Epidemiology of Canine Vector-Borne Pathogens (CVBPs) of Dogs in Nigerian Communities

By

Ternenge Thaddaeus, Apaa

Supervisors: Dr Rachael Tarlinton (BVSc, PhD, MRCVS)

Dr Stephen Dunham (BVSc, MRCVS, PhD)

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School of veterinary Medicine and Science

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Abstract

Canine vector-borne pathogens (CVBPs) have a significant impact on the health and welfare of dogs and humans. There is extremely little data on the prevalence of tick species, CVBPs and their impact on Nigerian dogs. The main aim of this study was to identify ticks, CVBPs and associated risk factors in Nigerian dogs using point-of-care (POC) test, PCR, metagenomics profiling and multivariate modelling. A total of 259 canine blood and 122 hard tick samples were examined in this study.

POC and PCR-based tests showed that *Borrelia burgdorferi* is unlikely to be present in Nigerian dogs, while an unexpectedly low prevalence of *Dirofilaria immitis* was reported. The low prevalence of *Anaplasma* species and high prevalence of *Ehrlichia canis* was reported via both POC and PCR test. While high prevalence of *Babesia* species (*B. vogeli* and *B. rossi*) were reported via PCR test on canine blood, this report confirmed the absence of *B. gibsoni*, *B. canis* and *B. vulpes* in canine blood sample examined. Risk factor analysis using PCR results showed that exotic dogs were more likely to test positive to CVBPs during the rainy season. While risk factors analysis from the POC test results showed that older and tick infested dogs were more likely to test positive to CVBPs, implying that older dogs would have a higher lifetime exposure risk than younger dogs. An increased risk of tick infestation was reported in male dogs which may be due to increased roaming activities.

The tropical lineage of *Rhipicephalus sanguineus* (s.l.) and *Haemaphysalis leachi* were the most prevalent tick species infesting dogs, while *Haemaphysalis* spp. tentatively identified as *H. longicornis* were demonstrated to infest *Gallus domesticus*.

Metagenomics profiling of ticks indicated the presence of *Leishmania, Plasmodium, Ehrlichia, Anaplasma* and *Candidatus Coxiella* species, demonstrating the risk of canine disease burden and human zoonosis. While additional studies are required to confirm these reports, the inclusion of these pathogens in differential diagnosis during clinical examination of dogs, and adherence to strategic tick control is recommended.

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Table of Abbreviations

ABBREVIATION	MEANING
AMR	Antimicrobial Resistance Genes
ANI	Average Nucleotide Identity
ANVI'O	Advanced Analysis and visualization Platform for 'Omics Data
BHAV	Bhanja Virus Fever
CBC	Complete Blood Count
CCHF	Crimean-Congo Haemorrhagic Fever
CELISA	Competitive Enzyme-Linked Immunosorbent Assay
CFT	Complement Fixation Test
CGA	Canine Granulocytic Ehrlichiosis
CGView	Circular Genome Viewer
CI	Confidence Interval
COGs	Cluster of Orthologous Genes
CVBPs	Canine Vector-Borne Pathogens
DB	Database
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide Triphosphates
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
GAAS	Genome Relative Abundance and Average Size
GC	Guanine-Cytosine
GTDB-TK	Genome Taxonomic Database Toolkit
HE	Human Granulocytic Ehrlichiosis
HTML	Hypertext Markup Language file format
IFAT	Immunofluorescent Antibody Test
LFD	Lateral Flow Device
MSA	Multiple Sequence Alignment
NGS	Next Generation Sequencing
NCBI	National Center for Biotechnology Information
OTU	Operational Taxonomic Units
PCR	Polymerase Chain Reaction
POC	Point-of-Care test
QPCR	Quantitative Polymerase Chain Reaction
rRNA	Ribosomal Ribonucleic Acid
rMAP2	Recombinant Major Antigenic Protein 2
SCG	Single Copy Gene
SFTS	Severe Fever with Thrombocytopenia Syndrome
S. l	Sensu lato
SNPs	Single Nucleotide Polymorphisms
TBDs	Tick-Borne Diseases
TBPs	Tick-Borne Pathogens
TBEV	Tick-Borne Encephalitis Virus
TBRF	Tick-Borne Relapsing Fever
USA	United States of America
VAMB	Variational Autoencoders for Metagenomics Binning

Chapter 1: Introduction

1.1 Nigerian states, geopolitical zones, and population

Nigeria covers a landmass of 923,768 square kilometres (Ali-Akpajiak and Pyke, 2003). It is a multi-ethnic and multi-cultural nation consisting of over 200 ethnic groups and no fewer than 374 languages are spoken across Nigeria (Eze et al., 2014). Nigeria is divided into 36 states, with Abuja as the federal capital territory (figure 1). All 36 states are classified into six geopolitical zones (figure 1), consisting of people with similar ancestral origin, ethnic and cultural heritage. These zones include the North Central (NC), North East (NE), North West (NW), South East (SE), South-South (SS) and the South West (SW) (Eze et al., 2014). Nigeria has an estimated human population of 211 million, making up 2.64% of the total world population. About 52% of Nigeria's total population live in urban areas (Worldometer, 2021).

Previous poverty assessment in Nigeria (1996) showed that the North West, North East, and North Central geopolitical zones had higher poverty levels, while the southern zones were comparatively less affected by poverty (Ogwumike, 2002). The World Bank 2013 policy note on Nigeria stated that Nigeria's Northern states were consistently poorer than the Southern states. According to the World Bank (WorldBank, 2013), stagnation of urbanization and fewer economic activities present in this region may be responsible for the elevated level of poverty recorded in this region. Consistent insecurity in the northern parts of Nigeria ranging from herder attacks on farmers and attacks by Boko-haram are factors responsible for higher poverty recorded in these areas. Education has been defined as one of the determinant factors of poverty reduction, however, the northern part of Nigeria is also known to be drastically left behind in terms of education and human resource development (Okojie, 2002). In addition, unemployment, corruption, non-diversification of the

economy or over-dependence on oil, inequality and a poor education system have been proposed to be key contributory factors of extreme poverty in Nigeria.

Two major seasons (rainy and dry) define the Nigerian climate. The annual duration of the rainy season is about seven months (April to October). while dry seasons usually lasts about five months (November to March) (Omotosho et al., 2000). The tropical savannah in the northern part of the country is characterized by one distinct peak of rainy and dry seasons. The mean average ambient temperature is >18^{o}C during the entire year, with hot and dry air during the dry seasons spanning from December to March yearly. Tropical rainforest is the prevailing climatic condition in the southern part of Nigeria. This is defined by cooler temperature ranges during the entire year and a combination of winds or storms due to its close location to the equator. Montane or highland climate is recorded in regions located >1520 metres above sea level and this is characterized by minimum and maximum mean average temperatures of 16^{o}C and 27.6^{o}C, respectively. Overall, Nigeria has an average annual ambient temperature range between 21^{o}C (minimum) and 32.8^{o}C (maximum), while the mean average annual relative humidity is estimated at 62% (Eludoyin et al., 2014).

A good understanding of the geography and climate of Nigeria is essential for understanding of Canine Vector-Borne Pathogens (CVBPs) distribution and transmission in Nigeria. Seasonal changes in climate have been demonstrated to potentiate the distribution of vectors and transmission of pathogens in domestic animals in Nigeria. Studies on the seasonal distribution and abundance of canine tick infestations in North Central Nigeria have indicated an increasing and decreasing abundance of ticks in the rainy (April to October) and dry season (January to April), respectively (Arong et al., 2011). The map of Nigeria (Figure 1.1) below shows Nigerian thirty-six states and the six geopolitical zones (created using google earth pro-7.3.1.4507). The map areas at the top coloured in purple and dark green indicate Northwest and Northeast, respectively. While

areas in white and brown represent the North central and South-west, respectively. The blue and light green areas at the bottom of the map represent South-east and South-south, respectively.

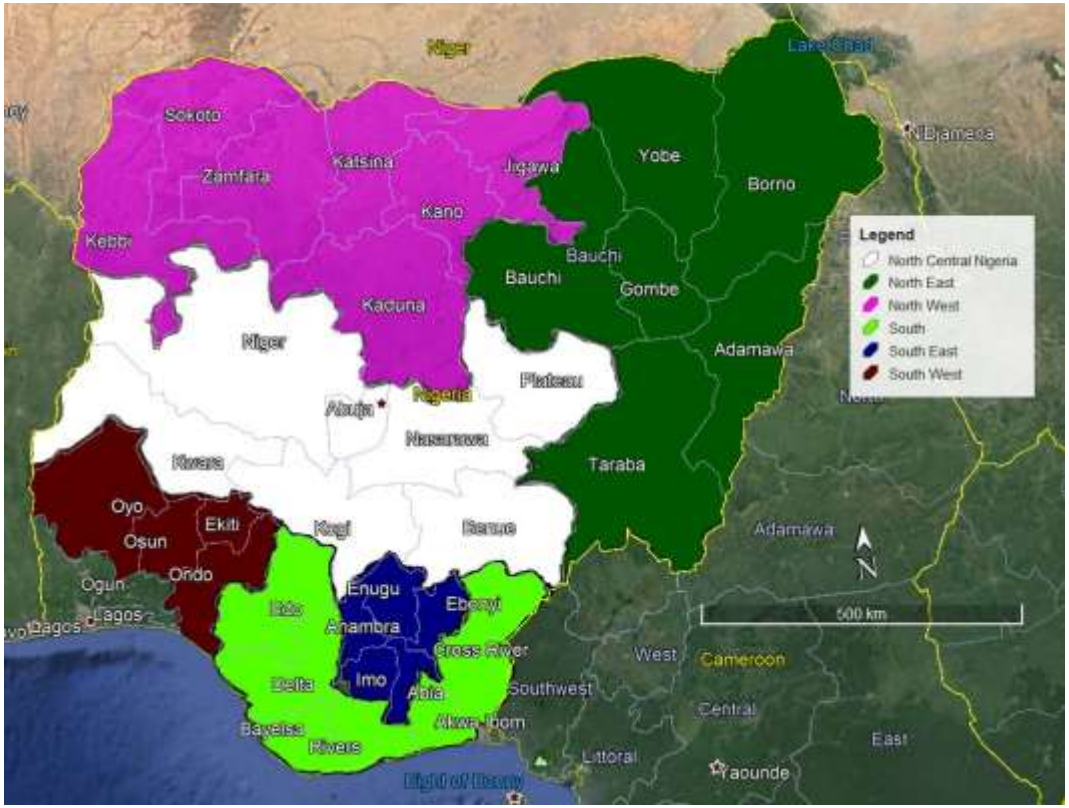


Figure 1.1: Map of Nigeria showing names of the thirty-six states and the six geopolitical zones**.**

1.2 Nigerian dog breeds

Different unrecognized indigenous dog breeds define the Nigerian domestic dog population. Indigenous dog breeds are owned by people with low socio-economic status, with a general assumption that indigenous dog breeds are cheap to obtain and care for. They are known for being hardy, requiring little or no care in terms of feeding and healthcare. Nigerian dog breeds share similarities with the African hound group or breed of dog called Basenji originating from Congo, officially recognized by the Kennel club. They are described as intelligent but hard-to-train small-to-medium-size dogs, with erect ears, short hair coat and muscular bodies (Figure 1.2) (KennelClub, 2021).

A mixture of local and exotic (imported) breeds of dog are common in Nigerian communities (Otolorin et al., 2014, Apaa et al., 2016, Garba et al., 2017, Kwaghe et al., 2019). Indigenous and some exotic dog breeds may be kept for breeding, hunting, as pets, or guard dogs in Nigeria (Kwaghe et al., 2019). Exotic breeds in Nigeria also serve as a status symbol for their owners as reported in other parts of the world (Fielding and Mather, 2001, Maher and Pierpoint, 2011, Omudu et al., 2010). The most common exotic dog breeds in Nigeria include the hound (dachshund and greyhound, beagle), pastoral (German shepherd, Caucasian shepherd), working (Rottweiler, Samoyed, Doberman, Bull Mastiff, Boerboel, Pitbull), non-sporting (Chow-Chow, bulldogs, Lhasa Apso), and terrier (Chihuahua) groups (Oboegbulem and Nwakonobi, 1989, Shima et al., 2015a, Oghenemega and Aderonke, 2018). Due to the perceived status of exotic breeds and the more likelihood of their owners to afford veterinary services, they are over-represented in the population of dogs presented for veterinary care. For example, there are reports in previous studies from the south-eastern part of Nigeria demonstrating that 66.3% of dogs presented for veterinary care were "exotic breeds" (Otolorin et al., 2014). In general, the Nigerian canine population

in both rural and urban areas is dominated by the indigenous dog breeds (Garba et al., 2017, Kwaghe et al., 2019).



Figure 1.2: Typical Nigerian indigenous dog breeds with a mature female adult (left) and young dogs between the ages of three to six months (right). Photo credit: Dr F.A Gberindyer.

1.3 Nigerian dog population, management practices, and usage

Currently, there is little information on the total dog population in Nigeria available to serve as a reference to researchers in this area. Recent studies have demonstrated that several exotic and indigenous breeds of dog are present in urban communities in Nigeria (Omudu et al., 2010, Aiyedun and Olugasa, 2012b, Otolorin et al., 2014, Hambolu et al., 2014, Ikpeze, 2017, Garba et al., 2017, Mshelbwala et al., 2018). While the study by Kwaghe et al. (2019) provided summary data on the estimated canine population and dog-to-human ratio, other previous localized studies mentioned here estimated canine population and dog-to-human ratio with more emphasis on urban communities. To-date the study by Luga et al. (2018) remains the only localized study providing estimated canine population and dog-to human ratio in both urban and rural areas. The Nigerian livestock resources survey of 1992, carried out to determine the characteristics and distribution of major species and breeds of domestic animals, estimated the Nigerian dog population to be 4.5 million (Bourn et al., 1994). The study employed a low-level aerial survey technique of systematic reconnaissance flights and a complementary ground technique covering various parts of Nigeria. This study remains the only comprehensive dog population census in Nigeria (Bourn et al., 1994). Several smaller studies on dog ecology have been carried out in various parts of Nigeria, but most of these studies were restricted to individual villages, towns, cities, states, or geopolitical zones. Thus, data generated from such studies may not be comprehensive enough to provide an accurate estimate of the total dog population in Nigeria (Table 1.1). Most of these studies used questionnaires, house to house or street counts of dogs (Luga et al., 2018, Ikpeze, 2017, Hambolu et al., 2014, Otolorin et al., 2014, Aiyedun and Olugasa, 2012b, Omudu et al., 2010, Garba et al., 2017), and only one such localized study differed in terms of the methodology, where photographic capture and recapture method was used (Mshelbwala et al., 2018). In summary, the mean average dog-to-human ratio in Nigerian communities can be estimated

to be 1:4 in urban and 1: 220 in rural communities (Table 1.1). This implies that one out of every four persons in Nigerian urban settlements owns a dog, while one out of every 220 people in rural settlements owns a dog. This shows that there is more contact between dogs and humans in urban than rural areas.

The present Nigerian human population is estimated to be 206 million people, 52% of the population is urban (107,112,526), while 48% (99,027,063) of the population lives in rural areas (Worldometer, 2021). By extrapolating dog population using the estimated urban (1:4) and rural (1:220) dog-to-human ratios, one may assume that the Nigerian urban communities provide shelter to over twenty-six (26,778,131) million dogs, while the rural communities provide shelter to over four hundred thousand (450,123) dogs. This extrapolation supports the popular Nigerian saying that urban dwellers are more likely to own dogs, than rural dwellers.

Table 1.1: Details of regional dog population census and methods used by different studies in Nigeria.

Location	Method	Dog population density	Dog population	Dog-to-human ratio	Reference
Kaduna (ABU/Bomo)	questionnaires	Nil	185	1:7.6 (urban) 1:219.5 (rural)	(Luga et al., 2018)
Abuja (Gwagwalada)	photographic capture/recapture	Nil	103, 758	1:3.7 (urban)	(Mshelbwala et al., 2018)
Enugu State (Enugu)	house to house count method	90-dogs/180 km^2	16, 152	N/A	(Ikpeze, 2017)
Lagos State (Lagos)	Questionnaires/ household/street counts	Nil	1, 527, 718	1:5.6 (urban)	(Hambolu et al., 2014)
Abia state (Aba north and south LGA)	compound questionnaires/direct street count	Nil	68, 121	1:7.8 (urban)	(Otolorin et al., 2014)
Kwara state (Ilorin)	questionnaires/direct street count/aerial photography	1-dog/13 households	1, 258	Nil	(Aiyedun and Olugasa, 2012b)
Benue state (Makurdi)	household census and street observation	1.43-dogs/households	290	1:4 (urban)	(Omudu et al., 2010)
Niger state (Mina, Bida, Suleja, Kontagora)	questionnaires	Nil	732,476	1:5.4 (urban)	(Garba et al., 2017)
Nasarawa state (three senatorial districts)	questionnaires	Nil	462,586	1.1:6 (urban)	(Kwaghe et al., 2019)

Key: Not applicable (N/A).

