella* spp. DNA from the blood of a dog represents the first report of its kind, with our sequence sharing 95% identity with a sequence previously detected in horse blood (accession #: MW261750.1).

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

The kinetoplastid targeting protocol, apart from the single *T. evansi* infection, discovered a large number of samples across all field sites with diverse ASVs classified to genera in the order Bodonida, including *Parabodo* (7%), *Bodo* (4%) and *Neobodo* (6%) (Moreira et al., 2004). These genera are typically recognised as being free-living organisms, however, a growing body of literature has identified them in a range of different animals, occasionally associated with pathology. For example, ASVs in the present study were found to be 99% identical to *Parabodo caudatus* (accession #: DQ207591.1) a species that has been found, via cystocentesis, in the urine of a dog with haematuria (Vandersea et al., 2015) as well as in the blood of a Thai dog (Huggins et al., 2019b). In addition, DNA from the genus *Bodo* has been previously found in the blood of an Algerian dog (Medkour et al., 2020), Brazilian bats (Dario et al., 2017) and the Australian marsupial *Bettongia penicillate* (woylie) (Northover et al., 2019). In the present study negative controls were free from Bodonida DNA and the number of reads from Bodonida organisms was in some cases much higher than would be expected if skin/needle-stick contamination was the cause. Additionally, further indications on the potential vector-borne transmission of Bodonida organisms comes from Szöke et al. (2017) which found larval ticks that had fed exclusively on cohabiting bats to be PCR positive for *Bodo* organisms (Szöke et al., 2017). Nonetheless, identification of DNA presence alone is not sufficient to ascertain the role of an arthropod in the transmission of a given pathogen. Another sequence had a low similarity to any entries in GenBank, with the highest being an 88% identity match with *Herpetomonas* spp. (accession #: AJ843897.1) and *Blechomonas pulexsimulantis* (accession #: KF054129.1), both genera are associated with parasites of arthropod vectors, such as fleas and midges, as well as free-living forms (Podlipaev et al., 2004; Votýpka et al., 2013).

Our study's statistical modelling identified the presence of ectoparasites, abnormal mucous membrane condition, elevated total protein, and anaemia (low PVC) to be significant predictors for canine VBP exposure. Of these, the odds ratio for VBP exposure in dogs was highest in those that had an abnormal mucous membrane condition (odds ratio 2.43, CI 1.29-4.92), with pallor the most common abnormality identified. This is not unexpected given that many canine VBPs generate anaemia (Otranto et al., 2009; Harrus and Waner, 2011). This indicator was further corroborated by the higher odds ratios in VBP exposed dogs that were found with ectoparasites (odds ratio 2.18, CI 1.33-3.59) or classified as anaemic (odds ratio 1.74, CI 1.01-3.085), based on PCV results (Hayes et al., 2011; Riond et al., 2014). In addition, we identified that a total protein increase of one unit was associated with a higher odds ratio for VBP exposure (odds ratio 1.60, CI 1.35-1.92). Hyperproteinemia is a common consequence of a chronic immune response to VBP as total protein increasing with host immunoglobulin production in response to infection (Shaw et al., 2001; Hayes et al., 2011).

Comparison of serological testing with NGS analysis identified moderate agreement (K = 0.517) for *Ehrlichia* spp. and poor agreement (K = 0.162) for *Anaplasma* spp. with serology identifying a greater number of positives for both pathogens than our metabarcoding method (Table 7). This is not surprising given that serology measures pathogen exposure via the production of antibodies that can remain in the blood for months to years, whilst circulating pathogen DNA can

be reduced below the limit of detection for many molecular diagnostics (Little, 2010; Wong et al., 2011; Mahmoud et al., 2015; Kidd, 2019). Cyclical production of antibodies against Rickettsiales pathogens has been identified in dogs before, specifically for the species *R. felis* (Ng-Nguyen et al., 2020). Ng-Nguyen et al. (2020) identified an inverse and fluctuating relationship between levels of IgG antibody against *R. felis* and PCR positivity in experimentally infected dogs. Peaks of IgG production coincided with buffy coat PCR negativity, whilst reductions in IgG titers correlated with PCR positivity. Such mechanisms could provide some explanation as to why NGS methods in the current study failed to detect rickettsial pathogens that were identified as positive using SNAP 4Dx tests.

In contrast, some dogs were found to be NGS positive and SNAP 4Dx negative for both *Anaplasma* spp. (67 samples) and *Ehrlichia* spp. (17 samples), see Table 7. Such results could be due to dog sampling having been conducted too soon after infection by the relevant VBP and thus production of host antibodies may not have yet commenced, generating SNAP 4Dx negative results, whilst circulating pathogen DNA was detectable via our NGS method (Wong et al., 2011).

The discrepancy between methods highlights the need for multiple diagnostic methods to be used in an integrated manner so as to more accurately characterise the apparent prevalence and exposure to different VBPs (Kidd, 2019). The identification of one sample serologically positive to *B. burgdorferi* sensu lato was unexpected, particularly given that no *Borrelia* DNA was detected by deep-sequencing. However, seropositive dogs to this important zoonotic bacterium have been identified in Indonesia and the Philippines before (Colella et al., 2020). The lack of *D. immitis* antigen positive dogs was also surprising, particularly as 15.8% of dogs were previously found positive to this filarial pathogen via cPCR in northern Cambodia (Inpankaew et al., 2016).

Conclusion

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

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Ethics Statement

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. Ethical committee approval was sought and obtained from the the University of Melbourne Animal Ethics Committee and approved under Permit: 1814620.1.

Conflicts of Interest

The authors declare no conflict of interest.

References

Alvar, J., I.D. Vélez, C. Bern, M. Herrero, P. Desjeux, J. Cano, J. Jannin, and M. de Boer, (2012). Leishmaniasis worldwide and global estimates of its incidence. PLoS One, 7, e35671, *DOI*: 10.1371/journal.pone.0035671

Álvarez-Fernández, A., E.B. Breitschwerdt, and L. Solano-Gallego, (2018). *Bartonella* infections in cats and dogs including zoonotic aspects. Parasites and Vectors, 11, 624, *DOI*: 10.1186/s13071-018-3152-6

Aregawi, W.G., G.E. Agga, R.D. Abdi, and P. Büscher, (2019). Systematic review and meta-analysis on the global distribution, host range, and prevalence of *Trypanosoma evansi*. Parasites and Vectors, 12, 67, *DOI*: 10.1186/s13071-019-3311-4

Baneth, G., J.S. Mathew, V. Shkap, D.K. Macintire, J.R. Barta, and S.A. Ewing, (2003). Canine hepatozoonosis: Two disease syndromes caused by separate *Hepatozoon* spp. Trends in Parasitology, 19, 27–31, *DOI*: 10.1016/S1471-4922(02)00016-8

Barameechaithanun, E., P. Suwannasaeng, N. Boonbal, S. Pattanee, and S. Hoisang, (2009). Treatment of trypanosomiasis in dog. Veterinary Medicine, 4, 51–60

1 Barker, E.N., D.A. Langton, C.R. Helps, G. Brown, R. Malik, S.E. Shaw, and S. Tasker, (2012)
2 Haemoparasites of free-roaming dogs associated with several remote Aboriginal communities in
3 Australia. BMC Veterinary Research, 8, *DOI*: 10.1186/1746-6148-8-55

4 Belkhiria, J., B.B. Chomel, T. Ben Hamida, R.W. Kasten, M.J. Stuckey, D.A. Fleischman, M.M.
5 Christopher, H.-J. Boulouis, and T.B. Farver, (2017). Prevalence and potential risk factors for
6 *Bartonella* infection in Tunisian stray dogs. Vector-Borne Zoonotic Diseases, 17, 388–397, *DOI*:
7 10.1089/vbz.2016.2039

8 Breitschwerdt, E.B., R.G. Maggi, B.B. Chomel, and M.R. Lappin, (2010). Bartonellosis: An emerging
9 infectious disease of zoonotic importance to animals and human beings. Journal Veterinary
10 Emergency and Critical Care, 20, 8–30, *DOI*: 10.1111/j.1476-4431.2009.00496.x

11 Breitschwerdt, E.B., (2014). Bartonellosis: One health perspectives for an emerging infectious
12 disease. Institute for Laboratory Animal Research Journal, 55, 46–58, *DOI*: 10.1093/ilar/ilu015

13 Brook, B.W., N.S. Sodhi, and C.J.A. Bradshaw, (2008). Synergies among extinction drivers under
14 global change. Trends Ecololgy & Evolution, 23, 453–460, *DOI*: 10.1016/j.tree.2008.03.011

15 Bryan, L.K., S.D. Clark, J. Díaz-Delgado, S.D. Lawhon, and J.F. Edwards, (2017). *Rhodococcus equi*
16 Infections in Dogs. Veterinary Pathology, 54, 159–163, *DOI*: 10.1177/0300985816650244

17 Buhl, M., M. Willmann, J. Liese, I.B. Autenrieth, and M. Marschal, (2016). *Prevotella colorans* sp.
18 Nov., isolated from a human wound. International Journal of Systematic and Evolutionary
19 Microbiology, 66, 3005–3009, *DOI*: 10.1099/ijsem.0.001134

20 Bui, K.L., D.H. Duong, D.T.A. Bui, V.L. Nguyen, T. Do, T.L.A. Le, and K.T. Tran, (2021). A case of
21 *Trypanosoma evansi* in a German Shepherd dog in Vietnam. Parasitology International, 80,
22 102198, *DOI*: 10.1016/j.parint.2020.102198

23 Callahan, B.J., P.J. McMurdie, M.J. Rosen, A.W. Han, A.J.A. Johnson, and S.P. Holmes, (2016).
24 DADA2: high-resolution sample inference from Illumina amplicon data. Nature Methods, 13, 581,
25 *DOI*: 10.1038/nmeth.3869

26 Calvani, N.E.D., L. Bell, A. Carney, C. De La Fuente, T. Stragliotto, M. Tunstall, and J. Šlapeta,
27 (2020). The molecular identity of fleas (Siphonaptera) carrying *Rickettsia felis*, *Bartonella*
28 *clarridgeiae* and *Bartonella rochalimae* from dogs and cats in Northern Laos. Heliyon, 6, e04385,
29 *DOI*: 10.1016/j.heliyon.2020.e04385

30 Cantas, H., M. Pekarkova, H.S. Kippenes, E. Brudal, and H. Sorum, (2011). First reported isolation
31 of *Neisseria canis* from a deep facial wound infection in a dog. Journal of Clinical Microbiology,
32 49, 2043–2046, *DOI*: 10.1128/JCM.02610-10

33 Chaudhry, U., Q. Ali, I. Rashid, M.Z. Shabbir, M. Ijaz, M. Abbas, M. Evans, K. Ashraf, I. Morrison, L.
34 Morrison, and N.D. Sargison, (2019). Development of a deep amplicon sequencing method to

determine the species composition of piroplasm haemoprotozoa. Ticks & Tick-Borne Disease, 10, *DOI*: 10.1016/j.ttbdis.2019.101276

Chomel, B.B., and R.W. Kasten, (2010). Bartonellosis, an increasingly recognized zoonosis. Journal of Applied Microbiology 109, 743–750, *DOI*: 10.1111/j.1365-2672.2010.04679.x

Cobiella, D., D. Gram, and D. Santoro, (2019). Isolation of *Neisseria dumasiana* from a deep bite wound infection in a dog. Veterinary Dermatology, 30, 556-e168, *DOI*: 10.1111/vde.12791

Colella, V., V.L. Nguyen, D.Y. Tan, N. Lu, F. Fang, Y. Zhijuan, J. Wang, X. Liu, X. Chen, J. Dong, W. Nurcahyo, U.K. Hadi, V. Venturina, K. B.Y. Tong, Y.-L. Tsai, P. Taweethavonsawat, S. Tiwananthagorn, T.Q. Le, K.L. Bui, M. Watanabe, P.A.M.A. Rani, G. Annoscia, F. Beugnet, D. Otranto, and L. Halos, (2020). Zoonotic vectorborne pathogens and ectoparasites of dogs and cats in Asia. Emerging Infectious Diseases, 26, *DOI*: 10.3201/eid2606.191832

Criado-Fornelio, A., A. Martinez-Marcos, A. Buling-Saraña, and J.C. Barba-Carretero, (2003). Presence of *Mycoplasma haemofelis*, *Mycoplasma haemominutum* and piroplasmids in cats from southern Europe: a molecular study. Veterinary Microbiology, 93, 307–317, *DOI*: 10.1016/S0378-1135(03)00044-0

Dario, M.A., R.M.M. da Rocha, P. Schwabl, A.M. Jansen, and M.S. Llewellyn, (2017). Small subunit ribosomal metabarcoding reveals extraordinary trypanosomatid diversity in Brazilian bats. PLoS Neglected Tropical Diseases, 11, e0005790, *DOI*: 10.1371/journal.pntd.0005790

Defontis, M., J. Richartz, N. Engelmann, C. Bauer, V.M. Schwierk, P. Büscher, and A. Moritz, (2012). Canine *Trypanosoma evansi* infection introduced into Germany. Veterinary Clinical Pathology, 41, 369–374, *DOI*: 10.1111/j.1939-165X.2012.00454.x

Díaz-Regañón, D., B. Agulla, B. Piya, N. Fernández-Ruiz, A. Villaescusa, M. García-Sancho, F. Rodríguez-Franco, and Á. Sainz, (2020). Stray dogs in Nepal have high prevalence of vector-borne pathogens: A molecular survey. Parasites and Vectors, 13, *DOI*: 10.1186/s13071-020-04057-7

Diaz, M.H., Y. Bai, L. Malania, J.M. Winchell, and M.Y. Kosoy, (2012). Development of a novel genus-specific real-time PCR assay for detection and differentiation of *Bartonella* species and genotypes. Journal of Clinical Microbiology, 50, 1645–1649, *DOI*: 10.1128/JCM.06621-11

Esson, C., L.F. Skerratt, L. Berger, J. Malmsten, T. Strand, Å. Lundkvist, J.D. Järhult, J. Michaux, T.N. Mijiddorj, R. Bayrakçısmith, C. Mishra, and Ö. Johansson, (2019). Health and zoonotic Infections of snow leopards *Panthera unica* in the South Gobi desert of Mongolia. Infection Ecology & Epidemiology, 9, 1604063, *DOI*: 10.1080/20008686.2019.1604063