Nigerian dog owners adopt several dog management practices, though there are insufficient data on which is the most popular dog management system observed across different parts of Nigeria. Depending on location, recent studies on dog management systems in Nigeria have shown that over 70% of dogs may be free-roaming, with less than 30% of dogs kept intensively or kennel-confined (Aiyedun and Olugasa, 2012a, Kwaghe et al., 2019). Many kennel-confined dogs mentioned in the Aiyedun and Olugasa (2012a) study are kept in residential dwellings where individuals with high income live. While the free-roaming dogs may be kept by hunters, dog meat consumers or those with low income who leave their dogs to seek shelter and food for themselves in villages, towns, and cities (Aiyedun and Olugasa, 2012a, Kwaghe et al., 2019).

Dog usage in Nigerian communities varies from region to region, diverse cultural groups in Nigeria have different beliefs about dogs and their usage A small proportion of herders in the north and other parts of the country may use dogs to protect their cattle and sheep from wild animals during shepherding. In rural communities, dogs are used for hunting wild animals and as guard dogs. On the other hand, most urban dwellers keep dogs as guard dogs; only a small proportion of dog owners keep dogs as pets (Oboegbulem and Nwakonobi, 1989, Aiyedun and Olugasa, 2012a, Garba et al., 2017, Kwaghe et al., 2019). In other parts of Nigeria, dogs have become a source of food as dog meat comprises a cooked delicacy by the locals. According to previous studies, meat from dogs is popularly known as 'dogmycin' or 'four-o-four (404) by the local consumers. Consumers provided these nicknames in the belief that dog meat is medicinal, and dogs can run as fast as Peugeot branded four wheeled cars. Dog meat consumption is particularly popular in regions like Plateau state, Kaduna (north central Nigeria) and south-eastern parts of Nigeria. However, tribes and individuals across various parts of Nigeria are also known for dog meat consumption (Elisha and Soloman, 2008, Ukamaka et al., 2020).

Although, limited information on dog population, breeds, management practices and usage adopted by Nigerians confirms the close association between people

and dogs especially in Nigerian urban areas. For this reason, owned dogs may play a significant role in transmitting zoonotic diseases to their owners. Total elimination of dogs to avoid these challenges may not only cause emotional trauma to owners, but also leave them with no alternative pets to play the roles usually carried out by these dogs.

1.4 Common diseases of dogs in Nigeria

Although many diseases are reported to be prevalent in the tropics affecting both humans and animals, there is inadequate information on the total prevalence and distribution of diseases affecting dogs in Nigeria. Notwithstanding, studies focusing on villages, cities or states have provided some insight into the prevalence of canine diseases in Nigeria. Helminthosis, mange, canine parvovirus gastroenteritis, dermatitis, aural haematoma, poisoning, and myiasis have been reported to be common clinical conditions affecting Nigerian owned dogs (Shima et al., 2015b). Helminthosis is reported to be the most prevalent canine disease in Nigeria, followed by tick infestation, and haemoparasite infection, including canine babesiosis (Karshima et al., 2010, Shima et al., 2015b, Ola-Fadunsin, 2018, Kamani et al., 2021). A ten-year retrospective study in Jalingo, in North Eastern Nigeria demonstrated that over 49% (n = 6088) of dogs presented to veterinary clinics had tick infestation and haemoparasite infection, including *Babesia* spp. (Karshima et al., 2010). A similar ten-year retrospective study in South Western Nigeria reported that over 42% (n= 2820) of dogs presenting to major veterinary clinics were diagnosed with tick infestation, with canine babesiosis (Ola-Fadunsin, 2018) comprising the second most reported parasitic disease of dogs. Infestation of dogs with ticks and tick-borne pathogens is a commonly encountered challenge for dog owners and veterinarians in the tropics (Irwin and Traub, 2006), and Nigeria is not an exception.

1.5 Classification of ticks

Ticks are known to transmit various infectious pathogens and diseases such as piroplasmosis (*Theileria and Babesia*), ehrlichiosis, anaplasmosis, borreliosis, hepatozoonosis and tick-borne encephalitis virus (TBEV) in both rural and urban communities of tropical and temperate regions of the world (Shaw et al., 2001, Chomel, 2011, Biernat et al., 2014, Balling et al., 2015, de Marco et al., 2017, Ogo et al., 2012). There are two major families of ticks, hard (*Ixodidae*) and soft (*Argasidae*) ticks, comprising of approximately 877 species globally (Magnarelli, 2009). Tick genera from the family *Argasidae* include *Argas*, *Ornithodoros* and *Otobius* species. Although a few members of this family have been known to transmit pathogens in humans and livestock, *Argasidae* are mostly known to transmit pathogens in birds. Hard ticks are responsible for most of the pathogens transmitted to humans, livestock, and companion animals. They remain a major challenge in the control of livestock and companion animal vector-borne pathogens or zoonosis (Jongejan and Uilenberg, 2004). The most abundant genera in the family *Ixodidae* infesting dogs and other domestic animal include the genus *Ixodes* (comprising of over 245 species), *Haemaphysalis* (with over 164 species), *Rhipicephalus*, and *Amblyomma*, consisting of over 131 species (Magnarelli, 2009, Saleh et al., 2021).

1.6 Global distribution of canine vector-borne disease and vectors

The damage and diseases caused by canine vectors including ticks and their pathogens has created a substantial impact on canine, human health, and the global livestock economy (Jongejan and Uilenberg, 2004). With the frequent movement of animals such as dogs, horses and birds across the globe coupled with the dynamic global climatic condition, the distribution of ticks and other vectors may not be restricted to areas originally known to be favourable for certain ticks and vector species (Hansford et al., 2019). Recent literature shows that over 867 species of ticks serve as vectors for various disease-causing

pathogens in humans, domestic, and wild animal species. Table 1.2 below shows common canine vector-borne diseases and the global distribution of potential tick and other vectors.

Table 1.2: Common canine vector-borne diseases, aetiology, potential vectors, distribution, and transmission routes.

Diseases	**Causative agent (s)**	**Potential vector (s)**	**Distribution**	**Transmission route**	**References**
Selected parasitic canine vector-borne diseases					
Canine piroplasmosis	Small *Babesia* species				
	B. gibsoni	*R. sanguineus (s.l.), H. hystricis, H. longicornis, *H. bispinosa*	Worldwide, United states, Europe, Australia/New Zealand, Asia, Africa	Tick bite, blood transfusion, direct dog-to-dog transmission via bite wounds, saliva, or ingestion of blood in fighting dogs, sharing of surgical equipment, and transplacental transmission is suspected	(Jongejan et al., 2018, Iwakami et al., 2014, Birkenheuer et al., 2020, Barash et al., 2019, Jain Jose et al., 2018, Panti-May and Rodriguez-Vivas, 2020, Yeagley et al., 2009)
	B. conradae	**R. sanguineus*	America (California), Asia	Tick bite	(Dear et al., 2018)
	B. vulpes n. sp *(Babesia microti-like, Theileria annae)*	*Ixodes hexagonus, D. reticulatus, *Ixodes ricinus, *Ixodes canisuga, *R. sanguineus*	North America, Europe, Asia	Described to be similar with *B. gibsoni*	(Barash et al., 2019, Checa et al., 2018, Sang et al., 2021, Baneth et al., 2019)
	B. negevi n. sp.,	**Ornithodoros tholozani*	Middle East (Israel, Palestine)	Tick bite	(Baneth et al., 2020)
	Large *Babesia* species				
	B. vogeli	*R. sanguineus*	Worldwide, Europe, Asia, Africa, America, Australia/New Zealand	Tick bite,	(Birkenheuer et al., 2020, Cardoso et al., 2016, Ngoka et al., 2021, Li et al., 2020, Manoj et al., 2020, Di

Diseases	Causative agent (s)	Potential vector (s)	Distribution	Transmission route	References
					Cataldo et al., 2020, Panti-May and Rodriguez-Vivas, 2020)
	B. rossi	*H. leachi, H. elliptica*	Africa, Asia (South-eastern), America (South/North, Latin America, and the Caribbean), Western Europe (France, Germany, Switzerland)	Tick bite, blood transfusion	(Shabangu et al., 2021, Birkenheuer et al., 2020, Ciuca et al., 2021, Kamani, 2021a, Adamu et al., 2014)
	B. canis	*D. reticulatus, R. sanguineus* (s.l.)	Europe, America (North America), Asia		(Birkenheuer et al., 2020, de Marco et al., 2017)
Canine hepatozoonosis	*Hepatozoon canis*	*R. sanguineus* (s.l.), *R. turanicus, R. apendiculatus, R. simus, H. longicornis, H. flava, etc.*	worldwide	Primarily via ingestion of *H. canis* infected *R. sanguineus,* vertical transmission from dam to puppies and transstadial transmission from nymph to adult ticks	(Baneth et al., 2007, Greay et al., 2018, Kamani et al., 2019a, Arnaout et al., 2022)
	Hepatozoon americanum	*Amblyomma maculatum*	South/Eastern United States	Direct ingestion of *H. americanum* infected *A. maculatum* or parts of the ticks containing matured oocysts and cyst found in tissue of intermediate hosts during predation	(Allen et al., 2011, Johnson et al., 2009)

Diseases	Causative agent (s)	Potential vector (s)	Distribution	Transmission route	References
Canine filariasis	Aetiology of canine heartworm disease: *D. immitis*	*Aedes, Anopheles and Culex* mosquitoes	Africa, America, Australia, Europe, Asia	Transmission of infective third stage (L3) larva via mosquito bite. Filaraemic blood transfusion is not associated with heartworm disease in the recipient dogs, but may interfere with diagnostic testing	(Anvari et al., 2020, Orr et al., 2020, Wardrop et al., 2016)
	Aetiology of cutaneous dirofilariasis: *Dirofilaria repens Candidatus Candidatus Dirofilaria hongkongensis sp. nov*	Same as in *D. immitis*	Europe, Asia, Africa	Same as in *D. immitis*	(Rjeibi et al., 2017, Genchi and Kramer, 2020, Maggi and Kramer, 2019, Pradeep et al., 2019)
Canine Leishmaniosis	Aetiology of Visceral leishmaniosis: *L. donovani* (Asia and Africa) *L. infantum*	Female sand flies (*Lutzomyia)*	South America, Europe, Africa, Asia	During blood meal on animals, sexual and transplacental transmission, and blood transfusion	(Dantas-Torres et al., 2019, Marcondes and Day, 2019, Svobodova et al., 2017, Carvalho Junior et al., 2017, Cantacessi et al., 2015, Usman et al., 2020, Kone et al., 2019)
	Aetiology of cutaneous leishmaniosis: *L. braziliensis, L. amazonensis*	*Same as in visceral leishmaniosis*	South America, Asia	During blood meal on animals	(Marcondes and Day, 2019, Lima et al., 2017, Baneth et al., 2016, Cantacessi et al., 2015)

Diseases	Causative agent (s)	Potential vector (s)	Distribution	Transmission route	References
	L. major (Asia)				
Canine trypanosomosis	*T. cruzi and T. rangeli* (Chagas or American form) *T evansi* (sleeping sickness or African form), *T. brucei and T. congolense* (Africa and Asia), *T. evansi* (Africa)	biting flies (tabanids, Stomoxys) vampire bats (America),	South America, Africa, Asia, Europe	Ingestion of infected vector is more common. Transmission via blood transfusion, sharing of needles, surgical blades etc., and organ transplant as well as vertical transmission.	(Eloy and Lucheis, 2009, Bui et al., 2021, Umeakuana et al., 2019, Leschnik et al., 2021, Aregawi et al., 2019)
Selected bacterial canine vector-borne diseases					
Canine ehrlichiosis	Canine monocytic ehrlichiosis				
	E. canis	*R. sanguineus* (s.l.), *D. variabilis?*	Worldwide	Tick bite and blood transfusion	(Kamani et al., 2019a, Taques et al., 2020, Bezerra-Santos et al., 2021, Ojeda-Chi et al., 2019, Perez-Macchi et al., 2019, Mylonakis et al., 2019, Qurollo et al., 2014, Neave et al., 2022, Wardrop et al., 2016)
	Canine granulocytic ehrlichiosis				
	E. ewingii	*A. americanum*	Worldwide	Tick bite and blood transfusion	(Han and Na, 2018, Kamani et al., 2019a, Qurollo et al., 2014, Aguiar et al., 2020, Wardrop et al., 2016)
	A. phagocytophilum	*I. ricinus, I capularis, I. pacificus*	Worldwide	Tick bite and blood transfusion	(Kolo et al., 2016, Cui et al., 2017, De Arcangeli et al., 2018, Qurollo et al.,

Diseases	Causative agent (s)	Potential vector (s)	Distribution	Transmission route	References
					2014)(Wardrop et al., 2016)
	Canine infectious cyclic thrombocytopenia				
	A. Platys (erstwhile E. platys)	*R. sanguineus* (s.l.), *Hyalomma truncatum, D. auratus*	Worldwide	Tick bite and blood transfusion	(Perez-Macchi et al., 2019, Kamani et al., 2019a, Wardrop et al., 2016)
Canine borreliosis	*B. burgdorferi* (s.l.)	*Ixodes spp., Dermacentor nitens, R. sanguineus* (s.l.)	America, Europe, Asia, Africa,	Tick bite	(Krupka and Straubinger, 2010, Rudenko et al., 2011)
Haemotropic mycoplasmosis (erstwhile haemobartonellosis and eperythrozoonosis)	*Mycoplasma haemocanis, Mycoplasma haematoparvum*	blood sucking arthropods including *R. sanguineus* suggested	South America, Asia, Europe, Australia,	Blood transfusion and direct contact with infected blood during aggressive interaction between dogs	(Kim et al., 2020, Cortese et al., 2020, Aktas and Ozubek, 2017, Barker et al., 2012, Barbosa et al., 2021)

Note: Not well understood (*).

1.6.1 Importance of canine tick infestation

Ticks of the family *Ixodidae* are important disease vectors due to their global distribution and contact with a wide range of companion animals, humans, livestock, and wildlife. (Troughton and Levin, 2007). The direct and indirect impact of tick infestation on canine, human and other animal hosts is remarkable, making ticks the second most important disease transmitting vector after mosquitoes. The presence of ticks on dogs may result in a variety of clinical signs including irritation and scratching (hypersensitivity reactions), skin wounds or ulcers. Successive colonization of these lesions by mites and fungi may also result in pain, chronic wounds, and poor body condition score. Hair loss (alopecia), tick paralysis and anaemia, and weight loss may be recorded in dogs with severe tick infestation (Kryda et al., 2020). In addition, over 60 tick species are known to cause paralysis and death in dogs, humans, and other domestic animals globally, including *Hyalomma truncatum, Ixodes holocyclus, Ixodes cornuatus, etc.* (Guernier et al., 2016, Rajput et al., 2006, Eppleston et al., 2013). Ticks are responsible for the transmission of a wide range of pathogens to dogs and other animals (Table 1.2) (Maggi and Kramer, 2019, Magnarelli, 2009). The impact of ticks on human health must be re-emphasized as numerous canine tick-borne diseases are known to be zoonotic (Springer et al., 2020). The presence of ticks and the likely role of infected dogs as reservoir host constitutes risk of human infection and significant threat to public health (Arroyave et al., 2020).

1.6.2 Distribution of tick species in Nigeria

There is a paucity of data on the distribution of tick species in Nigeria. Limited reports available from recent studies may not be enough to make a conclusive statement concerning the epidemiology of tick species in Nigeria. Nigeria is such a large country and generating data on the epidemiology and distribution of tick species from two to three states may not be enough to definitively say whether

a particular tick or pathogen is present or not. Also, a lot of data available on tick species identification have relied solely on morphological features of ticks (Table 1.3); these methods of identification may be deficient considering similarities among the distinct species of ticks studied. According to previous literature, *Amblyomma* and *Rhipicephalus* species are the most common tick species infesting domestic animals in Nigeria. Most studies on ticks and tick-borne pathogens in Nigeria have been carried out in cattle and not dogs. *Rhipicephalus (Boophilus) microplus* has been identified in Nigeria from cattle, using morphological and molecular techniques in the south east, south west and north east regions of Nigeria (Eyo et al., 2014, Kamani et al., 2017). Previous studies have shown that this species of tick has exhibited an eastward movement in recent years, and movement of people and trade activities between the Republic of Benin and Nigeria may be responsible for this (Kamani et al., 2017). Review of canine tick infestation in this study showed that *R. sanguineus* (s.l.), *R. turanicus*, and *H. leachi* are the common tick species infesting Nigerian dogs (Ogo et al., 2012, Lorusso et al., 2013, Kamani et al., 2013a, Ofukwu et al., 2008, Adang et al., 2015, Opara M.N et al., 2017, Emmanuel et al., 2017, Kamani, 2021b).