Flaherty, B.R., J. Barratt, M. Lane, E. Talundzic, and R.S. Bradbury, (2021). Sensitive universal detection of blood parasites by selective pathogen-DNA enrichment and deep amplicon sequencing. Microbiome, 9, 1, *DOI*: 10.1186/s40168-020-00939-1

1 Flaherty, B.R., E. Talundzic, J. Barratt, K.J. Kines, C. Olsen, M. Lane, M. Sheth, and R.S. Bradbury,
2 (2018). Restriction enzyme digestion of host DNA enhances universal detection of parasitic
3 pathogens in blood via targeted amplicon deep sequencing. Microbiome, 6, 164, *DOI*:
4 10.1186/s40168-018-0540-2

5 Gasser, R.B., (2006). Molecular tools - advances, opportunities and prospects. Veterinary
6 Parasitology, 136, 69–89, *DOI*: 10.1016/j.vetpar.2005.12.002

7 Gestal, C., and C. Azevedo, (2005). Ultrastructure of *Goussia cruciata* (Apicomplexa: Coccidia)
8 infecting the liver of horse mackerel, *Trachurus trachurus* (L.), from Ibero-Atlantic waters. Journal
9 of Fish Diseases, 28, 125–132, *DOI*: 10.1111/j.1365-2761.2005.00611.x

10 Ghafar, A., A. Khan, A. Cabezas-Cruz, C.G. Gauci, S. Niaz, S. Ayaz, L. Mateos-Hernández, C. Galon,
11 N. Nasreen, S. Moutailler, R.B. Gasser, and A. Jabbar, (2020). An assessment of the molecular
12 diversity of ticks and tick-Borne microorganisms of small ruminants in Pakistan. Microorganisms,
13 8, 1428, *DOI*: 10.3390/microorganisms8091428

14 Ghafar, A., A. V. Koehler, R.S. Hall, C.G. Gauci, R.B. Gasser, and A. Jabbar, (2021). Targeted next-
15 generation sequencing and informatics as an effective tool to establish the composition of bovine
16 piroplasm populations in endemic regions. Microorganisms, 9, 1–14, *DOI*:
17 10.3390/microorganisms9010021

18 Glidden, C.K., A. V. Koehler, R.S. Hall, M.A. Saeed, M. Coppo, B.R. Beechler, B. Charleston, R.B.
19 Gasser, A.E. Jolles, and A. Jabbar, (2020). Elucidating cryptic dynamics of *Theileria* communities
20 in African buffalo using a high-throughput sequencing informatics approach. Ecology and
21 Evolution, 10, 70–80, *DOI*: 10.1002/ece3.5758

22 Goodwin, S., J.D. McPherson, and W.R. McCombie, (2016). Coming of age: ten years of next-
23 generation sequencing technologies. Nature Reviews Genetics, 17, 333–351, *DOI*:
24 10.1038/nrg.2016.49

25 Greay, T.L., A.W. Gofton, A. Paparini, U.M. Ryan, C.L. Oskam, and P.J. Irwin, (2018). Recent
26 insights into the tick microbiome gained through next-generation sequencing. Parasites and
27 Vectors, 11, *DOI*: 10.1186/s13071-017-2550-5

28 Harrus, S., and T. Waner, (2011). Diagnosis of canine monocytotropic ehrlichiosis (*Ehrlichia canis*):
29 an overview. Veterinary Journal, 187, 292–296, *DOI*: 10.1016/j.tvjl.2010.02.001

30 Hayes, G.M., K. Mathews, A. Floras, and C. Dewey, (2011). Refractometric total plasma protein
31 measurement as a cage-side indicator of hypoalbuminemia and hypoproteinemia in hospitalized
32 dogs. Journal of Veterinary Emergency and Critical Care, 21, 356–362, *DOI*: 10.1111/j.1476-
33 4431.2011.00647.x

34 Hii, S., R. Traub, M. Thompson, J. Henning, C. O'Leary, A. Burleigh, S. McMahon, R. Rees, and S.
35 Kopp, (2015). Canine tick-borne pathogens and associated risk factors in dogs presenting with

1 and without clinical signs consistent with tick-borne diseases in northern Australia. Australian
2 Veterinary Journal, 93, 58–66, *DOI*: 10.1111/avj.12293

3 Hornung, B.V.H., R.D. Zwittink, and E.J. Kuijper, (2019). Issues and current standards of controls
4 in microbiome research. FEMS Microbiology Ecology, 95, *DOI*: 10.1093/femsec/fiz045

5 Huggins, L.G., A. V. Koehler, D. Ng-Nguyen, S. Wilcox, B. Schunack, T. Inpankaew, and R.J. Traub,
6 (2019a). Assessment of a metabarcoding approach for the characterisation of vector-borne
7 bacteria in canines from Bangkok, Thailand. Parasites and Vectors, 12, 394, *DOI*: 10.1186/s13071-
8 019-3651-0

9 Huggins, L.G., A. V. Koehler, D. Ng-Nguyen, S. Wilcox, B. Schunack, T. Inpankaew, and R.J. Traub,
10 (2019b). A novel metabarcoding diagnostic tool to explore protozoan haemoparasite diversity in
11 mammals: a proof-of-concept study using canines from the tropics. Scientific Reports, 9, 12644,
12 *DOI*: 10.1038/s41598-019-49118-9

13 Huggins, L.G., A. V. Koehler, B. Schunack, T. Inpankaew, and R.J. Traub, (2020). A host-specific
14 blocking primer combined with optimal DNA extraction improves the detection capability of a
15 metabarcoding protocol for canine vector-borne bacteria. Pathogens, 9, 258, *DOI*:
16 10.3390/pathogens9040258

17 Inpankaew, T., S.F. Hii, W. Chimnoi, and R.J. Traub, (2016). Canine vector-borne pathogens in
18 semi-domesticated dogs residing in northern Cambodia. Parasites and Vectors, 9, 253, *DOI*:
19 10.1186/s13071-016-1552-z

20 Irwin, P.J., and R. Jefferies, (2004). Arthropod-transmitted diseases of companion animals in
21 Southeast Asia. Trends in Parasitology, 20, 27–34, *DOI*: 10.1016/j.pt.2003.11.004

22 Ivanov, A., and I. Tsachev, (2008). *Hepatozoon canis* and hepatozoonosis in the dog. Trakia
23 Journal of Sciences, 6, 27–35.