The origin of the name *R. sanguineus* can be traced back to the 1806 study where Latreille described *Ixodes sanguineus*, Koch later assigned the genus *Rhipicephalus* changing the name to *R. sanguineus* after seven decades (1884). Russian researchers working under the leadership of Pomerantzev initiated the idea of *R. sanguineus* species group between 1952 to 1997 (Gray et al., 2013). Reports from multiple molecular and phylogenetic analysis studies across the globe revealed many questions regarding the traditional tick identification using morphological features. In the last two decades, two separate lineages of *R. sanguineus* consisting *of R. sanguineus* sensu lato (s.l.) "temperate lineage" and *R. sanguineus* s.l. "tropical lineage" has been described within the *R. sanguineus* species complex. Many authors have doubts about the specific identity of *R. sanguineus* sequences available in the NCBI database (Slapeta et al., 2021).

Evidence from previous studies revealed the prevalence of the nominal *R. sanguineus* sensu stricto (s.s.) (Latreille) Europe, and America, it is now renamed as "temperate lineage" *R. sanguineus* based on morphological and genetic description (Nava et al., 2018). The identification and description of *R. sanguineus* (s.l.) "tropical lineage" was described in a recent study utilizing tick samples originating from Australia. Using morphological and genetic descriptions, findings from this study proposed replacement of the name *R. sanguineus* (s.l.) "tropical lineage" with *Rhipicephalus linnaei* (Slapeta et al., 2021) to ensure simple naming for the tropical lineages. However, several studies have failed to recognize this new proposal, the name *R. sanguineus* (s.l.) "tropical lineage" is still used in most recent publications (Kamani, 2021b). Reports on the genetic diversity, and geographic distribution of *R. sanguineus* complex in America confirms that *R. sanguineus* (s.s.) corresponds to "temperate lineage." Previous South American studies reporting the presence of the tropical lineage in northern parts of Argentina and the temperate lineage in in temperate and cold regions of Argentina, Chile and Uruguay confirms that both lineages can be present within a geographical region with varying climatic condition (Cicuttin et al., 2015, Sánchez-Montes et al., 2021).

Recent studies in Nigeria using hard tick 16S rRNA gene PCR confirmed the abundance of *R. sanguineus* (s.l.) identified to have 98% similarity with the *R. sanguineus* (s.l.) "tropical lineage" (Kamani, 2021b). The abundance of the *R. sanguineus* (s.l.) "tropical lineage" in Nigeria coincides with reports in several tropical parts of America (Sánchez-Montes et al., 2021) including Columbia (Paez-Triana et al., 2021), Brazil (Sanches et al., 2016), and warmer parts of Southern Europe e.g. Portugal/Italy (Sanches et al., 2018, Dantas-Torres et al., 2018). The geographical similarity between certain parts of America (especially the southern part of America with mean average ambient temperature of 32^{o}C), Portugal (Europe) and Sub-Sahara Africa including Nigeria may be responsible for the similarity in the distribution of the *R. sanguineus* (s.l.) "tropical lineage" across these regions.

Table 1.3: Review of regional distribution of common species of hard ticks in Nigeria communities.

Tick species Identified	**Animal host species**				**Tick distribution based on geopolitical zones in Nigeria**						References
	Dog	**Cattle**	**Sheep**	**Goat**	**NC**	**NE**	**NW**	**SE**	**SS**	**SW**	
Rhipicephalus (Boophilus) decoloratus		✓	✓	✓	✓	✓	✓	✓		✓	(Ogo et al., 2012, Ofukwu et al., 2008, Lorusso et al., 2013, Kamani et al., 2017)
R. (Boophilus) microplus		✓	✓	✓	✓	✓	✓	✓		✓	(Lorusso et al., 2013, Eyo et al., 2014, Kamani et al., 2017, Emmanuel et al., 2017)
R. guilhoni		✓			✓						(Lorusso et al., 2013)
R. (Boophilus) geigyi		✓			✓	✓	✓	✓		✓	(Lorusso et al., 2013, Kamani et al., 2017)
R. simus		✓			✓						(Lorusso et al., 2013)
R. evertsi		✓	✓	✓	✓				✓		(Ofukwu et al., 2008, Ibe et al., 2012)
R. apendiculatus			✓	✓	✓						(Ofukwu et al., 2008)
R. turanicus	✓	✓			✓		✓				(Lorusso et al., 2013, Kamani et al., 2013a)
R. sanguineus	✓	✓		✓	✓	✓	✓				(Ogo et al., 2012, Lorusso et al., 2013, Kamani et al., 2013a, Ofukwu et al., 2008, Adang et al., 2015, Opara M.N et al., 2017, Emmanuel et al., 2017)
R. (Boophilus) muhsamae		✓	✓	✓	✓		✓			✓	(Kamani et al., 2017)
Hyalomma truncatum		✓			✓	✓	✓	✓		✓	(Lorusso et al., 2013, Adang et al., 2015, Emmanuel et al., 2017, Kamani et al., 2017)

Tick species Identified	Animal host species				Tick distribution based on geopolitical zones in Nigeria						References
	Dog	Cattle	Sheep	Goat	NC	NE	NW	SE	SS	SW	
Hyalomma rufipes		✓			✓	✓	✓				(Lorusso et al., 2013, Adang et al., 2015, Kamani et al., 2017)
Hyalomma impressum		✓					✓		✓		(Kamani et al., 2017)
Amblyomma variegatum		✓	✓	✓	✓		✓	✓	✓	✓	(Ofukwu et al., 2008, Ogo et al., 2012, Lorusso et al., 2013, Adang et al., 2015, Ibe et al., 2012, Eyo et al., 2014, Emmanuel et al., 2017, Kamani et al., 2017)
A. maculatum		✓						✓			(Eyo et al., 2014)
Haemaphysalis leachi	✓		✓	✓	✓		✓				(Ofukwu et al., 2008, Kamani et al., 2013a)
Ixodes ricinus			✓			✓					(Adang et al., 2015),

1.7 Life cycle of ticks infesting dogs

Knowledge of the life cycle of ticks is necessary to understand the transmission and distribution of vector-borne pathogens in dogs. The description of the life cycle of ticks in this review is limited to hard ticks as they are the major vectors of diseases in dogs. Four stages including egg, larva, nymph, and adult are associated with one, two or three-host tick species life cycles. The frequency of moulting (ecdysis) and development to successive stages such as larva, nymph, and adult, in the life span of ticks may vary, depending on whether one, two or three-host species ticks are being considered. For example, canine one-host ticks are known to undergo moulting entirely on one animal host species (Figure 1.3), at two consecutive intervals during their life span. Following laying of eggs and hatching of the larvae within the environment, the larvae creep and find habitation on flora where they wait for their host; this process of waiting for a host is called questing. For certain species of ticks such as *Haemaphysalis sp.*, the larvae are found attached to the flora, near to the earth surface, within a height of 0 to 29 centimetres (Mejlon and Jaenson, 1997).

Upon attaching to the host, completion of a blood meal and engorgement, they continue to cling to the animal host where moulting results in a nymph. The nymph also continues to cling and feed on the same animal host. Nymphal stages in one-host ticks will later moult and develop in adult ticks that will also feed on the same animal host. *Hyalomma scupense* and members of the genus *Boophilus* subgenus *Rhipicephalus* are good examples of one-host tick species. Again, during the life cycle of two-host ticks, akin to that of one host ticks, only the larvae and the nymph feed on the same animal host. Successive adults following moulting of engorged nymphs may cling to another animal host (Figure 1.4). This life cycle is common with members of the genus *Hyalomma*. In this type of life cycle, larvae develop from eggs that hatch after several weeks, the larvae cling to the host and drop off seeking habitation within vegetation cover or soil, after completion of only one blood meal and then moult. The resulting nymph

finds and attaches to a second host, where they also take a single blood meal. The nymph eventually becomes engorged, dropping off the host with successive moulting and developing into an adult tick. Adult females quest for another host where they take only one blood meal and lay numerous eggs, followed by death (Figure 1.5). Male tick species such as *R. sanguineus* (s.l.) have interrupted feeding activities including motivation to seek new animal hosts in search of females. While death in female tick occurs following egg-laying, starvation and dehydration could result to death in both sexes (Tahir et al., 2020). The most important tick species of livestock, companion animals and humans fall under the three-host tick category. They include *Ixodes, Haemaphysalis, Amblyomma, Dermacentor* and *Rhipicephalus* (except for *Rhipicephalus evertsi,* a two-host tick). Also, a sizable number of the genera *Hyalomma* are three-host ticks, apart from *Hyalomma scupense (*a one host tick) (Jongejan and Uilenberg, 2004, Walker, 2003, Troughton and Levin, 2007).

The total life cycle of hard ticks of veterinary importance has been established under laboratory conditions to occur between a minimum of 162 days (about 5 and a half months) and a maximum of 229 days (about 7 and a half months). This total life cycle duration covers female feeding, gestation, egg incubation, larval stage (feeding and moulting) and nymphal stage (feeding and moulting) (Troughton and Levin, 2007, Chen et al., 2012).

Numerous determinants have been described to influence the life cycle, survival, and distribution of ticks. These factors include climate (rainfall, temperature, and humidity), presence or absence of hosts (animal), vegetation cover or hiding spots and predation, among others. For example, previous studies from Sub-Saharan African regions (East Africa and South Africa) vulnerable to climate-induced changes in tick distribution and tick-borne disease reported potentials for increase in range of tick species expansion following examination of 30 *Rhipicephalus* species (Olwoch et al., 2007). Also, previous reports on the effect of climate change on tick (*I. ricinus*) distribution in Europe shows that increased ambient temperature recorded during summer may favour the survival and

distribution of ticks (Gray et al., 2009, Alkishe et al., 2017). Thus, with the high ambient temperature in Nigeria, wide distribution and abundance of dog tick species is likely.

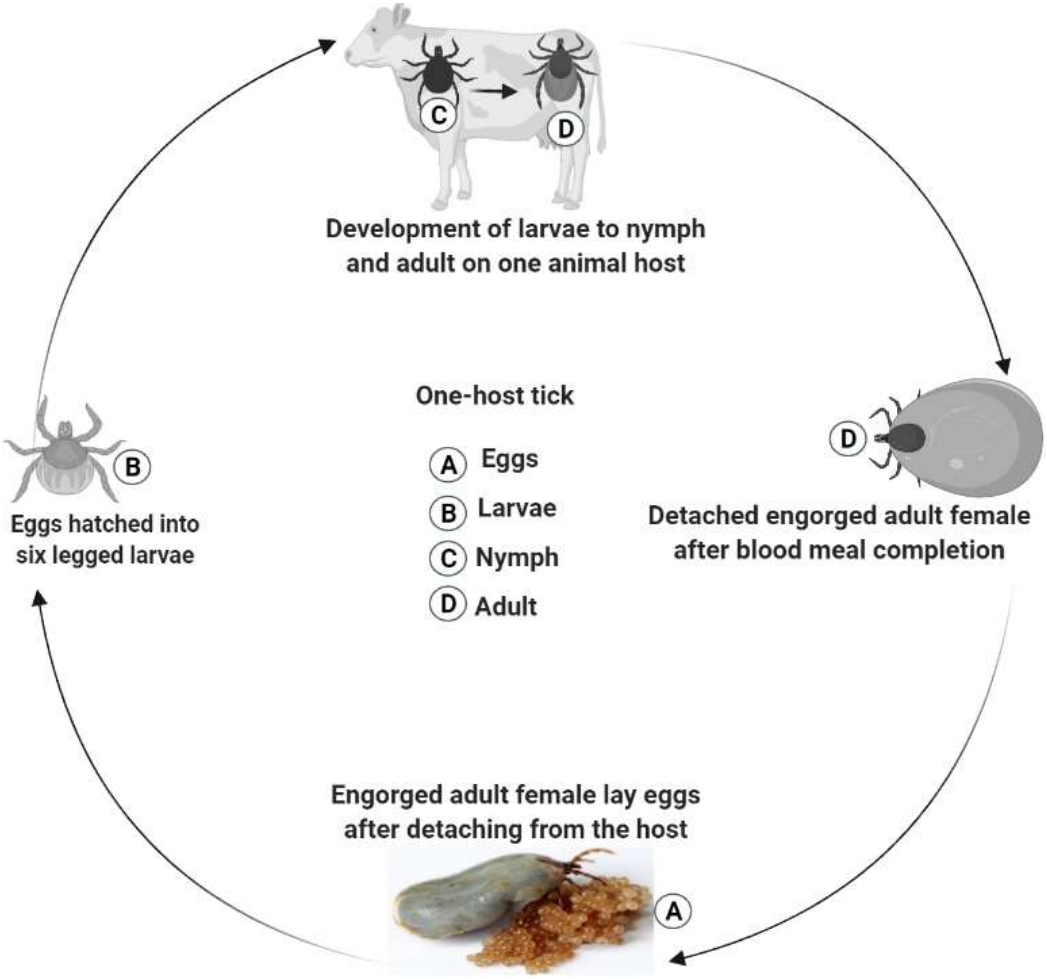


Figure 1.3: Life cycle of one-host hard ticks, showing the emergence of larvae from eggs/gravid females and maturation to adult ticks on one host. Adapted from Centers for Disease Control and Prevention, USA.

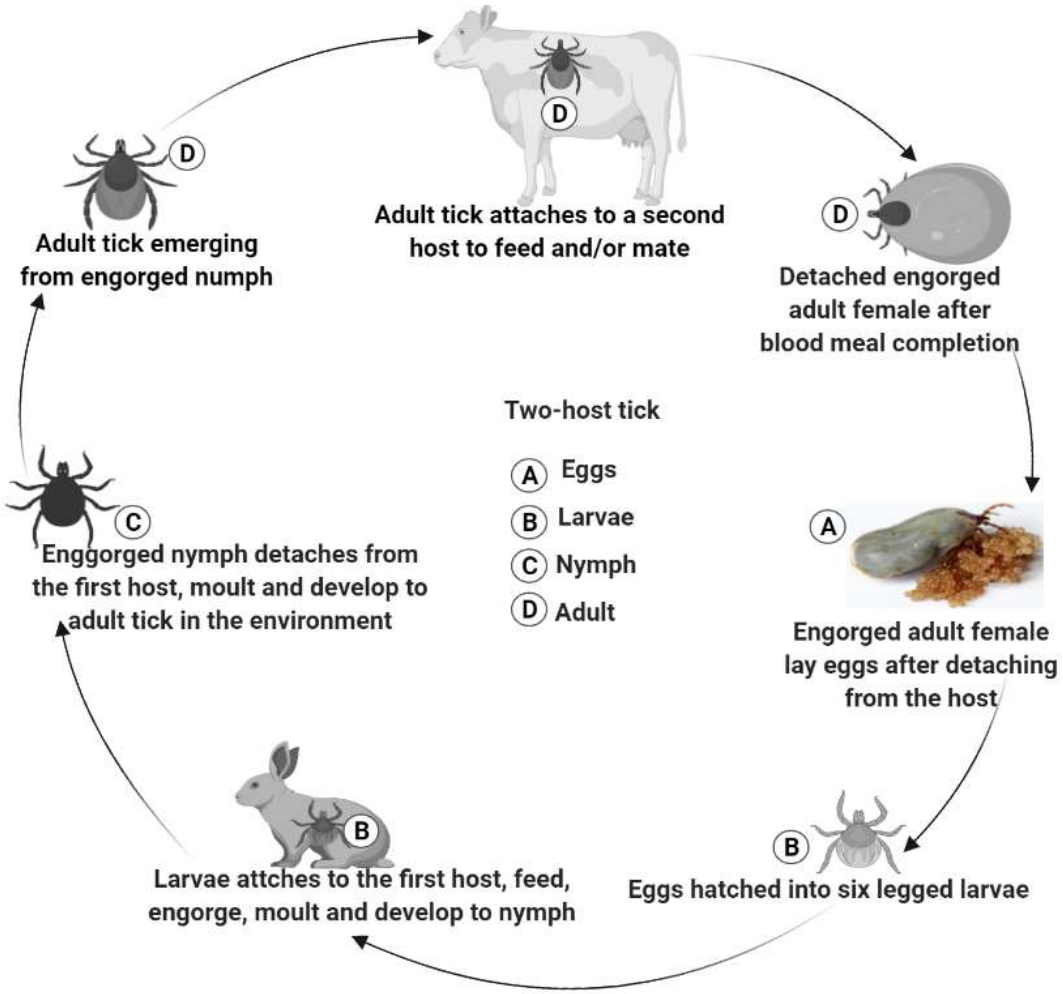


Figure 1.4: Life cycle of two-host hard ticks, showing the developmental stages of hard ticks from eggs to engorged adult ticks on two separate hosts. Adapted from Centers for Disease Control and Prevention, USA.