24 Jiang, J.F., R.R. Jiang, Q.C. Chang, Y.C. Zheng, B.G. Jiang, Y. Sun, N. Jia, R. Wei, H.B. Liu, Q.B. Huo,
25 H. Wang, M.E. von Fricken, and W.C. Cao, (2018). Potential novel tick-borne *Colpodella* species
26 parasite infection in patient with neurological symptoms. PLoS Neglected Tropical Diseases, 12,
27 e0006546, *DOI:* 10.1371/journal.pntd.0006546

28 Jirků, M., M. Jirků, M. Oborník, J. Lukeš, and D. Modrý, (2009). *Goussia* Labbé, 1896 (Apicomplexa,
29 Eimeriorina) in Amphibia: Diversity, biology, molecular phylogeny and comments on the status
30 of the genus. Protist, 160, 123–136, *DOI*: 10.1016/j.protis.2008.08.003

31 Katoh, K., and D.M. Standley, (2013). MAFFT multiple sequence alignment software version 7:
32 improvements in performance and usability. Molecular Biology and Evolution, 30, 772–780, *DOI*:
33 10.1093/molbev/mst010

34 Kernif, T., C. Socolovschi, K. Wells, M.B. Lakim, S. Inthalad, G. Slesak, N. Boudebouch, J.-C.
35 Beaucournu, P.N. Newton, D. Raoult, and P. Parola, (2012). *Bartonella* and *Rickettsia* in

1 arthropods from the Lao PDR and from Borneo, Malaysia. Comparative Immunology,
2 Microbiology & Infectious Diseases, 35, 51–57, *DOI*: 10.1016/j.cimid.2011.10.003

3 Khoo, J.J., F.S. Lim, F. Chen, W.H. Phoon, C.S. Khor, B.L. Pike, L.Y. Chang, and S. Abubakar, (2016).
4 *Coxiella* detection in ticks from wildlife and livestock in Malaysia. Vector-Borne Zoonotic
5 Diseases, 16, 744–751, *DOI*: 10.1089/vbz.2016.1959

6 Kidd, L., (2019). Optimal vector-borne disease screening in dogs using both serology-based and
7 polymerase chain reaction–based diagnostic panels. Veterinary Clinics of North America: Small
8 Animal Practice, 49, 703–718, *DOI*: 10.1016/j.cvsm.2019.02.011

9 Kim, D., C.E. Hofstaedter, C. Zhao, L. Mattei, C. Tanes, E. Clarke, A. Lauder, S. Sherrill-Mix, C.
10 Chehoud, J. Kelsen, M. Conrad, R.G. Collman, R. Baldassano, F.D. Bushman, and K. Bittinger,
11 (2017). Optimizing methods and dodging pitfalls in microbiome research. Microbiome, 5, 52, *DOI*:
12 10.1186/s40168-017-0267-5

13 Kirk, S.K., J.K. Levy, and P.C. Crawford, (2017). Efficacy of azithromycin and compounded
14 atovaquone for treatment of *Babesia gibsoni* in dogs. Journal of Veterinary Internal Medicine,
15 31, 1108–1112, *DOI*: 10.1111/jvim.14777

16 Knight, R., A. Vrbanac, B.C. Taylor, A. Aksenov, C. Callewaert, J. Debelius, A. Gonzalez, T. Kosciolek,
17 L.I. McCall, D. McDonald, A. V. Melnik, J.T. Morton, J. Navas, R.A. Quinn, J.G. Sanders, A.D.
18 Swafford, L.R. Thompson, A. Tripathi, Z.Z. Xu, J.R. Zaneveld, Q. Zhu, J.G. Caporaso, and P.C.
19 Dorrestein, (2018). Best practices for analysing microbiomes. Nature Reviews Microbiology, 16,
20 410–422, *DOI*: 10.1038/s41579-018-0029-9

21 Koh, F.X., C. Panchadcharam, and S.T. Tay, (2016). Vector-borne diseases in stray dogs in
22 peninsular Malaysia and molecular detection of *Anaplasma* and *Ehrlichia* spp. from *Rhipicephalus*
23 *sanguineus* (Acari: Ixodidae) ticks. Journal of Medical Entomology, 53, 183–187, *DOI*:
24 10.1093/jme/tjv153

25 Laidoudi, Y., J. Lou Marié, D. Tahir, S. Watier-Grillot, O. Mediannikov, and B. Davoust, (2020).
26 Detection of canine vector-borne filariasis and their *Wolbachia* endosymbionts in French Guiana.
27 Microorganisms, 8, 770, *DOI*: 10.3390/microorganisms8050770

28 Langille, M.G.I., J.T. Nearing, G.M. Douglas, and A.M. Comeau, (2018). Denoising the denoisers:
29 an independent evaluation of microbiome sequence error-correction approaches. PeerJ, 8,
30 e5364, *DOI*: 10.7717/peerj.5364

31 Little, S.E., (2010). Ehrlichiosis and anaplasmosis in dogs and cats. Veterinary Clinics of North
32 America: Small Animal Practice, 40, 1121–1140, *DOI*: 10.1016/j.cvsm.2010.07.004

33 Liu, M., N. Ruttayaporn, V. Saechan, C. Jirapattharasate, P. Vudriko, P.F.A. Moumouni, S. Cao, T.
34 Inpankaew, A.P. Ybañez, H. Suzuki, and X. Xuan, (2016). Molecular survey of canine vector-borne
35 diseases in stray dogs in Thailand. Parasitology International, 65, 357–361, *DOI*:
36 10.1016/j.parint.2016.04.011

1 Ma, G.C., J.M. Norris, K.O. Mathews, S. Chandra, J. Šlapeta, K.L. Bosward, and M.P. Ward, (2020).
2 New insights on the epidemiology of *Coxiella burnetii* in pet dogs and cats from New South Wales,
3 Australia. Acta Tropica, 205, 105416, *DOI*: 10.1016/j.actatropica.2020.105416

4 Maggi, R.G., M.C. Chitwood, S. Kennedy-Stoskopf, and C.S. DePerno, (2013). Novel hemotropic
5 *Mycoplasma* species in white-tailed deer (*Odocoileus virginianus*). Comparative Immunology,
6 Microbiology & Infectious Diseases, 36, 607–611, *DOI*: 10.1016/j.cimid.2013.08.001

7 Mahmoud, M.S., O.M. Kandil, S.M. Nasr, S.H.M. Hendawy, S.M. Habeeb, D.M. Mabrouk, M.G.
8 Silva, and C.E. Suarez, (2015). Serological and molecular diagnostic surveys combined with
9 examining hematological profiles suggests increased levels of infection and hematological
10 response of cattle to babesiosis infections compared to native buffaloes in Egypt. Parasites and
11 Vectors, 8, 319, *DOI*: 10.1186/s13071-015-0928-9

12 Matsimbe, A.M., V. Magaia, G.S. Sanches, L. Neves, E. Noormahomed, S. Antunes, and A.
13 Domingos, (2017). Molecular detection of pathogens in ticks infesting cattle in Nampula province,
14 Mozambique. Experimental and Applied Acarology, 73, 91–102, *DOI*: 10.1007/s10493-017-0155-
15 5

16 McHugh, M.L., (2012). Interrater reliability: The kappa statistic. Biochemia Medica, 22, 276–282,
17 *DOI*: 10.11613/bm.2012.031Medkour, H., B. Davoust, F. Dulieu, L. Maurizi, T. Lamour, J.-L. Marié,
18 and O. Mediannikov, (2019). Potential animal reservoirs (dogs and bats) of human visceral
19 leishmaniasis due to *Leishmania infantum* in French Guiana. PLoS Neglected Tropical Diseases,
20 13, e0007456, *DOI*: 10.1371/journal.pntd.0007456

21 Medkour, H., M. Varloud, B. Davoust, and O. Mediannikov, (2020). New molecular approach for
22 the detection of kinetoplastida parasites of medical and veterinary interest. Microorganisms, 8,
23 356, *DOI*: 10.3390/microorganisms8030356

24 Megat Abd Rani, P.A., P.J. Irwin, M. Gatne, G.T. Coleman, and R.J. Traub, (2010). Canine vector-
25 borne diseases in India: a review of the literature and identification of existing knowledge gaps.
26 Parasites and Vectors, 3, 28, *DOI*: 10.1186/1756-3305-3-28

27 Mohamed, W.M.A., A.O. Ali, H.Y.A.H. Mahmoud, M.A. Omar, E. Chatanga, B. Salim, D. Naguib,
28 J.L. Anders, N. Nonaka, M.A.M. Moustafa, and R. Nakao, (2021). Exploring prokaryotic and
29 eukaryotic microbiomes helps in detecting tick-borne infectious agents in the blood of camels.
30 Pathogens, 10, 351, *DOI*: 10.3390/pathogens10030351

31 Molnár, K, (1989). Nodular and epicellular coccidiosis in the intestine of cyprinid fishes. Diseases
32 of Aquatic Organisms, 7, 1–12, *DOI*: 10.3354/dao007001

33 Molnár, Kálmán, G. Ostoros, D. Dunams-Morel, and B.M. Rosenthal, (2012). *Eimeria* that infect
34 fish are diverse and are related to, but distinct from, those that infect terrestrial vertebrates.
35 Infection, Genetics and Evolution, 12, 1810–1815, *DOI*: 10.1016/j.meegid.2012.06.017

1 Moreira, D., P. López-García, and K. Vickerman, (2004). An updated view of kinetoplastid
2 phylogeny using environmental sequences and a closer outgroup: Proposal for a new
3 classification of the class Kinetoplastea. International Journal of Systematic and Evolutionary
4 Microbiology, 54, 1861–1875, *DOI*: 10.1099/ijs.0.63081-0

5 Ng-Nguyen, D., S.F. Hii, M.T.T. Hoang, V.A.T. Nguyen, R. Rees, J. Stenos, and R.J. Traub, (2020).
6 Domestic dogs are mammalian reservoirs for the emerging zoonosis flea-borne spotted fever,
7 caused by *Rickettsia felis.* Scientific Reports, 10, 1–10, *DOI*: 10.1038/s41598-020-61122-y

8 Nguyen, V.L., V. Colella, R. Iatta, K.L. Bui, F. Dantas-Torres, and D. Otranto, (2019). Ticks and
9 associated pathogens from dogs in northern Vietnam. Parasitology Research, 118, 139–142, *DOI*:
10 10.1007/s00436-018-6138-6

11 Nises, J., A. Rosander, A. Pettersson, and A. Backhans, (2018). The occurrence of *Treponema* spp.
12 in gingival plaque from dogs with varying degree of periodontal disease. PLoS One, 13, e0201888,
13 *DOI*: 10.1371/journal.pone.0201888

14 Norman, A.F., R. Regnery, P. Jameson, C. Greene, and D.C. Krause, (1995). Differentiation of
15 *Bartonella*-like isolates at the species level by PCR- restriction fragment length polymorphism in
16 the citrate synthase gene. Journal of Clinical Microbiology, 33, 1797–1803, *DOI*:
17 10.1128/jcm.33.7.1797-1803.1995

18 Northover, A.S., S.S. Godfrey, S. Keatley, A.J. Lymbery, A.F. Wayne, C. Cooper, L. Pallant, K. Morris,
19 and R.C.A. Thompson, (2019). Increased *Trypanosoma* spp. richness and prevalence of
20 haemoparasite co-infection following translocation. Parasites and Vectors, 12, 126, *DOI*:
21 10.1186/s13071-019-3370-6

22 Nyholm, S. V, McFall-Ngai, M.J., (2004). The winnowing: Establishing the squid-*Vibrio* symbiosis.
23 Nature Reviews Microbiology, 2, 632–642, *DOI*: 10.1038/nrmicro957

24 Otranto, D., F. Dantas-Torres, and E.B. Breitschwerdt, (2009). Managing canine vector-borne
25 diseases of zoonotic concern: part two. Trends in Parasitology, 25, 228–235, *DOI*:
26 10.1016/j.pt.2009.02.005

27 Païssé, S., C. Valle, F. Servant, M. Courtney, R. Burcelin, J. Amar, and B. Lelouvier, (2016).
28 Comprehensive description of blood microbiome from healthy donors assessed by 16S targeted
29 metagenomic sequencing. Transfusion, 56, 1138–1147, *DOI*: 10.1111/trf.13477

30 Panigrahi, P.N., K. Mahendran, S.C. Jena, P. Behera, S. Mahajan, K. Arjun, and S. Dey, (2015).
31 *Trypanosoma evansi* infection in a German shepherd dog — Apparent successful treatment using
32 serial low dose of diminazene aceturate. Veterinary Parasitology: Regional Studies and Reports,
33 1–2, 70–74, *DOI*: 10.1016/j.vprsr.2016.03.003

34 Parola, P., O.Y. Sanogo, K. Lerdthusnee, Z. Zeaiter, G. Chauvancy, J.P. Gonzalez, R.S. Miller, S.R.
35 Telford, C. Wongsrichanalai, and D. Raoult, (2003). Identification of *Rickettsia* spp. and *Bartonella*

spp. in fleas from the Thai-Myanmar border. Annals of the New York Academy of Sciences, 990, 173–181, *DOI*: 10.1111/j.1749-6632.2003.tb07359.x

Parsons, S.D.C., R.M. Warren, T.H.M. Ottenhoff, N.C. Gey van Pittius, and P.D. van Helden, (2012). Detection of *Mycobacterium tuberculosis* infection in dogs in a high-risk setting. Research in Veterinary Science, 92, 414–419, *DOI*: 10.1016/j.rvsc.2011.03.026

Pedregosa, F., G. Varoquaux, A. Gramfort, V. Michel, B. Thirion, O. Grisel, M. Blondel, P. Prettenhofer, R. Weiss, V. Dubourg, J. Vanderplas, A. Passos, D. Cournapeau, M. Brucher, M. Perrot, and É. Duchesnay, (2011). Scikit-learn: machine learning in Python. Journal of Machine Learning Research, 12, 2825–2830.

Podlipaev, S., J. Votýpka, M. Jirků, M. Svobodová, and J. Lukeš, (2004). *Herpetomonas ztiplika* n. sp. (Kinetoplastida: Trypanosomatidae): A parasite of the blood-sucking biting midge *Culicoides kibunensis* Tokunaga, 1937 (Diptera: Ceratopogonidae). Journal of Parasitology, 90, 342–347, *DOI*: 10.1645/GE-156R

Portillo, A., R. de Sousa, S. Santibáñez, A. Duarte, S. Edouard, I.P. Fonseca, C. Marques, M. Novakova, A.M. Palomar, M. Santos, C. Silaghi, L. Tomassone, S. Zúquete, and J.A. Oteo, (2017). Guidelines for the detection of *Rickettsia* spp. Vector-Borne Zoonotic Diseases, 17, 23–32, *DOI*: 10.1089/vbz.2016.1966

Price, M.N., P.S. Dehal, and A.P. Arkin, (2010). FastTree 2 - approximately maximum-likelihood trees for large alignments. PLoS One, 5, e9490, *DOI*: 10.1371/journal.pone.0009490

Rawangchue, T., and S. Sungpradit, (2020). Clinicopathological and molecular profiles of *Babesia vogeli* infection and *Ehrlichia canis* coinfection. Veterinary World, 13, 1294–1302, *DOI*: 10.14202/vetworld.2020.1294-1302