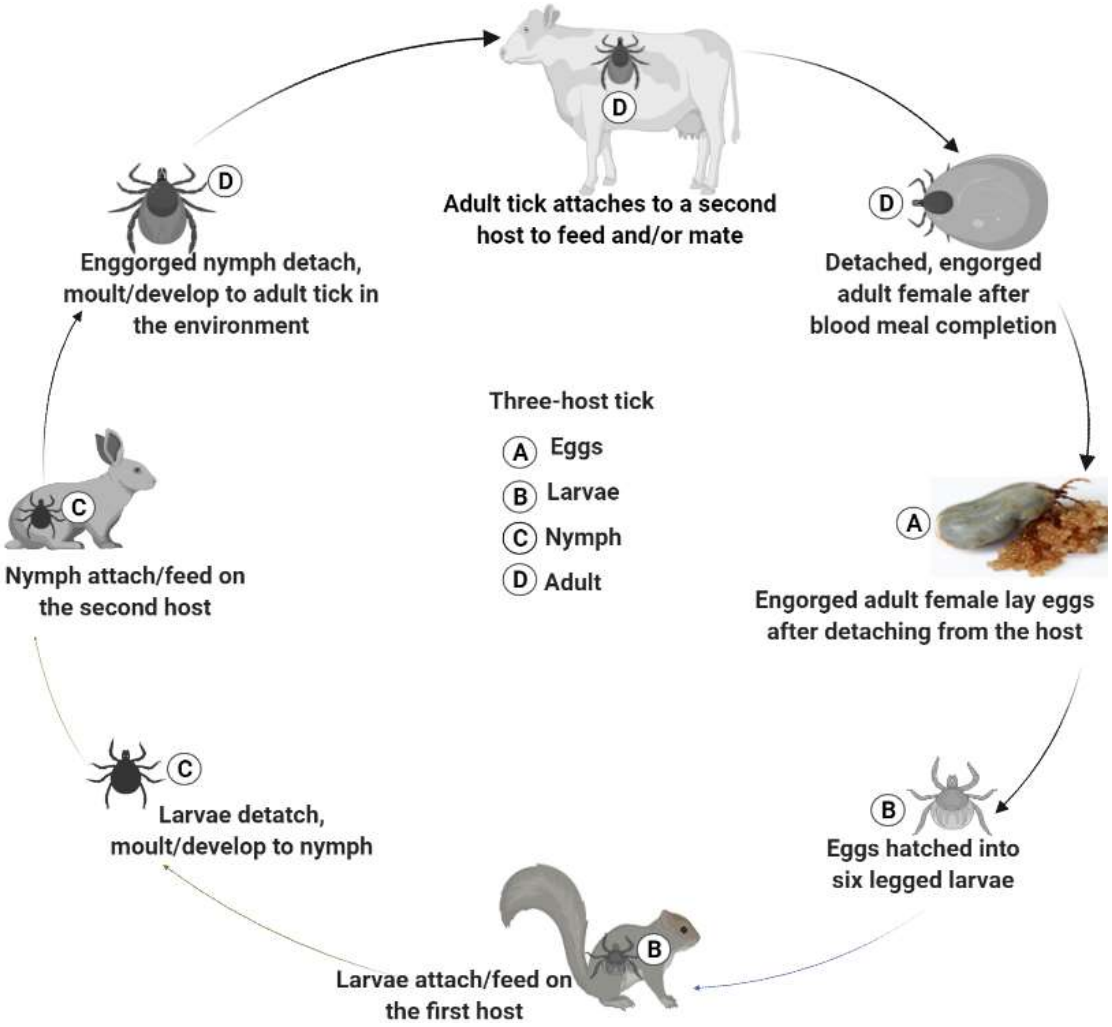


Figure 1.5: Life cycle of three-host hard ticks, showing the developmental stages of hard ticks from eggs to engorged adult ticks on three separate hosts. Adapted from Centers for Disease Control and Prevention, USA.

1.7.1 Canine tick control methods

Tick control refers to the use of insecticides and other integrated management practices such as reduction of host populations, vaccination, grooming, and biological methods, to curtail or eradicate tick populations infesting animal hosts or within the environment (Rozendaal, 1997). In general, insecticides used for tick or insect control have been observed to be the most common and cheapest method of tick control following mechanical removal.

Mechanical control methods involve removal and destruction of ticks from the affected animal, using gloved hands or portable hand tools. It is a frequent practice by farmers and dog owners to attempt to remove ticks from animals using bare hands. Portable appliances developed for safe removal of ticks may be manually operated or electrically driven. The manually operated appliances consist of long arms with free narrow and curved ends, which when pressed together can enclose and remove ticks attached to an animal (Childs III, 1990). Electrically driven devices consist of jaws resembling that of a tweezer, coated with a non-insulating material. When connected to an electricity source, voltage passing through the device will cause the tick to be electrocuted, becoming detached and can be easily removed (Farnsworth, 2016).

Chemical control methods involve the use of acaricides that target the respiratory or nervous system of ticks causing paralysis and death. Common chemicals used include organophosphates, macrocyclic lactones (ivermectin), formamidine (amitraz), carbamates, phenylpyrazole (fipronil), pyrethrin and pyrethroids (cypermethrin, permethrin, cyfluthrin, flumethrin, allethrin, deltamethrin fenvalerate) and isoxazolines (Otranto et al., 2005, Shafer et al., 2005, Zhou et al., 2022). Acaricides are also reported to be lethal to both animals and tick and pet owners are advised to always follow the recommended dosage and instruction (Lorsirigool et al., 2022). They may be prepared in different formulations or products including collars, sprays, dips or shampoo,

dusts, chewable tablets, and topical spot-on treatments for dogs (Zhou et al., 2022). Resistance to insecticides has been demonstrated in the ticks and fleas infesting dogs as well as cattle ticks *Rhipicephalus (Boophilus) microplus*; this has created a challenge for the use of insecticides to control ticks and other ectoparasites in dogs and domestic animals (Morgan et al., 2009, Shaw, 1966, Coles and Dryden, 2014).

Although, there is a drastic reduction in the use of organophosphates and pyrethroids/pyrethrin for canine ectoparasites control in developed countries, these products are commonly used for tick control in developing countries including Nigeria. Organophosphates cause paralysis and death in both mammals and insects by binding to the active serine residue site of acetylcholinesterase (AchE), thereby inhibiting acetylcholine metabolism. The absence of selective toxicity to only insects has resulted in numerous cases of organophosphate poisoning in animals (Poirier et al., 2021) and humans (Eddleston, 2020), they are not currently recommended for tick control. Pyrethroid (cypermethrin, permethrin) and pyrethrin are repellent acaricides causing paralysis and death by delaying the closure of voltage-gated sodium channels in insect nerve cells, this results in continuous nerve firing, lack of coordination, paralysis, and death in ticks. Even though pyrethroids have better selective toxicity for insects than organophosphates, neurotoxicity in other species including dogs may occur if a high effective dose is administered (Wolansky and Harrill, 2008). Irrespective of these side effects, organophosphate and pyrethroids appear to be the most common chemical products for arthropod control in developing countries.

The use of phenylpyrazole acaricides including fipronil for canine tick control has been effective. Fipronil causes neurotoxicity in mammals including dogs via inhibition of gamma-aminobutyric acid (GABA) glutamate-gated chloride. Cases of fipronil poisoning resulting from over dosage and accidental ingestion in healthy animals, application in aged, pregnant, sick, kittens, and puppies occur.

Inadequate selective toxicity of fipronil for ectoparasites and their shorter duration of activity on the host and the environment make this class of compounds less than ideal for tick control.

Another recent development is the evaluation and approval of isoxazolines (e.g., fluralaner) chewable tablets with similar mechanism of action and potency as fipronil. It has been approved in over 70 countries for safe and effective canine flea, tick, and mite control (Comas and Armstrong, 2018). The use of isoxazolines for canine tick control is recommended in line with the approved usage information provided by Zhou et al. (2022). Isoxazolines causes hyperexcitation, paralysis, and death in invertebrate nervous system via a highly potent and specific inhibition of arthropod GABA and significant but less effective inhibition of arthropod glutamate-gated chloride channel. The inhibition of arthropod specific GABA-gated chloride channel by isoxazolines is more efficacious than phenylpyrazole (fipronil). Convenient routes of administration (chewable tablets), adequate client compliance, long duration of activity (months), and sufficient canine and feline tolerance have been reported in clinical studies on companion animal ectoparasite control using isoxazolines (Rohdich et al., 2014). Isoxazolines single and combination products including NexGard®, Bravecto®, Simparica®, Credelio® are effective for the control of mites, fleas, and different tick species including species prevalent in Nigeria. They have been approved for the control of ectoparasite in dogs and cats in the United States (US), Europe (EU), Canada, Australia (AU), and New Zealand. According to Chen et al. (2018), "The dose makes the poison" and a few adverse effects such as muscular tremors, ataxia, seizures, and convulsion have been linked to the use of isoxazolines in dogs in these countries.

A list of registered animal health products and agrochemicals provided by the National Agency for Food and Drugs Administration and Control (NAFDAC) between 2016 — 2018 (NAFDAC, 2018) has shown that pyrethroids (cypermethrin, deltamethrin), pyrethrin, and phenylpyrazole (fipronil) are the

currently available products for canine tick control in Nigeria, as there is little or no published research data to provide this information. It is likely Nigerian veterinarians may have used isoxazolines (fluralaner) for canine tick control in Nigeria, as the list for the registered animal health products provided by NAFDAC in Nigeria appear not to have been updated in the last 3 — 4 years. The use of fipronil, and isoxazolines (e.g., fluralaner) is likely to be the first recommended chemical treatment for safe and effective canine tick control in Nigeria.

In the absence of information on official approval and availability of isooxazoline in Nigeria, the use of pyrethroids, permethrin, and phenylpyrazole (fipronil) are likely to be suggested as alternative acaricides for canine tick control. The use of organophosphates is not recommended in this study. For effective tick control, strategic tick control including monthly treatment of dogs infested with ticks using fipronil is recommended. This can be combined with treatment of dog kennels or infested environments using pyrethroids/pyrethrin acaricides for at least four consecutive times at 3 — 4-week intervals.

Resistance to acaricides poses a great challenge to tick control in both companion and farm animals, there are also limited number of natural predators for tick control. These two challenges explain why ticks remain common and important vectors for domestic animal infectious disease. The development of vaccines to address tick vector and infectious pathogens transmitted by ticks is an important prospect (de la Fuente and Merino, 2013). Vaccines may be easy, precise, and safe to deliver to animals, at little cost and with no environmental contamination (Otranto and Wall, 2008). Although, the BM86 (TickGARD®) vaccine is known to cross protect against other species of *Rhipicephalus* in cattle, no vaccine is currently available for canine tick control (de la Fuente and Merino, 2013, Otranto and Wall, 2008).

1.8 Canine vector-borne diseases (CVBDs)

TBDs have been demonstrated to have severe effects on the well-being of companion animals, especially dogs in different parts of the world. This has been linked to the global distribution of ticks, the emerging nature of tick pathogens and their potential to cause infections in both animals and humans. The most prevalent tick-borne diseases in dogs include ehrlichiosis, anaplasmosis, babesiosis, borreliosis, hepatozoonosis and borreliosis (Elsheikha, 2016, Shaw et al., 2001, Little et al., 2021).

1.8.1 Canine piroplasmosis

Canine piroplasmosis is an emerging protozoan infection of dogs caused by species of intracellular protozoan parasites with similar characteristics (*Babesia* and *Theileria*) transmitted by hard tick species (Nuttali, 1904, Solano-Gallego et al., 2016). It is clinically characterized by fever, haemolytic anaemia, and kidney failure. The highest number of cases are recorded in tropical and subtropical regions, though it is also reported, albeit at a lower prevalence, in temperate climates (Matijatko et al., 2012, Bourdoiseau, 2006, Matjila et al., 2008). Apart from dogs, *Babesia and Theileria* species parasitize a wide range of wild and domestic animals globally including humans (Hornok et al., 2018, Stockham et al., 2000, Zanet et al., 2014).

The first reports of *Babesia* as the main cause of febrile illness, red urine (haemoglobinuria) and mortality in cattle in North America were as early as 1810. However, pathogens known to cause this disease in cattle were later demonstrated to be transmitted by ticks at the beginning of the 20th century (Florin-Christensen et al., 2014). *Theileria* was first discovered in South Africa in the year 1904 and was named *Piroplasma parvum* by Arnold Theiler, based on the pyriform shape of the bacteria. Further classification placed *Piroplasma parvum* into the genus *Theileria* by Franca and Borges in 1907 (DuToit and Jackson, 1936).

1.8.1.1 Canine babesiosis

Canine babesiosis is caused by *Babesia* species belonging to the order *Piroplasmida*, classified under the phylum *Apicomplexa*. Based on morphological features, *Babesia* species infecting dogs are placed into two categories including large (3–5µm) and small (1–3µm) forms of *Babesia* (Máthé et al., 2006, Adaszek et al., 2016). The first naming of large *Babesia canis* species was documented in Italy in 1932. Three genetically related large *Babesiae* species including *Babesia canis, Babesia rossi, and Babesia vogeli* (Uilenberg et al., 1989) are known. Since then, many other species have been demonstrated to be infectious to dogs globally (Table 1.4) under field and laboratory conditions, such species include *Babesia gibsoni, B. vulpes* n. sp *(*erstwhile *B. microti-like* and *T. annae), Babesia conradae* (Schoeman, 2009, Hornok et al., 2018, Chan et al., 2016, Zahler et al., 2000), and *Babesia negevi* n. sp. (Baneth et al., 2020). Previous studies in the USA have also demonstrated the presence of novel *Babesia* species in dogs with signs of clinical canine babesiosis, sharing more identity with nucleotide sequence of *B. bigemina* (93.9%) and *B. caballi*, (93.5%) than *Babesia* species known to infect dogs (91.2–91.6% identity) (Birkenheuer et al., 2004).

Tick vectors for canine babesiosis transmission include species belonging to the genera *Rhipicephalus*, *Haemaphysalis* and *Dermacentor* (Table 1.2) (Jongejan et al., 2018, Jain Jose et al., 2018, Barash et al., 2019, Cardoso et al., 2016, Panti-May and Rodriguez-Vivas, 2020, Li et al., 2020, Manoj et al., 2020, Di Cataldo et al., 2020, Ngoka et al., 2021, Kamani, 2021a, de Marco et al., 2017, Adamu et al., 2014). Other tick species classified under the genera *Ixodes*, and *Ornithodoros* (soft tick) have also been reported to transmit canine babesiosis (Table 1.2) including *B. vulpes* n. sp (Zahler et al., 2000), and *Babesia negevi* n. sp. (Baneth et al., 2020). *Rhipicephalus* species have global distribution, they are more abundant in the tropics, while *Ixodes* and *Dermacentor* species are more common in temperate parts of the world (Nijhof et al., 2005, Jongejan et al., 2015, Jongejan et al., 2011, Taenzler et al., 2015, Bourdoiseau, 2006, Nijhof

et al., 2007, de Marco et al., 2017, Abd Rani et al., 2011). *B. gibsoni* can also be transmitted via blood transfusion (Stegeman et al., 2003), while both *B. gibsoni* and *B. vulpes* n. sp have been reported to be transmitted via bite wounds, saliva, or ingestion of blood in fighting dogs (Radyuk and Karan, 2020, Tuska-Szalay et al., 2021). Vertical transmission of canine babesiosis from bitches to puppies have been reported in a recently whelped asymptomatic/symptomatic bitches and puppies (Mierzejewska et al., 2014, Adaszek et al., 2016) in Poland. The report of death of four puppies whelped by an asymptomatic bitch, following infection with *Babesia gibsoni,* 2—5 weeks postpartum suggests that vertical transmission of canine babesiosis occurs via the uterine route and not the transmammary route (Fukumoto et al., 2005).