Riond, B., F. Steffen, O. Schmied, R. Hofmann-Lehmann, and H. Lutz, (2014). Total protein measurement in canine cerebrospinal fluid: Agreement between a turbidimetric assay and 2 dye-binding methods and determination of reference intervals using an indirect *a posteriori* method. Veterinary Clinical Pathology, 43, 78–88, *DOI*: 10.1111/vcp.12107

Rjeibi, M.R., T. Ben Hamida, Z. Dalgatova, T. Mahjoub, A. Rejeb, W. Dridi, and M. Gharbi, (2015). First report of surra (*Trypanosoma evansi* infection) in a Tunisian dog. Parasite, 22, *DOI*: 10.1051/parasite/2015004

Rojas, A., D. Rojas, V. Montenegro, R. Gutiérrez, D. Yasur-Landau, and G. Baneth, (2014). Vector-borne pathogens in dogs from Costa Rica: First molecular description of *Babesia vogeli* and *Hepatozoon canis* infections with a high prevalence of monocytic ehrlichiosis and the manifestations of co-infection. Veterinary Parasitology, 199, 121–128, *DOI*: 10.1016/j.vetpar.2013.10.027

Rozo, M., K.L. Schully, C. Philipson, A. Fitkariwala, D. Nhim, T. Som, D. Sieng, B. Huot, S. Dul, M.J. Gregory, V. Heang, A. Vaughn, T. Vantha, A.M. Prouty, C.C. Chao, Z. Zhang, T. Belinskaya, L.J.

1 Voegtly, R.Z. Cer, K.A. Bishop-Lilly, C. Duplessis, J. V. Lawler, and D. V. Clark, (2020). An
2 observational study of sepsis in Takeo province Cambodia: An in-depth examination of pathogens
3 causing severe infections. PLoS Neglected Tropical Diseases, 14, e0008381, *DOI*:
4 10.1371/journal.pntd.0008381

5 Rucksaken, R., C. Maneeruttanarungroj, T. Maswanna, M. Sussadee, and P. Kanbutra, (2019).
6 Comparison of conventional polymerase chain reaction and routine blood smear for the
7 detection of *Babesia canis*, *Hepatozoon canis*, *Ehrlichia canis*, and *Anaplasma platys* in Buriram
8 Province, Thailand. Veterinary World, 12, 700–705, *DOI*: 10.14202/vetworld.2019.700-705

9 Ruby, E.G., Urbanowski, M., Campbell, J., Dunn, A., Faini, M., Gunsalus, R., Lostroh, P., Lupp, C.,
10 McCann, J., Millikan, D., Schaefer, A., Stabb, E., Stevens, A., Visick, K., Whistler, C., Greenberg,
11 E.P., (2005). Complete genome sequence of *Vibrio fischeri*: A symbiotic bacterium with
12 pathogenic congeners. Proceedings of the National Academy of Sciences of the U.S.A., 102,
13 3004–3009. *DOI*: 10.1073/pnas.0409900102

14 Sam-Yellowe, T.Y., K. Addepalli, R. Yadavalli, and J.W. Peterson, (2020). New trichrome stains
15 identify cysts of *Colpodella* sp. (Apicomplexa) and *Bodo caudatus*. International Microbiology, 23,
16 303–311, *DOI*: 10.1007/s10123-019-00104-1

17 Santibáñez, S., A. Portillo, P. Santibáñez, A.M. Palomar, and J.A. Oteo, (2013). Usefulness of
18 rickettsial PCR assays for the molecular diagnosis of human rickettsioses. Enfermedades
19 Infecciosas y Microbiología Clínica, 31, 283–288, *DOI*: 10.1016/j.eimc.2012.08.001

20 Schulz, B.S., S. Kurz, K. Weber, H.J. Balzer, and K. Hartmann, (2014). Detection of respiratory
21 viruses and *Bordetella bronchiseptica* in dogs with acute respiratory tract infections. Veterinary
22 Journal, 201, 365–369, *DOI*: 10.1016/j.tvjl.2014.04.019

23 Shapiro, A.J., J.M. Norris, J. Heller, G. Brown, R. Malik, and K.L. Bosward, (2016). Seroprevalence
24 of *Coxiella burnetii* in Australian dogs. Zoonoses and Public Health, 63, 458–466, *DOI*:
25 10.1111/zph.12250

26 Shaw, S.E., M.J. Day, R.J. Birtles, and E.B. Breitschwerdt, (2001). Tick-borne infectious diseases of
27 dogs. Trends in Parasitology, 17, 74–80, *DOI*: 10.1016/S1471-4922(00)01856-0

28 Shrestha, A., C. Schindler, P. Odermatt, J. Gerold, S. Erismann, S. Sharma, R. Koju, J. Utzinger, and
29 G. Cissé, (2018). Intestinal parasite infections and associated risk factors among schoolchildren
30 in Dolakha and Ramechhap districts, Nepal: A cross-sectional study. Parasites and Vectors, 11,
31 532, *DOI*: 10.1186/s13071-018-3105-0

32 Silva, B.T.G. da, A.M. de Souza, S.D.E. Campos, D. de B. Macieira, E.R.S. de Lemos, A.R. de M.
33 Favacho, and N.R.P. Almosny, (2019). *Bartonella henselae* and *Bartonella clarridgeiae* infection,
34 hematological changes and associated factors in domestic cats and dogs from an Atlantic rain
35 forest area, Brazil. Acta Tropica, 193, 163–168, *DOI*: 10.1016/j.actatropica.2019.02.026

Song, N., W. Zhang, Y. Ding, D. Wu, Z. Dai, L. Xu, and Y. Cao, (2021). Preliminary characterization of dog derived pathogenic strains of *Leptospira interrogans* serovar australis in Nanchang of Jiangxi province, China. Frontiers in Veterinary Science, 7, *DOI*: 10.3389/fvets.2020.607115

Sor, S., M. Higuchi, M.A.B. Sarker, and N. Hamajima, (2018). Knowledge of rabies and dog-related behaviors among people in Siem Reap Province, Cambodia. Tropical Medicine and Health, 46, 20, *DOI*: 10.1186/s41182-018-0102-0

Squarre, D., Y. Nakamura, K. Hayashida, N. Kawai, H. Chambaro, B. Namangala, C. Sugimoto, and J. Yamagishi, (2020). Investigation of the piroplasm diversity circulating in wildlife and cattle of the greater Kafue ecosystem, Zambia. Parasites and Vectors, 13, 1–11, *DOI*: 10.1186/s13071-020-04475-7

Suksawat, J., Y. Xuejie, S.I. Hancock, B.C. Hegarty, P. Nilkumhang, and E.B. Breitschwerdt, (2001). Serologic and molecular evidence of coinfection with multiple vector-borne pathogens in dogs from Thailand. Journal of Veterinary Internal Medicine, 15, 453–462, *DOI*: 10.1111/j.1939-1676.2001.tb01574.x

Szöke, K., A.D. Sándor, S.A. Boldogh, T. Görföl, J. Votýpka, N. Takács, P. Estók, D. Kováts, A. Corduneanu, V. Molnár, J. Kontschán, and S. Hornok, (2017). DNA of free-living bodonids (euglenozoa: Kinetoplastea) in bat ectoparasites: Potential relevance to the evolution of parasitic trypanosomatids. Acta Veterinaria Hungarica, 65, 531–540, *DOI*: 10.1556/004.2017.051

Takai, S., R.J. Martens, A. Julian, M.G. Ribeiro, M.R. De Farias, Y. Sasaki, K. Inuzuka, T. Kakuda, S. Tsubaki, and J.F. Prescott, (2003). Virulence of *Rhodococcus equi* isolated from cats and dogs. Journal of Clinical Microbiology, 41, 4468–4470, *DOI*: 10.1128/JCM.41.9.4468-4470.2003

Takhampunya, R., A. Korkusol, C. Pongpichit, K. Yodin, A. Rungrojn, N. Chanarat, S. Promsathaporn, T. Monkanna, S. Thaloengsok, B. Tippayachai, N. Kumfao, A.L. Richards, and S.A. Davidson, (2019). Metagenomic approach to characterizing disease epidemiology in a disease-endemic environment in Northern Thailand. Frontiers in Microbiology, 10, 319, *DOI*: 10.3389/fmicb.2019.00319

Tamburini, F.B., T.M. Andermann, E. Tkachenko, F. Senchyna, N. Banaei, and A.S. Bhatt, (2018). Precision identification of diverse bloodstream pathogens in the gut microbiome. Nature Medicine, 24, 1809–1814, *DOI*: 10.1038/s41591-018-0202-8

Taylor, M.J., C. Bandi, and A. Hoerauf, (2005). *Wolbachia* bacterial endosymbionts of filarial nematodes. Advances in Parasitology, 60, 245–284, *DOI*: 10.1016/S0065-308X(05)60004-8

Van Vinh Chau, N., L. Buu Chau, M. Desquesnes, S. Herder, N. Phu Huong Lan, J.I. Campbell, N. Van Cuong, B. Yimming, P. Chalermwong, S. Jittapalapong, J.R. Franco, N.T. Tue, M.A. Rabaa, J. Carrique-Mas, T.P.T. Thanh, N. Tran Vu Thieu, A. Berto, N. Thi Hoa, N. Van Minh Hoang, N. Canh Tu, N. Khac Chuyen, B. Wills, T. Tinh Hien, G.E. Thwaites, S. Yacoub, and S. Baker, (2016). A clinical and epidemiological investigation of the first reported human infection with the zoonotic

parasite *Trypanosoma evansi* in Southeast Asia. Clinical Infectious Diseases, 62, 1002–1008, *DOI*: 10.1093/cid/ciw052

Vandersea, M.W., A.J. Birkenheuer, R.W. Litaker, S.L. Vaden, J.S. Renschler, and J.L. Gookin, (2015). Identification of *Parabodo caudatus* (class Kinetoplastea) in urine voided from a dog with hematuria. Journal of Veterinary Diagnostic Investigation, 27, 117–120, *DOI*: 10.1177/1040638714562827

Votýpka, J., E. Suková, N. Kraeva, A. Ishemgulova, I. Duží, J. Lukeš, and V. Yurchenko, (2013). Diversity of Trypanosomatids (Kinetoplastea: Trypanosomatidae) parasitizing fleas (Insecta: Siphonaptera) and description of a new genus *Blechomonas* gen. n. Protist, 164, 763–781, *DOI*: 10.1016/j.protis.2013.08.002

Williams, M., L. Izzard, S.R. Graves, J. Stenos, and J.J. Kelly, (2011). First probable Australian cases of human infection with *Rickettsia felis* (cat-flea typhus). Medical Journal of Australia, 194, 41–43, *DOI*: 10.5694/j.1326-5377.2011.tb04145.x

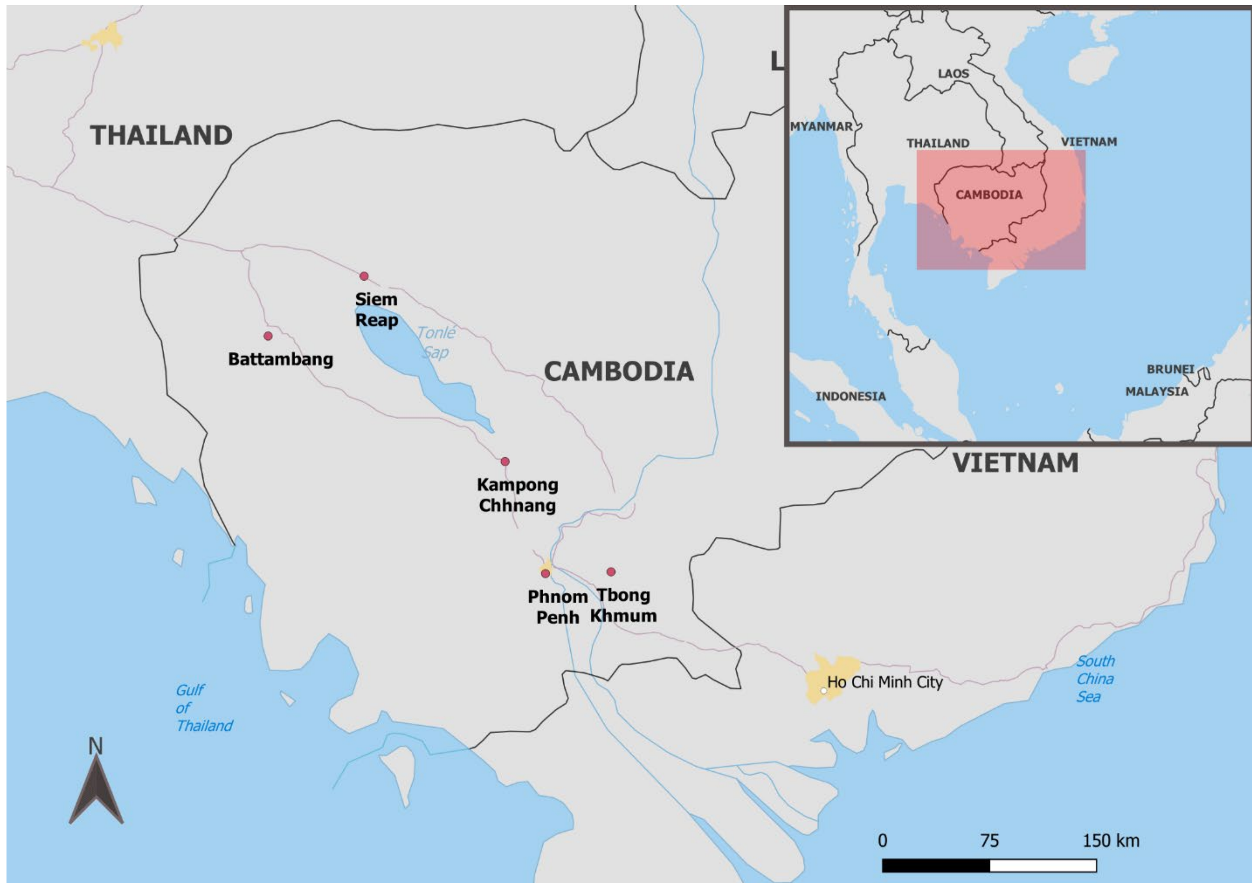
Wong, S.S.Y., J.L.L. Teng, R.W.S. Poon, G.K.Y. Choi, K.H. Chan, M.L. Yeung, J.J.Y. Hui, and K.Y. Yuen, (2011). Comparative evaluation of a point-of-care immunochromatographic test SNAP 4Dx with molecular detection tests for vector-borne canine pathogens in Hong Kong. Vector-Borne Zoonotic Diseases, 11, 1269–1277, *DOI*: 10.1089/vbz.2010.0265