Previous reports from Sub-Sahara Africa have shown that *Rhipicephalus sanguineus (s.l.)* is a major vector in the transmission of *B. vogeli* (Uilenberg et al., 1989, Kamani et al., 2019a). In Nigeria, there is no detailed information on the tick species responsible for transmission of *Babesia* species in dogs, apart from the Kamani (2021a) study which suggested *H. leachi* as the principal vector for *B. rossi* in Nigeria. However, studies using molecular techniques have established the presence of *B. vogeli* and *B. rossi* in Nigerian dogs (Sasaki et al., 2007, Kamani et al., 2013a, Adamu et al., 2014). *B. canis*, *B. gibsoni*, and *B. vulpes* n. sp have not been reported in in Nigeria (Obeta et al., 2020, Kamani et al., 2019a). This is likely due to the absence of *D. reticularis* reported to transmit *B. canis* and *B. vulpes* n. sp in Europe, America, and Asia (Mierzejewska et al., 2018, Birkenheuer et al., 2020, Barash et al., 2019, Checa et al., 2018, Sang et al., 2021).

Dogs of all ages and breeds may present with canine babesiosis, while severe clinical canine babesiosis is often reported in young dogs, the subclinical form of the disease is common in adult dogs. Age, infection with other pathogens, present state of immunity and species of *Babesia* infecting dogs may determine the course or outcome of disease in dogs. Depending on the species of *Babesia* affecting dogs, the period between infection and the manifestation of clinical

disease (incubation period) may differ. Duration of infection with *B. canis*, and *B. gibsoni* may be between 2 to 3 and 2 to 4 weeks, respectively (Schoeman, 2009).

Table 1.4: Morphology, severity of clinical presentation of *Babesia* and *Theileria* species infecting dogs adapted from Ayoob et al. (2010).

Babesia/Theileria species	Size and Morphology	Severity	Reference
B. vogeli	Large, single, or paired pyriform	Mild	(Birkenheuer et al., 2005, Ayoob et al., 2010, Jain et al., 2018, Sasaki et al., 2007)
B. gibsoni	Small, oval, single or pair	Moderate	(Birkenheuer et al., 2005, Ayoob et al., 2010, Davitkov et al., 2015, Jain et al., 2018)
B. canis	Large, single, or paired pyriform	Moderate	(Cacciò et al., 2002, Davitkov et al., 2015, Uilenberg et al., 1989, Ćoralić et al., 2018)
B. rossi	Large, single, or paired	Severe	(Uilenberg et al., 1989, Sasaki et al., 2007, Kamani, 2021a)
B. conradae	Small, single maltese, tetrad	Moderate to severe	(Ayoob et al., 2010, Kjemtrup et al., 2006)
T. equi	Small	Not well known	(Beck et al., 2009, Inacio et al., 2019, Chomel, 2011, Rosa et al., 2014)
T. annulata	Small		(Chomel, 2011)
B. vulpes n. sp *(*erstwhile *T. anne or B. microti–like* spp.*)*	1–2 µm in diameter, single, ring-shaped resembling *B. gibsoni*, dark dot (nucleus) with a variable amount of visible cytoplasm	Same to other small Babesia spp.	(Falkenö et al., 2013, Dixit et al., 2010, Barash et al., 2019)
B. negevi n. sp.	Distinct small form of piroplasm with life cycle stages larger than that of other small Babesia spp. (*B. gibsoni, B. conradae, B. vulpes* n. sp), but smaller than the large canine *Babesia* spp. (*B. canis*, *B. vogeli*, *B. rossi*)	Associated with severe clinical disease	(Baneth et al., 2020)

Variable clinical signs are reported in canine babesiosis irrespective of the size and morphology (large or small) of *Babesia* species infecting dogs. Peracute, acute, and chronic infections which may be subclinical or result in multiple organs failure or death may be associated with different *Babesia* species. General clinical signs in acute canine babesiosis include fever, tachycardia, haemolytic anaemia (characterized by pale mucous membranes), lethargy, loss of appetite or vomiting, jaundice (characterized by haemoglobinuria, and icterus) and hypotension (Davitkov et al., 2015, Ćoralić et al., 2018). Seropositive asymptomatic dogs with chronic infection are often reported, they can also serve as carriers or reservoirs of parasites as reported in *B. canis* infection (Petra et al., 2018). Based on clinical management, canine babesiosis may be uncomplicated due to parasitic infection and lysis of erythrocytes and complicated because of multi-organ damage. Both intravascular and extravascular haemolytic anemia are reported in canine babesiosis.

Anaemia is associated with *Babesia* spp. infection and lysis of erythrocytes by trophozoite and merozoite developmental stages of the parasite, causing elimination of infected and damaged red blood cells. Autoimmune haemolytic anaemia characterized by destruction of the host erythrocytes as a sequalae to systemic production of anti-erythrocyte antibodies is reported in dogs with complicated babesiosis. A spectrum of clinical signs accompanying erythrocyte damage, which is not often associated with the level of parasitaemia and anaemia may be observed (Petra et al., 2018). High red blood cell counts attributed to haemocentration occurring secondary to vasculitis and outward movement of intravascular fluid into the extravascular tissues have been reported to increase the likelihood of multiple organ failure (e.g., renal failure) and death (Welzl et al., 2001). Haemorrhage is an uncommon haematological finding despite the presence of severe thrombocytopenia (due to immune-mediated thrombocyte destruction, splenic sequestration, or disseminated intravascular coagulation) reported in most cases (Welzl et al., 2001, Dubova et al., 2020).

1.8.1.2 Canine theileriosis

Canine theileriosis is an emerging febrile tick-borne disease of dogs caused by *Theileria* species. Both canine theileriosis and babesiosis have similar clinical presentations and could be considered as differential diagnosis, depending on which disease is diagnosed (Rosa et al., 2014). Cases of canine theileriosis are characterized by lethargy, anaemia (pale mucous membrane), splenomegaly and hepatomegaly, loss of appetite or vomiting, haemoglobinuria, and jaundice (Rosa et al., 2014). Although thrombocytopenia is common in canine babesiosis the likelihood of haemorrhage is rare, bleeding tendencies have been reported in dogs (5/6) with coinfection of *T. equi* and an unknown *Theileria* species (Rosa et al., 2014). Dogs and a wide range of wild and domestic animal hosts are infected by *Theileria* species. Ticks of the genera *Amblyomma, Haemaphysalis, Hyalomma* and *Rhipicephalus* are known to transmit canine theileriosis (Stockham et al., 2000). Most cases of *Theileria* species infection are diagnosed in dogs with concurrent haemoparasitic infections, and a clear association between canine theileriosis and clinical presentation requires further consideration.

1.8.1.3 Diagnosis of canine piroplasmosis

Suspicion of canine piroplasmosis is based on history of tick infestation, travel to tick-infested locations, receipt of blood transfusion from a suspected dog or abnormal findings following clinical and haematological examination. The most common diagnosis is by visualization of the piroplasms in infected erythrocytes via microscopic examination of thin blood smears stained with Giemsa, Wright, or Diff-Quick stain (modified Wright-Giemsa stain). Use of serological and PCR-based tests are also recommended (Irwin and Hutchinson, 1991, Petra et al., 2018).

Although, non-specific clinicopathological changes may be seen in the uncomplicated phase of canine babesiosis, a critical review of the haematological and clinicopathological findings is essential for the establishment of tentative

diagnosis and treatment of affected dogs. Thrombocytopenia is common in almost all cases of canine babesiosis (Furlanello et al., 2005, Petra et al., 2018, Defauw et al., 2020), making canine ehrlichiosis a differential diagnosis. Normocytic hypochromic anaemia is recorded during the early phase of canine babesiosis and may advance to macrocytic, hypochromic, and regenerative anaemia depending on the severity of the disease or in the absence of medical treatment (Petra et al., 2018). Varying changes in leucocyte counts including eosinophilia, neutropaenia, leucopaenia, and/or leukocytosis are also reported from dogs diagnosed with canine babesiosis (Furlanello et al., 2005, Nalubamba et al., 2015, Petra et al., 2018). A normal serum biochemistry is observed in most cases however, low serum protein, hypokalaemia due to low level of potassium intake and hypoglycaemia due to anorexia have been reported (Petra et al., 2018). Serum biochemistry from dogs with severe and complicated babesiosis, organ damage, and intravascular haemolysis may indicate azotaemia (changes in BUN; blood urea nitrogen, and Cr; serum creatinine), and metabolic acidosis (due to serum electrolyte imbalance) (Petra et al., 2018, Defauw et al., 2018, Defauw et al., 2020).

Microscopic examination of thin blood smears is often regarded as the most affordable and available test for the morphological identification of piroplasms (Figure 1.6). However, animals with long standing or subclinical infection may be misdiagnosed due to low level of parasitaemia, making PCR a preferential test (Irwin, 2010). Although light microscopy is commonly used for the basic identification of piroplasm species (large and small form), differentiation of *Babesia* species using this method is often not feasible due to shared morphological similarity across different forms of *Babesia* species (Dos Santos et al., 2021). For example, *B. negevi* n. sp. recently described have similar morphology with other small forms of *Babesia* including *B. conradae*, *B. gibsoni* and *B. vulpes* (Baneth et al., 2020). Both thick and thin blood smears are routinely used for laboratory identification of piroplasms. Thick blood smear consists of a concentrated layer of blood elements comprising haemoparasites

and dehaemoglobinized erythrocytes. The presence of a concentrated layer of blood elements improves efficiency and sensitivity for piroplasm detection over thin blood smear where less blood elements may be present. However, thick blood smears are inadequate for piroplasm species differentiation and the use of thin blood smears to confirm piroplasm species identification following thick blood smear examination is recommended. Examination of the edges of blood smears is also established to increase the chances of obtaining positive results as infected erythrocytes may move towards the edges of the slide during thin smear preparation (Dos Santos et al., 2021). According to Dos Santos et al. (2021), microscopy examination of piroplasmids (combination of thick and thin smear) is reliable for diagnosis and treatment of canine piroplasmosis, and it could be useful in Nigeria where PCR-based test is not easily accessible. In addition, the combination of microscopic examination and PCR-based diagnostic methods is recommended for efficient diagnosis and management of canine babesisosis (Huber et al., 2017). Several factors are established to increase the chances of piroplasm identification in blood sample during laboratory microscopy including collection of blood samples from body parts consisting of a network of small blood vessels supplying the muscle (capillary beds) such as the ear tips, and toe capillaries (Dos Santos et al., 2021). Microscopic examination of the segment of erythrocytes located immediately after the sealant and before the buffy coat parts of the haematocrit tube following centrifugation is shown to increase morphological identification of piroplasms (Otranto et al., 2010b).

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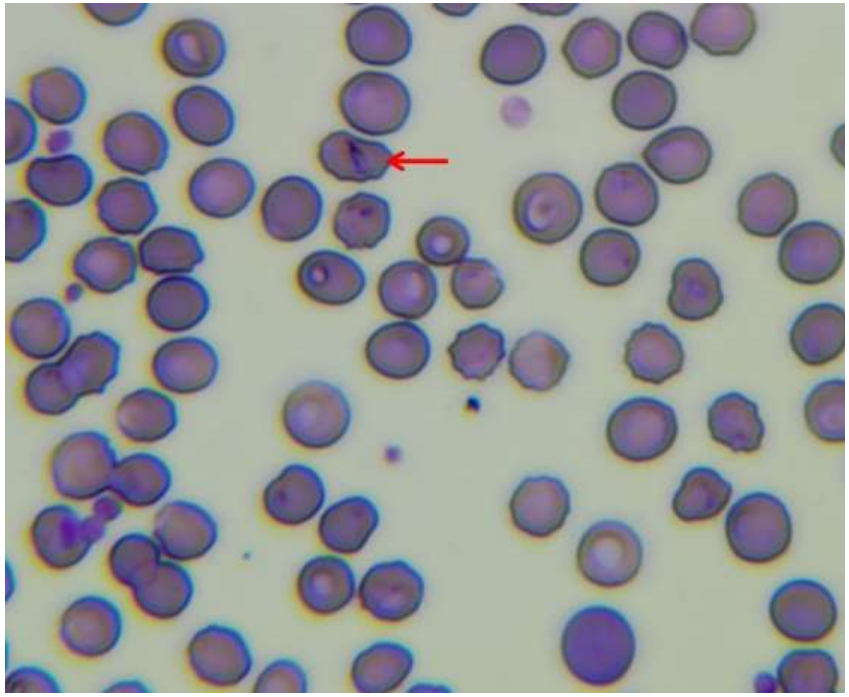


Figure 1.6: An example of stained thin blood smear (magnification: X40) prepared using blood sample collected from Nigerian indigenous dog during sample collection in this study, showing *Babesia* species (red arrow).

Serological diagnostic tests such as immunofluorescence antibody test, enzyme-linked immunosorbent assay (ELISA) and complement fixation tests (CFT) have been used for the detection of *Babesia* and *Theileria* antibody from infected animal blood samples. IFAT involves the reaction between antigens of the parasite and the anti-canine serum with results viewed under a fluorescent microscope. Positive results are obtained following a significant amount of fluorescence recorded. The test is time consuming requiring production of antigen, interpretation of fluorescence is subjective and cross reaction between *Theileria*, *Babesia* and other species belonging to the phyla *Apicomplexa* occurs (Böse et al., 1995).

CFT was developed based on the principle of antigen-IgM antibody reaction. CFT faces similar constraints as seen in IFAT including low detection of piroplasm antibodies in patients with long-standing infections and it is no longer in use (Böse et al., 1995, Mahoney, 1962). IFAT is described as the most specific test to establish canine exposure to *Babesia* species, and several samples can be processed within a given period (Bicalho et al., 2004). However, interpretation of IFAT readings is subjective depending on the judgement of the person carrying out the test (Böse et al., 1995). Therefore, assays for *Babesia* species antibody detection including slide ELISA, sandwich ELISA, dot-ELISA, and competitive ELISA (cELISA) have been established to be less specific but more sensitive (Furuta et al., 2009, Eichenberger et al., 2017). Commercially available cELISA has been demonstrated to produce reliable results as against the IFAT and CFT in the diagnosis of both clinically and sub-clinically infected animals. As a result, cELISA kits for detection of piroplasms are replacing IFAT and CFT. Dot-ELISAs are produced commercially and have been used in the diagnosis of *Babesia* and *Theileria* in dogs. Antibody tests demonstrate the lifetime exposure of an animal to piroplasms, but do not necessarily demonstrate the present infection status of the animal tested as only antibodies are detected (Salim et al., 2008).

Polymerase chain reaction (PCR) remains the most reliable molecular technique for detection of piroplasm pathogens from dogs. PCR is specific and sensitive, positive results may be recorded in dogs with both clinical and sub-clinical infections. Several studies demonstrating the presence of *Babesia* species in canine blood at various stages of infection (peracute, acute, chronic) have proven that a PCR-based diagnostic approach is more reliable than blood smear (Huber et al., 2017, Happi et al., 2018, Dos Santos et al., 2021, Baneth et al., 2020, Krause et al., 1996). Most PCR techniques for detection of piroplasm are based on PCR amplification of ribosomal RNA genes 18S, 5.8S, 28S and other loci such as the P18 gene (Irwin, 2009, Gou et al., 2012). However, the 18S rRNA gene is most used for molecular detection of piroplasms (de la Fuente et al., 2004, de Marco et al., 2017, Falkenö et al., 2013, Matjila et al., 2008). Additionally, fast, and inexpensive loop-mediated isothermal amplification (LAMP) methods for detection of *Babesia* species (including *B. gibsoni*) designed to amplify *Babesia* species 18s RNA gene with sufficient specificity, sensitivity and efficiency have been developed (Mandal et al., 2015). The use of LAMP is suggested for the diagnosis of canine babesiosis in countries with less access to PCR-based tests or facilities.

1.8.1.4 Piroplasmosis as an emerging zoonotic disease

Babesiosis is considered an emerging zoonosis, and many human cases have been recorded in different parts of the world including Asia, Australia, Europe, North and South America (Yang et al., 2021). Many cases of human babesiosis have been recorded in the USA (Yang et al., 2021, Ingram and Crook, 2020). *B. microti,* a primary pathogen of rodents (Mardosaite-Busaitiene et al., 2021) with a highly similar morphology to *B. vulpes* n. sp infecting dogs (Zahler et al., 2000), has been implicated in most human cases of babesiosis in the USA. Case reports indicate that aged and asplenic people or those with impaired immune systems are highly susceptible (Kjemtrup and Conrad, 2000, Gorenflot et al., 1998, White et al., 1998). Babesiosis is presently recognized as a reportable

emerging disease in different parts of the world (Vannier and Krause, 2012). In Nigeria, antibodies against *Babesia* species were detected in 54% of serum samples collected from 173 apparently healthy men in Nigeria four decades ago using IFAT and sera samples and antigens originating from *B. bigemina*, *B. bovis*, and rodent *Babesia* species. *B. bovis* (63/173) recorded the highest seroprevalence, followed by rodent *Babesia* species and *B. bigemina* (Leeflang et al., 1976). The most recent case of human babesiosis in Nigeria diagnosed via light microscopy was reported in a nine-year old girl with underlying retroviral disease (Jimoh et al., 2020). It is probable that screening of people against *Babesia* infections has been conducted in Nigeria, but not reported. Apart from these two reports, no information is presently available to demonstrate the presence of *Babesia* antibodies in people at risk in Nigeria.

1.8.2 Canine Monocytic Ehrlichiosis (CME)

CME is a severe infectious disease of dogs and cats caused by a pleomorphic intracellular obligatory Gram-negative rickettsia infecting monocytes and macrophages called *Ehrlichia canis (E. canis)*. It was first demonstrated to infect dogs and humans in 1935; since then, it has been recorded in different parts of the world, especially in the tropics (Kamani et al., 2019a, Taques et al., 2020, Bezerra-Santos et al., 2021, Ojeda-Chi et al., 2019, Perez-Macchi et al., 2019, Mylonakis et al., 2019, Qurollo et al., 2014). *E. canis* multiplies in mononuclear macrophages having an increased affinity for cells responsible for blood formation including splenic, hepatic and lymph node cells (Mittal et al., 2017). CME is transmitted principally by *Rhipicephalus sanguineus* (the brown dog tick) with a global distribution, *Dermacentor variabilis* has also been demonstrated to transmit CME (Mittal et al., 2017, Montenegro et al., 2017, Harrus et al., 1999). Certain risk factors are known to predispose dogs to *E. canis* infection; studies have demonstrated that clinical disease with *E. canis* is more prevalent in dogs younger than 6 months than dogs older than 6 months, and infection with these bacteria is more common in summer than during the winter period of the year

(Milanjeet et al., 2014). Other studies have shown that older dogs may have increased contact with infected ticks or dogs and demonstrate higher seroprevalence than young dogs. Dogs living in urban centres and those known to have frequent contact with others are at an increased risk of contracting *Ehrlichia*. The vector *R. sanguineus* has been demonstrated to be more predominant in urban than rural areas (Guedes et al., 2015, Trapp et al., 2006, Melo et al., 2011). All dog breeds may be susceptible however, German shepherd dogs (GSD) and Huskies are highly susceptible to CME and more prone to severe clinical disease when compared to other dog breeds (Sainz et al., 2015). While the humoral immune system is not protective against *E. canis* infection in dogs, GSD is known to have weaker cellular immune response making them highly susceptibility to CME. The weakened cellular immune response is also described as exacerbating the pathogenesis of CME in GSD. In these dog breeds poor prognosis and death due to haemorrhage and concurrent infection are reported (Ansari-Mood et al., 2015, Lakkawar et al., 2003, Harrus and Waner, 2011, Sainz et al., 2015).

The prevalence of *Ehrlichia* species in Nigeria has been demonstrated using microscopic and serological diagnosis (Adamu et al., 2012). Studies using PCR-based techniques to identify canine *Ehrlichia species* are also available in Nigeria. The first molecular identification of *E. canis* was demonstrated in two different studies in 2013 from North central and South-south Nigeria (River state). Findings from these studies have shown a prevalence of 11% and 12.7% from a total of 100 and 180 sample sizes respectively, via conventional PCR (Kamani et al., 2013b, Kamani et al., 2013a). A further epidemiological survey of *E. canis* in North central Nigeria also demonstrated a 7% prevalence from a sample size of 100 dogs via conventional PCR (Adamu et al., 2014). A more recent study covering South West Nigeria indicated that out of 205 dogs examined, 22.9% tested positive for *E. canis* via nested PCR (Daramola et al., 2018). However, further studies covering a larger sample size and different regions of the country

are needed to give further information on the prevalence of *Ehrlichia* species in Nigeria.

CME incubation period is between 1—3 weeks and may present with rapid onset and a short course (acute form) marked by fever, anorexia, lethargy, lymphadenitis, and splenomegaly. Bleeding tendencies including epistaxis, pinpoint (petechial) haemorrhages or discolouration of the skin (ecchymoses) due to escape of blood from vessels into tissues may also be recorded (Harrus and Waner, 2011). Thrombocytopenia is a frequent clinical finding in over 90% of dogs diagnosed with acute CME (Harrus, 2015). Ocular clinical findings in acute CME include choroiditis, retinitis, and anterior uveitis. Infiltration of perivascular cells and haemorrhages into the retina. In addition, increased viscosity of ocular blood flow and detachment of sub-retinal tissues from the orbit may give rise to the occurrence of blindness in the affected dog (Harrus and Waner, 2011). It is thought that most dogs affected may advance into a chronic form of CME. The chronic form of CME may persist for a long time, although clinical manifestations may be more severe than the acute form. Severe and common clinical findings include reduced body weight, anaemia characterized by weakness and pale mucous membranes and bleeding (Harrus and Waner, 2011). Pancytopenia is also a common clinical finding in dogs with a severe chronic form of CME because of bone marrow depletion (Harrus, 2015).

Diagnosis of CME may involve several diagnostic tests including thin blood smear examination, serology, and molecular detection. Laboratory examination of thin blood smears under light microscopy is usually carried out to establish the presence of *E. canis* membrane-bound vacuoles heavily filled with *E. canis* bacteria within the cytoplasm of monocytes (morulae). Although this test is unreliable and lacks sensitivity, microscopic examination of buffy coat smears may significantly increase the chances of detecting *E. canis* in dogs with acute ehrlichiosis. Morulae may be more often seen in lymphocytes than in monocytes, as they are more present in circulation than the latter. Other blood elements such as platelets, lymphocytic azurophilic granules and nuclear materials

engulfed by phagocytes must be morphologically differentiated from *E. canis* morulae during thin blood smear examination (Harrus and Waner, 2011, Mylonakis et al., 2003, Happi et al., 2018). Also, knowledge of the epidemiology of other *Ehrlichia* species or other tick pathogens including their vectors within any given location must be established to differentiate *E. canis* from other species or mixed infection with other tick-borne pathogens. Complete blood count (CBC) is a core test in diagnosis of CME, as thrombocyte count is relevant to identify and manage dogs with thrombocytopenia (Harrus and Waner, 2011).

Although several valuable serological diagnostic methods have been established for *E. canis*, the indirect immunofluorescence antibody test (IFA) remains the most accurate available diagnostic tool for *E. canis* IgG antibody detection. In addition, three recommended commercial enzyme-linked immunosorbent assays (ELISA) including an ELISA kit based on the recombinant major antigenic protein 2 (rMAP2) and an Immunocomb dot-ELISA test (Biogal, Galed Laboratories) are available. Lateral flow test devices utilizing synthetic peptides derived from the major immunodominant *E. canis* proteins *P30* and *P30-1* (e.g., IDEXX lab, USA) are also presently in use for *E. canis* antibody detection. Though, these tests are said to be highly specific for *E. canis* (Harrus et al., 2002), they may be cross-reactions between *E. canis* antibodies and antibodies against other diverse species of *Ehrlichia*, and this remains a limitation for ELISA kits. In addition, *E. canis* antibody can be detected from infected dogs 7 — 28d days post infection. While previously infected dogs may be positive for *E. canis* antibody 6 — 9 months post infection and this may be considered as the limitations of *E. canis* serological tests (Neer et al., 2002, Waner, 2022). Western immunoblot has been established to be a more specific serological tool to detect *E. canis* in dogs. This technique has demonstrated the difference between various species of *Ehrlichia* including *E. canis, E. ewingii, E. chaffeensis* and *E. ruminatium* (Harrus and Waner, 2011, O'Connor et al., 2006). However, apart from the cost of reagents, western blotting is time-consuming and usually thought of as a research tool rather than a diagnostic tool. Culture and isolation may be labour

intensive requiring a long time but has been used by researchers to enhance diagnosis of *E. canis. E. canis* has been established to grow effectively in three continuous cell lines, including the canine macrophage cell line (DN82) (Dawson et al., 1991, Keysary et al., 1996, Romero et al., 2011), the mouse macrophage cell line (J774.A1) (Keysary et al., 2001), and the human microvascular endothelial immortal cell line (CDC/EU.HMEC-1) (Dawson et al., 1993, Dawson, 1995).

Molecular techniques including PCR and sequencing remain the most sensitive methods presently available to characterize distinct species of *E. canis*. *E. canis* has many genes of importance. However, the major outer membrane protein gene (*p30*) is known to be more abundant than any other gene present. This explains why most *E. canis* detection using PCR has targeted PCR amplification of *p30* genes (Stich et al., 2002, Ionita et al., 2013, Mylonakis and Theodorou, 2017), the amplification of *16S rRNA* genes has also yielded sensitive results (Romero et al., 2011, Keysary et al., 1996, Unver et al., 2001, Mohamed El-Dakhly et al., 2021, Mylonakis and Theodorou, 2017). However, major outer membrane protein p28 (Gusa et al., 2001, Nakaghi et al., 2010), disulphide bond (dsb) formation protein gene (Doyle et al., 2005), and VirB9 genes have also been amplified successfully. More sensitive results have been produced from *E. canis* PCR using samples from spleen than those performed on blood and bone marrow samples (Harrus et al., 2004). Serum samples may be used for PCR as a last resort if preferred sample types are unavailable (Harrus and Waner, 2011). Inexpensive, fast, and accessible alternative *E. canis* nucleic acid detection techniques using LAMP assay designed to amplify E. canis citrate synthase gene have been developed and reported to have no significant difference with PCR (Chua et al., 2020). This can be a valuable nucleic acid detection technique in areas with little or no access to PCR-based tests. *Ehrlichia* species known to infect dogs are zoonotic, several cases have been reported in patients diagnosed with human ehrlichiosis in different parts of the world including the USA

(Springer and Johnson, 2018, Dahm et al., 2020), and Columbia (Echeverri et al., 2021).

1.8.3 Canine anaplasmosis

1.8.3.1 Canine Granulocytic Anaplasmosis (CGA)

Canine granulocytic anaplamosis is a febrile illness of dogs caused by an obligate intracytoplasmic bacterium called *Anaplasma phagocytophilum*, classified under the family *Anaplasmatacea*. *A. phagocytophilum* is also known to cause a similar disease in humans called human granulocytic ehrlichiosis (HGE). For decades *A. phagocytophilum* has been known as *Ehrlichia equi* and *Ehrlichia phagocytophila*, until the year 2001 when it was renamed *A. phagocytophilum* based on *16S rRNA*, *groESL* and surface protein gene genetic analysis (Dumler et al., 2001). The most common blood cells infected by *A. phagocytophilum* are granulocytes, precisely neutrophils, but eosinophils can also be infected with a subsequent development of morulae (von Loewenich et al., 2003). The first case of CGA in dogs was diagnosed in the USA (California) in 1982 (Sainz et al., 2015). Since then, CGA has been recorded in different parts of the world (Tsachev, 2009), including Europe (Germany, England) (Kohn et al., 2008, Bexfield et al., 2005, von Loewenich et al., 2003, Dyachenko et al., 2017), Africa (South Africa) (Inokuma et al., 2005, Kolo et al., 2020), the USA (Ewing et al., 1997, Lester et al., 2005), and Asia/Russia (Kawahara et al., 2006, Rar et al., 2010, Yang et al., 2013) etc. Apart from *A. platys* and other non-specific *Anaplasma* species reported from camels, dogs, and cattle from Nigeria (Lorusso et al., 2016b, Kamani et al., 2013a, Lorusso et al., 2016a, Adamu et al., 2014), *A. phagocytophilum* has not been reported in Nigeria. Canine granulocytic anaplasmosis is transmitted by varied species of hard ticks of the genus *Ixodes* in different parts of the world, *Ixodes ricinus* is known to be the principal vector of *A. phagocytophilum* in Europe (Beugnet and Marié, 2009, Blanco and Oteo, 2002), while *Ixodes scapularis* and *Ixodes pacificus* are known to transmit this

pathogen in the USA (Kohn et al., 2008, Bakken and Dumler, 2000). In Asia and Russia, *Ixodes persulcatus* and *Dermacentor silvarum* have been reported to transmit CGA (Rar et al., 2010), infection in dogs may also occur following blood transfusion. While transmission via the placenta to the fetus has been reported in cattle (Pusterla et al., 1997), and transmission from mother to child immediately before or after birth is reported in humans (Horowitz et al., 1998).

Although susceptibility to infection with *A. phagocytophilum* is known to increase with age, dogs or animals of all age groups are known to be highly susceptible. The Presence of *Ixode*s tick species responsible for the transmission, progression of susceptible animals to areas known to be endemic and transfusion of infected blood to susceptible patients especially in humans are known risk factors for CGA transmission (Atif, 2015). In temperate climate, the presence of trees producing cones and evergreen needle (coniferous) may serve as a good shelter for ticks, while lack of regular inspection of self (for humans) or grooming (for animals) may also constitute a greater risk of infection (Walker et al., 2001).

Many cases of CGA are diagnosed as co-infections with other canine pathogens such as *Babesia*, *Ehrlichia* and *Borrelia*. Diagnosis of CGA may be difficult as the presentation is not characterized by specific clinical signs. Diagnosis may be based on observation of clinical signs, serology, PCR, and response to treatment following institution of treatment (Kohn et al., 2008). The laboratory microscopic identification of the characteristic morulae in granulocytes including neutrophils and eosinophils remains the most common and primary diagnostic test for *A. phagocytophilum* (Dagnone et al., 2009, Kohn et al., 2011).

Serological tests based on enzyme immunoassay techniques (e.g., SNAP® 4Dx®, IDEXX Laboratories, USA) have been used to detect *A. phagocytophilum* IgM and IgG antibodies with 99% sensitivity in both apparently healthy and clinically diseased animals (Montenegro et al., 2017, Chandrashekar et al., 2010). Indirect fluorescent antibody assay (IFA) and western immunoblotting have also been demonstrated as practical and useful tests for detection of *A.*

phagocytophilum antibodies (Park et al., 2003, Kohn et al., 2011). PCR remains the most sensitive test for the detection of *A. phagocytophilum* in animals, most PCR reactions are based on the amplification of the *16S rRNA* gene (Rar et al., 2010). However, a combination of *16S rRNA* gene and *p44* gene demonstrated to be more specific for *A. phagocytophilum* has been used with high sensitivity (Kawahara et al., 2006). The highly conserved major surface protein-2 (*msp2)* gene has been proven to be useful in the detection of *A. phagocytophilum* antigen from both clinically and non-clinically infected animals. In addition, a fast, inexpensive, and less time-consuming loop-mediated isothermal amplification (LAMP) assay using sets of primers designed to specifically amplify the citrate synthase *(gltA)* gene has shown to be valuable for rapid detection of *A. phagocytophilum* in dogs (LEE et al., 2012).

Human anaplasmosis is an emerging zoonotic disease cause by *A. phagocytophilum*. The disease was first recorded as a febrile illness with generalized symptoms in humans in the USA in 1994, where it was first called human granulocytic ehrlichiosis (HGE) (Bakken et al., 1994). In Europe it was first described in Slovenia and since then several cases of HGE have been diagnosed in different parts of Europe including Spain (Blanco and Oteo, 2002, Oteo et al., 2000), France (Remy et al., 2003), Italy etc. (Ruscio and Cinco, 2003). To date, no case of human anaplasmosis caused by *A. phagocytophilum* has been reported in Nigeria.