Xavier, R., J. Cardoso, R. Barroso, S. Nogueira, C. Cruz, A. Pereira, and A. Saraiva, (2020). Effects of *Goussia* infecting the blue whiting and phylogenetic placement of *Goussia* infecting marine fish off Northern Portugal. Parasitology Research, 119, 2139–2147, *DOI*: 10.1007/s00436-020-06727-y

Yuan, C.L., P.J. Keeling, P.J. Krause, A. Horak, S. Bent, L. Rollend, and X.G. Hua, (2012). *Colpodella* spp.- like parasite infection in woman, China. Emerging Infectious Diseases, 18, 125–127, *DOI*: 10.3201/eid1801.110716

1 **Figures**

2 **Figure 1:** Map of field areas from which canine samples were collected. Phnom Penh (PP, n = 148),
3 Kampong Chhnang (KC, n = 48), Siem Reap (SR, n = 155), Battambang (BB, n = 165) and Tbong Khmum (TK,
4 n = 156). Map created in QGIS 3.4 via QGIS.org, 2021. QGIS Geographic Information System. QGIS
5 Association.



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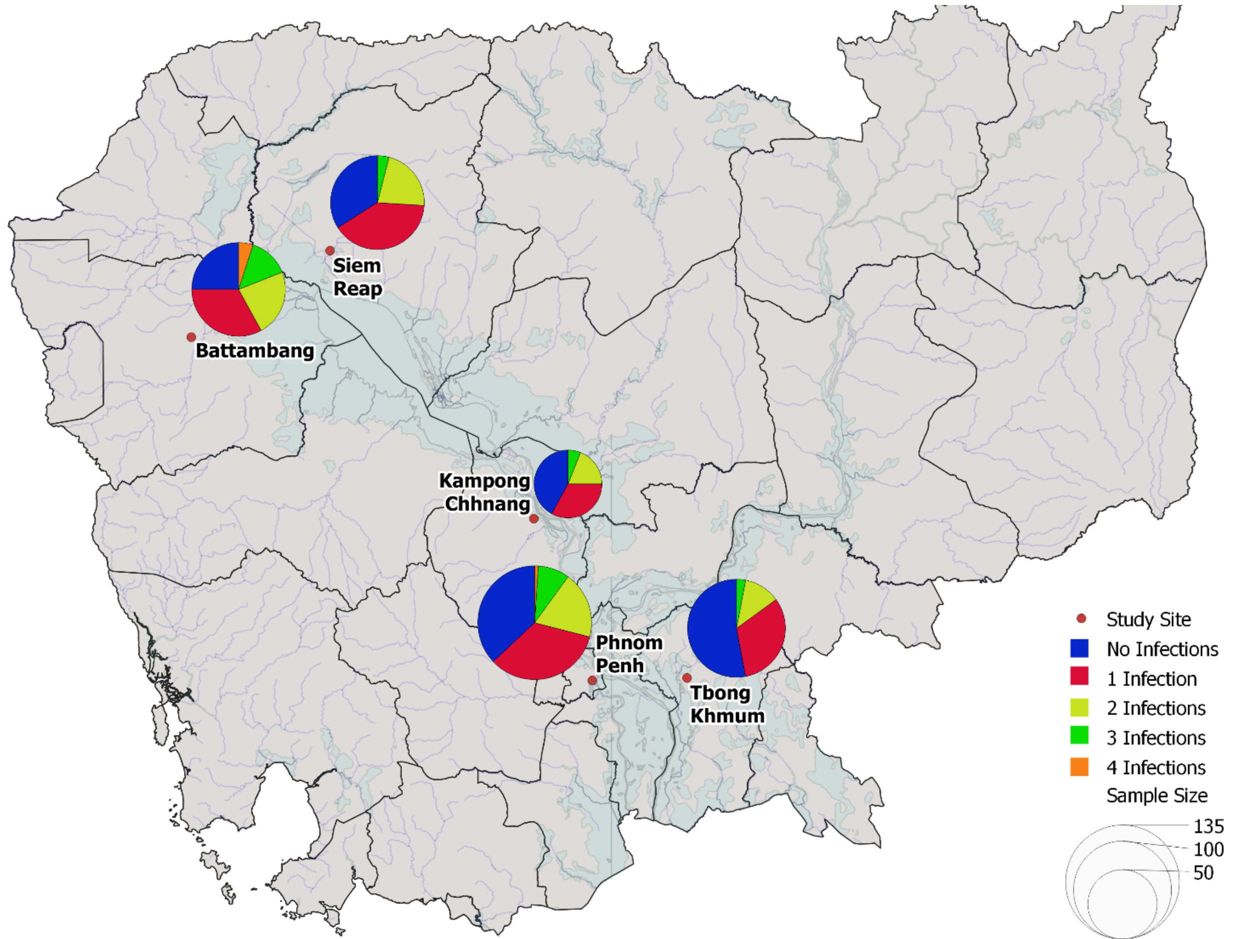
1 **Figure 2. Map with the relative proportions of uninfected, singularly infected and coinfected dogs at**
2 **five different Cambodian field sites.** Pie chart size is proportional to sample size for that study site. Dark
3 blue lines depict rivers whilst light blue areas demarcate lakes and regions prone to inundation. Map
4 created in QGIS 3.4 via QGIS.org, 2021. QGIS Geographic Information System. QGIS Association.



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1 **Supporting information: Sequences of our unique positive control gBlock DNA constructs.**

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

5 **5'-**
6 **GTGCCAGCAGCCGCGGTAA**TACGGAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGCATGCAGGTG
7 GTTCATTAAGTCAGATGTGAAAGCCCGGGGCTCAACCTCGGAACCGCATTTGAAACTGGTGAACTAGAGTGCTGT
8 AGAGGGGGGTAGAATTTCAGGTGTAGCGGTGAAATGCGTAGAGATCTGAAGGAATACCAGTGGCGAAGGCGGC
9 CCCCTGGACAGACACTGACACTCAGATGCGAAAGCGTGGGGAGCAAACAGG**ATTAGATACCCTGGTAGTCC - 3'**

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

13 **5'-**
14 **CAAGGAAGTTTAAGGCAATAACAG**TACGGAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGCATGC
15 AGGTGGTTCATTAAGTCAGATGTGAAAGCCCGGGGCTCAACCTCGGAACCGCATTTGAAACTGGTGAACTAGAGT
16 GCTGTA**AATCGTGAACGAGGAATGCCTAG – 3'**

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

20 **5' –**
21 **CAAACGATGACACCCATGAA**GGAACCGCATTTGAAACTGGTGAACTAGAGTGCTGTAGAGGGGGGTAGAATTTC
22 AGGTGTAGCGGTGAAATGCGTAGAGATCTGAAGGAATACCAGTGGCGAAGGCGGCCCCCTGGACAGACACTGAC
23 ACTCAGATGCGAAAGCGTGGGGAGCAAACAGG**GAGGTTACAGTCTCAGGGGG – 3'**

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