1.8.3.2 Canine Cyclical Thrombocytopenia (CCT)

This is a disease of dogs caused by *Anaplasma platys* (formerly called *Ehrlichia platys*), cats, humans and foxes are also known to be infected. *A. platys* was documented for the first time in the USA in 1978 and has been recorded throughout the world including Europe, Asia, Australia, and Africa (Dahmani et al., 2015). *R. sanguineus* is the known principal vector for the transmission of *A. platys* (Almazán et al., 2016). Tick infested dogs, and those with little or no veterinary care or tick treatment have an elevated risk of infection with CCT

(Selim et al., 2021b). The first molecular studies on *A. platys* in Nigeria were recorded between 2013 to 2016 from dogs and camels in Nigeria (Lorusso et al., 2016a, Kamani et al., 2019a, Kamani et al., 2013a, Adamu et al., 2014), camels have been reported to harbour *A. platys* without showing clinical signs in different, parts of the world (Atif et al., 2021). CCT is characterized by fever, anaemia (pale mucous membranes), epistaxis, petechiation of the skin, oral and ocular mucous membranes, splenomegaly, hepatomegaly, and lymphadenomegaly. Anaemia and thrombocytopenia are consistent abnormal haematological findings associated with all cases of CCT (Arun et al., 2017, Atif et al., 2021). *A. platys* infection, and replication, or immune-mediated response mechanisms may damage thrombocytes leading to thrombocytopenia. In cyclic thrombocytopenia, extreme periodic changes in thrombocyte counts may heighten the risk of haemorrhage (thrombocytopenia) fluctuating with the heightened risk of blood clotting (thrombosis), due to increase thrombocyte production following haematopoetic feedback mechanisms (Langlois et al., 2018). Haematological parameters of dogs diagnosed with CCT may show alternating increased and decreased platelet counts within 7 — 14 days, while platelet numbers decrease with every single successive cycle. (Arun et al., 2017). Direct light microscopic examination of stained (Giemsa, Wright) blood smears to identify *A. platys* morulae in thrombocytes is the cheapest, and quickest method of CCT diagnosis during the acute phase of CCT. Commercially available in-clinic test kits including SNAP® 4Dx test (proven to be more sensitive than the rest) (Evason et al., 2019, Kotwa et al., 2020, Liu et al., 2018b), VetScan® (Abaxis) (Liu et al., 2018b), FLEX4 Rapid Test (FLEX4) (Liu et al., 2018a), and CaniV-4 rapid test (QBiotech) (Arnaudov, 2021) etc. detect antibodies to canine *Anaplasma* species and are not specific for *A. platys*. However, they are convenient and useful for routine screening of dogs to guide clinicians during diagnosis, treatment, prevention, and control. Indirect immunofluorescent assay (IFA) (Liu et al., 2018b), and nucleic acid detection techniques such as conventional/real-time PCR, and loop-mediated isothermal

amplification (LAMP) are valuable (Atif et al., 2021). PCR-based tests to amplify *16s rRNA* (Arun et al., 2017) *gltA* (Maksimovic et al., 2021), *23S rRNA*, and *groEL* gene have proven reliable (Alhassan et al., 2021), but the *16s rRNA* is commonly used in molecular detection of canine *Anaplasma* species. The use of a novel real-time LAMP (RT-LAMP) methods and RT-LAMP primers designed to specifically amplify *A. platys citrate synthase* gene within a total thermal cycling period of 30 minutes without cross reactivity with *Ehrlichia* and other *Anaplasma* species has proven to be cheap, fast, and valuable (Li et al., 2014). Based on this review, the LAMP method could be a good recommendation for low and middle-income countries. Doxycycline administration is an effective chemotherapeutic treatment for both CGA and CCT. Oral administration for doxycycline at 5—10 mg/kg body weight for 4-weeks has proven to be an effective CCT treatment. Dexamethasone administration at 0.3 mg/kg body weight may be given where immune-mediated thrombocytopenia is anticipated (Arun et al., 2017, Atif et al., 2021). Information on *A. platys* as a zoonotic pathogen is not currently available in Nigeria. Zoonotic cases of *A. platys* have been documented in other parts of the world including reports of intravascular persistence of *A. platys*/*Ehrlichia* spp. in people (mother and daughter) and their ≥3 months old bitch in Chicago (Breitschwerdt et al., 2014), and two women (exposed to *R. sanguineus* tick bites) from Venezuela (Arraga-Alvarado et al., 2014).

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1.8.4 Canine borreliosis or Lyme disease

This is a disease caused by a group of spiral-shaped bacteria called *Borrelia burgdorferi sensu lato* belonging to the genus *Borrelia* and the family *Spirochaetaceae*. The *B. burgdorferi* (s.l.) complex consists of over 15 species of *Borrelia*, transmitted by different tick species mostly from the genus *Ixodes* and distributed in different parts of the world (Table 1.5) (Krupka and Straubinger, 2010, Rudenko et al., 2011). Lyme disease was first described in the 1970s at a place called Lyme in Connecticut, USA (Hahn et al., 1996), while borreliosis in dogs was first documented in the USA in the 1980s (Magnarelli et al., 1987, Skotarczak, 2002). Canine borreliosis has also been recorded in Europe including in the UK (May et al., 1991, Abdullah et al., 2018), Poland (Skotarczak et al., 2005), and Asia including Turkey (Bhide et al., 2008). The only member of *B. burgdorferi* (s.l.) recorded in North Africa is *B. lusitaniae* (Krupka and Straubinger, 2010, Rudenko et al., 2011). However, *B. crocidurae* transmitted by soft ticks (*Ornithodoros sonrai*) that live in burrows, homes, and bedrooms, is not a member of the *B. burgdorferi* (s.l.) but has been associated with tick-borne relapsing fever (TBRF) in humans in Senegal. TBRF due to *B. duttoni* and louse-borne relapsing fever caused by *B. recurrentis* are also reported to be prevalent in East African countries (Goubau, 1984, Vial et al., 2006).

B. burgdorferi, the aetiological agent of canine borreliosis is transmitted by ticks from the genus I*xodes*, in the USA; *I. scapularis, I. pacificus* and *I. neotomae* have been established to transmit *B. burgdorferi*, while *I. ricinus* is known to transmit *B. burgdorferi* in Europe and *I. persulcatus* in both Europe and Asia (Table 1.5) (Krupka and Straubinger, 2010, Rudenko et al., 2011). The transmission of this pathogen is not restricted to dogs as *B. burgdorferi* is not host specific; it has been documented in humans (Lopes et al., 2017), cats (Muz et al., 2021), horses (Socoloski et al., 2018, Bae, 2018), sheep, goat, camel, cattle (Ben Said et al., 2016), rodents (Rodríguez-Rojas et al., 2020), birds

(Cleveland et al., 2020, Buker et al., 2013) and other wild animals (Kucerova et al., 2019, Alonso et al., 2012). The bacteria are also unable to survive outside the host as it requires biochemical products of the host and temperature between 30°C and 42°C to survive. This property may reduce the risk of indirect infection through contact with inanimate surfaces (Krupka and Straubinger, 2010). The risk of infection may be higher for dogs undergoing blood transfusion (Wardrop et al., 2005). Dogs living in areas with lower altitudes (tick density is established to decrease at higher altitude) (Jouda et al., 2004, Angelou et al., 2019), rural areas, sex (male more affected due to wandering behavior), stray, hunting and shepherd dogs or dogs more than 1 year old, season of the year (e.g., spring and summer) and presence of ticks in the environment are considered as canine borreliosis risk factors (Merino et al., 2000, Galluzzo et al., 2020).

Table 1.5: Geographical distribution of tick vector and species of *Borrelia burgdorferi sensu lato* complex. Adapted from Rudenko et al (2011).

Species	Vector	Reservoir host	Distribution	References
**B. burgdorferi sensu stricto*	*I. ricinus, I. scapularis, I. pacificus*	Rodents, birds, lizards, carnivores	North America, Europe	(Halsey et al., 2018, Geurden et al., 2018, Abdullah et al., 2018)
**B. garinii*	*I. ricinus, I. uriae I. persulcatus, I. hexagonus, I. nipponensis*	Birds, lizards, rodents	Europe, Asia	(Geurden et al., 2018, Wang et al., 2019)
**B. afzelii*	*I. ricinus, I. persulcatus*	Rodents	Europe, Asia	(Geurden et al., 2018, Wang et al., 2019)
B. valaisiana	*I. ricinus, I. granulatus*	Birds, lizards, rodents	Europe, Asia	(Geurden et al., 2018, Khoo et al., 2018)
**B. spielmanii*	*I. ricinus*	Rodents	Europe	(Abdullah et al., 2018)
**B. lusitaniae*	*I. ricinus*	Rodents, lizards	Europe, North Africa	(Geurden et al., 2018, Norte et al., 2021)
B. andersonii	*I. dentatus*	Cotton tail rabbit, birds, rodents	North America	(Hamer et al., 2012)
**B. bissettii*	*I. ricinus, I. scapularis, I. pacificus, I. minor*	Rodents	North America, Europe, Asia	(Wang et al., 2019, Hanschmann et al., 2021, Hacker et al., 2018)
**B. bavariensis*	*I. ricinus, I. uriae, I. persulcatus*	Birds, rodents	Europe, Asia	(Hanschmann et al., 2021, Scholz et al., 2013)
**B. kurtenbachii*	*I. ricinus, I. scapularis*	rodents	Europe, North America	(Hanschmann et al., 2021, Margos et al., 2010, Margos et al., 2014)
B. tanukii	*I. tanuki*	Rodents	Asia (Japan)	
B. turdii	*I. turdus, I. nipponensis*	Birds	Europe, Asia	(Kang et al., 2013, Norte et al., 2013)
B. japonica	*I. ovatus, I. granulatus*	Rodents	Asia	(Khoo et al., 2018)
B. sinica	*I. ovatus*	Rodents	Asia (China)	(Masuzawa et al., 2001)

Species	Vector	Reservoir host	Distribution	References
B. californiensis	*I. pacificus,* *I. longiscutatus* *I. jellisonii,* *I. spinipalpis*	Kangaroo rat, mule deer	North America	(Dall'Agnol et al., 2017)
B. carolinensis	*I. minor,* *I. longiscutatus,* *I. ariadnae*	Rodents, birds,	North America, Europe	(Dall'Agnol et al., 2017, Michalik et al., 2020, Dunaj et al., 2021)
B. americana	*I. pacificus,* *I. minor,* *I. ricinus*	Birds	North/south America	(Dunaj et al., 2021, Nava et al., 2014)
(*): Suspected zoonotic spp.				

Canine borreliosis may present as a disease condition with similar signs as recorded in humans. Dogs are regarded as the most susceptible host for *B. burgdorferi* infection and clinical presentation in dogs has been used as sentinel or model to study human borreliosis (Krupka and Straubinger, 2010). Both field and experimental studies have shown that dogs seropositive to *B. burgdorferi* may not show signs of illness during presentation. Bilateral or unilateral inflammation of joints or limbs characterized by lameness is common but there is a transient recovery if antibiotic treatment is instituted on time. Nephritis is reported as the most serious clinical sign in canine borreliosis, and chronic nephritis in dogs is highly suggestive of *B. burgdorferi* infection (Littman et al., 2018). Inflammation of the inguinal or prescapular lymph nodes can be additional clinical findings in infected dogs (Skotarczak et al., 2005, Wardrop et al., 2005, Kornblatt et al., 1985). Additionally, fever, anorexia and weakness are possible clinical signs, though signs of nervous dysfunction and kidney diseases are rare in infected dogs (Skotarczak, 2002, Krupka and Straubinger, 2010). Infection has been shown to be persistent in infected dogs making them carriers for a considerable period (Straubinger et al., 1997, Littman et al., 2018).

Clinical suspicion of canine borreliosis is based on travel history of sick or seropositive dogs, presence of ticks or information indicating tick infestation, recovery of dogs from clinical signs following institution of antibiotic therapy (Littman et al., 2018). Laboratory microscopic examination of thin blood smears, serological tests and PCR have been used, with the latter mostly carried out for research purposes. Diagnosis based on microscopic examination of *Borrelia* bacteria may be challenging owing to its small size measuring 25 µm in length with a diameter of 0.2 µm. Its smaller size makes it almost impossible to detect it using light microscopy. However, the use of a dark-field microscope to view thin smears from suspected cases can produce useful results (Krupka and Straubinger, 2010).

Serological detection of *Borrelia* specific antibodies is the principal test in routine screening of clinically ill dogs and has been a widespread practice for human cases as well. IFAT and ELISA tests are regarded as the *B. burgdorferi* first and second-generation serological test, respectively. Both methods and commercially available lateral flow devices including SNAP4DxPlus (IDEXX), VetScan Rapid (Abaxis), Quant C (IDEXX) can potentially distinguish between vaccinal and natural exposure antibody (Littman et al., 2018). Western blotting, which also involves the use of recombinant antigens, is regarded as the only available serological test to detect specific antibodies without cross reactivity. It is mostly used to confirm the outcome of IFA and ELISA during diagnosis, and differentiate between acute and chronic infection (Skotarczak, 2002, Littman et al., 2018). *B. burgdorferi* (s.l.) vaccines stimulate sufficient immunoglobulin response to *Borrelia* antigenic proteins including the outer surface proteins (OspA and OspB). Most of these antigenic proteins are incorporated in commercially available assays. Therefore, identification of specific immunoglobulins reactive to *Borrelia* OspA and OspB antigenic proteins is used in distinguishing between vaccination and infection status in dogs. Western blotting has its limitation and challenges, given that bands of similar sizes may be identified in serum samples obtained from nonexposed/vaccinated and exposed/vaccinated dogs (O'Connor et al., 2004, Leschnik et al., 2010, Stillman et al., 2019). Immunofluorescent antibody (IFA) and Western blot testing are not recommended for use in the USA as cross reaction with other infections spirochaetes have been reported (Littman et al., 2018). Dogs with a previous history of tick infestation or treatment with antibiotics may still be positive to *Borrelia* serological tests. The use of serological tests in diagnosis of canine borreliosis may be deceptive and controversial, as not all seropositive dogs may be diseased (Bhide et al., 2008). Other cross reactions between *Borrelia* antigens and *spirochaete* bacteria species such as *Leptospira, Treponema* and *Bradyspira* species have also been reported (Skotarczak, 2002, Krupka and Straubinger, 2010).

PCR is sensitive and useful to detect canine borreliosis, and it is valuable for research purposes. Previous PCR methods have been carried out using species specific primers designed to target the *Borrelia 16S rRNA* gene with successful results (Marconi and Garon, 1992, Olsén et al., 1995). *B. burgdorferi* f*lagellin* (*flaB*) gene has been defined to be a conserved region that may be useful to amplify the *B. burgdorferi* pathogen from ticks (Johnson et al., 1992, Clark et al., 2005). Primers have also been developed targeting the *Borrelia 5S, 23S rRNA* and intergenic spacer genes, all of which are useful in identification of specific species of *Borrelia* (Wilhelmsson et al., 2010). Similarly, PCR methods targeting the *Borrelia* outer-surface protein A (*OspA*) gene have also been utilized (Nocton et al., 1994, Davies et al., 2017).

1.8.5 Canine hepatozoonosis

Canine hepatozoonosis is a severe febrile illness caused by *Hepatozoon canis* and *Hepatozoon americanum*, transmitted to dogs via ingestion of ticks harbouring infective *Hepatozoon* oocysts (Baneth et al., 2003). It was first described in India as *Leukocytozoon canis*, and since then it has been documented in Africa (Ezeokoli et al., 1983, Abakpa et al., 2021), Asia (Abd Rani et al., 2011), Europe (Baneth and Weigler, 1997), the Middle East and America (Mathew et al., 2001, Ewing et al., 2000). The brown dog tick (*R. sanguineus*) prevalent in both tropical and temperate climates is a principal vector for *H. canis* species although, other tick vectors such as *A. americanum* have been suggested to transmit *H. canis* in previous studies (Abd Rani et al., 2011). *Amblyomma maculatum* (gulf coast tick) nymphal or adult stages remains the principal vector known to transmit *H. americanum* (Ewing and Panciera, 2003). The aetiology of feline hepatozoonosis is *H. felis*, which has also been reported worldwide including in Africa (Nigeria, South Africa), Asia, USA, and Europe. Little or no information is available on *H. felis* life cycle or transmission, clinical manifestation, and clinical signs (Baneth et al., 2013).

Clinical presentation of canine hepatozoonosis may vary depending on the species of the parasite involved. *H. canis* may present as a mild or severe disease; the affected dog may show clinical signs such as fever, weakness, anaemia, and weight loss in chronic cases. The mild form of the disease characterized by low parasitaemia is more common in dogs, while the severe form is rare but may be seen in dogs with a high number of gamonts detected in neutrophils or parasitaemia. There may be 100% infection of neutrophils with gamonts during microscopic examination of blood collected from *H. canis* infected dogs (Baneth et al., 2003). In contrast, *H. americanum* in dogs may present as a serious febrile illness with signs such as weakness, lameness, muscular pain (myalgia) and ocular discharge, which may be mucous or purulent. Significant leukocytosis due to neutrophilia and increased bone proliferation may be common findings in *H. americanum* infection. Gamonts in infected neutrophils (parasitaemia) may be rare and death may occur if not treated (Panciera and Ewing, 2003, Baneth et al., 2003).

Diagnosis of *H. canis* is commonly done via examination of stained blood smears for the presence of *H. canis* gamonts located within the cytoplasm of neutrophils under a light microscope (Otranto et al., 2011). Microscopic examination of buffy coat has proven to be more valuable than blood smear, cytology on buffy coat is recommended to be used where PCR-based test is not available. (Otranto et al., 2011, Sasanelli et al., 2010). Serological tests such as the indirect fluorescent antibody test (Karagenc et al., 2006), real time (Pacifico et al., 2020) and conventional PCR assays designed to amplify the *18S rRNA* gene of *Hepatozoon* spp. have been used to detect *H. canis* in suspected dogs (Karagenc et al., 2006, Vojta et al., 2009, Helm et al., 2020, Prakash et al., 2018). PCR-based assay using buffy coat more also produces more positive PCR result than whole blood sample (Otranto et al., 2011). However, the multiplication of gamonts in neutrophils may be rare in *H. americanum* infection, examination of stained muscle biopsy becomes a useful technique for the diagnosis of *H. americanum*. Previous histopathological studies in provided evidence for the

presence of *Hepatozoon* meronts in the myocardium and skeletal muscle of cats, showing that the muscular organs are also destinations for *Hepatozoon* species (Baneth, 2003). Similarly, an indirect ELISA with 93% sensitivity has been developed as a gold standard test for diagnosis of *H. americanum* infection in dogs (Mathew et al., 2001). Diagnosis of *H. americanum* via PCR is also designed to amplify the 18S rRNA gene of *Hepatozoon* species as documented in previous studies on *H. canis* (Baneth et al., 2000). A simple, and rapid new generation nucleic acid amplification method such as LAMP and LAMP primers designed to amplify *Hepatozoon* species 18S rRNA gene, is also recommended for routine field examination where real time or routine conventional PCR is not achievable (Singh et al., 2019).

1.8.6 Canine dirofilariasis

Canine dirofilariasis is a parasitic disease of carnivores and primates, with filarial worms of the genus *Dirofilaria* as a causative agent. Canine dirofilariasis is not a tick-borne disease; however, some stages of the filarial worm lifecycle are present in canine blood and routine tests for the tick-borne pathogens (light microscopy and some of the lateral flow tests) will also detect this pathogen. Over 27 *Dirofilaria* species have been described; nonetheless, *D. hongkongensis* (Gowrishankar et al., 2019), *D. repens* and *D. immitis* remain the two significant species of veterinary and public health importance, due to their pathogenicity to dogs, cats, and humans. Mosquitoes are known to be principal vectors of transmission, except for *D. ursi* transmitted by simuliids (black fly) (Michalski et al., 2010). *Culex* species (e.g., *Culex quinquefasciatus, Cx. pipiens)* and *Aedes* species (e.g., *Ae aegypti, Ae. Scapularis, Ae taeniorhynchus)* have been demonstrated as mosquito vectors for the transmission of *D. immitis* and *D. repens.* However, other mosquito species may be considered as vectors for *Dirofilaria* transmission (Labarthe et al., 1998). A report of canine dirofilariasis infection that was documented in South America in 1875 seems to have been the first report of canine dirofilariasis. In addition, the 1887 case report of a child

with pulmonary dirofilariasis from Rio de Janeiro, Brazil has remained the first documented case of human pulmonary dirofilariasis (Vezzani et al., 2006). *D. repens* is the known aetiology of subcutaneous or ocular legions in humans in Asia, Africa, and Europe, while *D. immitis* is the common cause of canine dirofilariasis in the tropics (Pozgain et al., 2014, Venco et al., 2014, Serrano-Parreño et al., 2017, Kamalu, 1991). In Asia (including India) *Candidatus Dirofilaria hongongensis sp. nov* is reported to the cause of cutaneous dirofilariasis characterized by abdominal swelling, presence of nodules on the eyelid/subconjunctival/orbit, penis, head, neck, face, breast etc., in humans (Kumar et al., 2021).

In Nigeria, the first cases of canine dirofilariasis were documented between 1974 and 1975 where examination of blood from 369 indigenous dogs from northern Nigeria revealed a 9.3% prevalence of *D. repens* microfilariae (Van Veen and Blotkamp, 1975). Following this documentation, studies in southeastern Nigeria also demonstrated the presence of microfilariae characteristic of *D. repens* from peripheral blood and subcutaneous connective tissues (Kamalu, 1991). Recent studies in the north central and Eastern part of Nigeria have reported *D. immitis* microfilaria prevalence of 3.36% from 119 dogs and 2.15% from 186 dogs respectively (Ugochukwu et al., 2016, Ogbaje and Danjuma, 2016). Studies to establish transmission of dirofilariasis in Nigeria have not been documented apart from the study in Zaria, northern Nigeria where *Aedes aegypti* were suggested to be responsible for dirofilariasis transmission in dogs (Anyanwu et al., 2000). However, most of the studies demonstrating the prevalence of *D. immitis* or *D. repens* in Nigeria starting from 1974 to present were based on examination of blood smears via wet mount, buffy coat and modified Knott's techniques and serology (Joshua and Gonzlez-Miguel, 2020, Ezema et al., 2019). In addition, a recent study using High Resolution Melt (HRM) Real-time PCR and sanger sequencing from a total of 197 samples, reported the absence of *Dirofilaria* species (Kamani et al., 2019b).

Canine dirofilariasis is usually diagnosed as a long-standing (chronic) infection and affected dogs may not show evidence of clinical disease for several weeks, months or years. However, factors such as the presence of high number of adult filarial worms in the blood, type, or level of exercise the affected dog is subjected to and the dog's reactivity to the presence of the adult worms may determine the form of disease presentation. Whatever may be the case, gradual development of clinical signs may be recorded in canine dirofilariasis, with coughing as the first clinical sign seen in most dogs, difficulty in breathing (dyspnea), weakness and intermittent loss of consciousness following exercise or excitement (lipotimias) may follow. Right-sided congestive heart failure may occur following the presence of a heavy worm burden. Cardiac murmurs or abnormal cardiac rhythm due to tricuspid valve insufficiency and fibrillation, respectively, may be additional clinical findings. Death may occur if thromboembolism occurs, or the affected dog may continue to emaciate over a considerable period. Following pulmonary hypertension in small size dogs, adult worms may be displaced from the pulmonary arteries into the right chamber of the heart resulting into a fatal condition called "caval syndrome" defined by difficulty in breathing, heart murmurs and presence of haemoglobin in the urine (Venco et al., 2005).

Previous studies demonstrated that *D. immitis* infecting dogs may carry a symbiotic bacterium called *Wolbachia pipientis*. *W. pipientis* is proposed to play a role in the development of canine dirofilariasis, as antigens are commonly found circulating in the blood of infected dogs (Casiraghi et al., 2004). Moreover, recent studies indicate no difference in pathological lesions between *Wolbachia pipientis* seropositive and seronegative dogs/cats, establishing a lack of evidence to support the claim that *Wolbachia pipientis* play a role in canine dirofilariasis (Dingman et al., 2010).

Suspicion of canine heartworm is based on clinical history, clinical examination, and knowledge of vector distribution. Wet mount, buffy coat and modified Knott's procedures are useful for the detection of *Dirofilaria* via light microscopy.

During wet mount, a drop of sterile water is placed on a sterile glass slide, and after addition of sample material to be examined, the cover glass is first lowered carefully onto the portion of sample placed on the slide. However, wet mount is not recommended for light microscopic identification of filarial parasites due to its inability to differentiate between pathogenic and non-pathogenic dirofilarial species (Hays et al., 2020). Buffy coats are prepared following centrifugation of blood in a micro haematocrit tube and aseptic removal of the buffy coat segment. In the Knott's technique 10ml of 2% formalin is added to about 1ml of blood containing anticoagulant. The tube is covered or capped using a thumb finger and is mixed by inverting the tube severally. The mixture is then centrifuged at 500xg for 5 minutes, and the resulting supernatant discarded. An aliquot of 0.1% methylene blue and the sediments (e.g. 35µl:20µl) is mixed and viewed under light microscope (Girdan et al., 2014). These three basic procedures have been used in the diagnosis of Dirofilaria in dogs (Rojas et al., 2015).

POC test kits for *D. immitis* antigen detection have been developed; the kits are specific for the detection of intact *D. immitis* antigen and not antibodies. However, cross-reactivity with antigens from other canine nematodes including *D. repens*, *Spirocerca lupi*, and *Angiostrongylus vasorum* have been reported particularly in samples exposed to immune complex dissociation (ICD) (Panarese et al., 2020). These include DiroCHEK® or Witness® HW (Zoetis) and PetChek® or SNAP® (IDEXX Laboratories, Inc.). A recent study suggested that heat treatment of sera samples before testing using these commercial lateral flow devices (LFD) test kits may aid earlier detection of *Dirofilaria immitis* in dogs (Carmichael et al., 2017, Starkey et al., 2017). In addition to the commercial antigen test kits, thoracic radiography should be utilized for diagnosis of canine dirofilariasis, it is an excellent method of determining severity of canine disease and assessment of affected dogs post treatment (Bowman and Atkins, 2009).

It is relevant to perform both Knott's and antigen detection tests during diagnosis of all dogs suspected of canine heartworm disease as microfilaraemia may be seen in only about 30% of dogs infected. Both tests have their

advantages and limitations depending on the life cycle stage of canine heartworm parasite following infection. The Knott's technique is useful for the concentration, morphological identification, and differentiation of *D. repens*, *D. immitis*, and *Acanthocheilonema* microfilariae species in the presence of low worm numbers (Magnis et al., 2013). Due to the similarity in morphology, misidentification of microfilariae originating from *D. immitis, D. repens, D. hongkongensis, Acanthocheilonema reconditum* and *A. reconditum* is reported where co-infection is present and molecular methods may be required (Hays et al., 2020, Little et al., 2018, Magnis et al., 2013). It is a valuable tool to detect microfilaraemia following adulticide therapy, microfilaria infection acquired via blood transfusion, and where antigen cannot be detected in the blood sample due to improper handling and storage (Hays et al., 2020, Little et al., 2018). Due to the similarity in morphology, misidentification of microfilariae originating from *D. immitis, D. repens, D. hongkongensis, Acanthocheilonema reconditum* and *A. reconditum* is reported where co-infection with multiple microfilaria species is present and PCR may be required (Hays et al., 2020, Little et al., 2018, Magnis et al., 2013) The use of Knott's technique is also limited in the presence of single sex infection (no microfilariae in the presence of only female infection), early stage of infection with immature worms (absence of microfilaria less than seven months post exposure to adult worms), and in the absence of microfilaraemia due to monthly treatment with macrocyclic lactone microfilaricides (milbemycin, moxidectin, selamectin), or immune-mediated clearance of microfilaraemia (Hays et al., 2020, Little et al., 2018). While the antigen test is a valuable in the presence of single sex infection, early stage of infection with immature worms, and the absence of microfilaraemia. False negative canine heartworm antigen test results could be recorded when there is low female worm burden, systemic antigen clearance post adulticide therapy (in the presence of surviving microfilaria), and microfilariae infection acquired via blood transfusion (Hays et al., 2020, Little et al., 2018). Another possible reason for the presence of a false negative microfilaria antigen test is the presence of

unbound antigen due to antigen-antibody immune complexes. Preheating of whole blood sample prior to antigen test is recommended to produce true positive results antigen test result (Little et al., 2014).

The application of molecular techniques such as PCR have been used in molecular genotyping of *Dirofilaria* species. The internal transcribed spacer region 2 (*ITS2*) of the ribosomal DNA of either *D. immitis* or *D. repens* has been used in PCR-based tests (Rishniw et al., 2006, Borthakur et al., 2015). The mitochondrial gene cytochrome oxidase subunits 1 and *12S rDNA* have also been amplified to construct phylogenies for the filarial parasites including *D. hongkongensis* (Kumar et al., 2021). Identification of both filarial worms of dogs and *Wolbachia pipientis* as a symbiotic bacterium in dirofilariasis has been achieved through the *16S rRNA* gene sequence analysis (Casiraghi et al., 2004, Kronefeld et al., 2014).

1.8.7 Treatment of canine tick-borne disease

Most of the canine tick-borne diseases are treatable; Table 1.6 and Table 1.7 summarize the most common treatment protocols (along with that for heart worm disease).

Table 1.6: Treatment, management, and prevention of canine vector-borne diseases (CVBDs).

Canine Babesiosis/theileriosis	Treatment	Several drugs have been used in field or laboratory clinical studies for the treatment of canine babesiosis depending on the form of *Babesia* species identified. A single intramuscular or subcutaneous dose of imidocarb dipropionate at 5-7 mg/kg body weight (repeated after 2 weeks) is the recommended treatment for canine babesiosis associated with large *Babesia* species. A variety of drugs and treatment protocols with less efficacy and presence of clinical relapse of infection after treatment have been documented for the management of small forms of *Babesia* species including the use of Imidocarb diproprionate and diminazene aceturate which are not drugs of choice for babesiosis. However, simultaneous oral administration of synthetic naphthoquinones anti-malaria drugs (atovaquone PO at 13.3 mg/kg three times per day for 10 days or buparvaquone at 5 mg/kg IM twice 48 hours apart) and azithromycin (10 mg/kg body weight once per day for 10 days) has been recommended (Dixit et al., 2010, Irwin, 2010, Petra et al., 2018, Baneth, 2018, Checa et al., 2017). Personal communication and clinical experience (authors opinion) shows that most Nigerian vets are likely to recommend diminazene aceturate (3.5 mg/kg body weight once) for treatment of canine babesiosis. This drug has a narrow therapeutic range and side effects such as central nervous system toxicity in dogs (Petra et al., 2018, Baneth, 2018). It is not a recommended drug of choice for canine babesiosis.
	Prevention/control	Prevention of tick bite, infestation on dogs, and their habitat via use of acaricides. Oral chewable and systemic licensed isoxazoline acaricides approved in the USA, Canada, Europe, New Zealand, and USA are reported to be effective acaricide for canine tick control (Irwin, 2010, Petra et al., 2018). However, current studies showed that Isoxazoline are less effective than topical tick control products in the tropics and may not be recommended in this study (Huggins et al., 2022). Dog collars, spot-on, spray, pour-on containing ectoparasiticides are commercially available to protect dogs for a minimum of 4 weeks to 8 months depending on the product. Prophylactic treatment with imidocarb and doxycycline has been described but it is rarely used (Irwin, 2010, Petra et al., 2018). There are no vaccines for the prevention and control on canine babesiosis however, studies on new tick control strategies using anti-tick vaccines are being reported (Petra et al., 2018).

Canine ehrlichiosis	Treatment	A consensus oral dose of doxycycline at 10mg/kg body weight, daily for 4 weeks (regardless of the phase of CME phase). Varying doxycycline dose regimen (5 to 10 mg/kg), lack of tick control, and host immune status are known factors affecting the treatment of CME. Supportive therapy involving administration of crystalloid fluid and or temporal whole blood transfusion, and iron sulphate supplement (in CME dogs with myelosuppression) are essential in dogs with aplastic pancytopenia. (McClure et al., 2010, Villaescusa et al., 2015, Mylonakis et al., 2019)
	Prevention/control	A commercially available acaricide called Advantix® (containing imidacloprid and permethrin) is recommended for the prevention of *E. canis* transmission in dogs by *R. sanguineus* for 4 weeks (topical application) (Jongejan et al., 2016). Application of several other ectoparasiticides (acaricides/repellents: collar, pour-on, or spot-on) on pets/environment can prevent canine tick infestation and introduction of ticks to canine habitat. Mechanical removal of ticks on pets, knowledge of dog breeds predisposed to severe disease following *E. canis* infection (German Shepherd, Siberian Huskies), tick prevalence or seasonality are also essential. There are no commercial vaccines available for prevention of canine ehrlichiosis (Sainz et al., 2015).
Canine anaplasmosis cyclic thrombocytopenia	Treatment	An oral dose of doxycycline at 5mg/kg body weight 12-hourly for a duration of 10 to 14 days has been determined to be efficacious for *A. platys,* a minimum treatment regimen of 2 weeks is recommended (Kohn et al., 2008).
	Prevention/control	Tick prevention and control as mentioned under canine ehrlichiosis.
Canine borreliosis (Lyme disease)	Treatment	Systemic administration of antibiotics, supportive care, and tick control. The use of amoxicillin (20 mg/kg body weight 8 hourly) or doxycycline (10mg/kg body weight 12 hourly for 4 weeks) orally is recommended (Chomel, 2015, Littman et al., 2018).
	Prevention/control	The use of vaccines against Borreliosis in dogs is controversial as the efficacies of canine Lyme disease vaccines in previous field and laboratory studies are highly variable (Vogt et al., 2019). The American College of Veterinary Internal Medicine (ACVIM) could not also reach a consensus on the use of canine Lyme vaccine (Littman et al., 2018), most veterinarians are unlikely to recommend dog vaccination in *Borrelia* endemic areas (Littman et al., 2006). The use of commercially available tick repellents/acaricide and mechanical removal of ticks less than 48 hour following attachment are the measures recommended for the prevention and control of canine borreliosis (Chomel, 2015, Vogt et al., 2019).

Canine hepatozoonosis	Treatment	There is no effective drug of choice, the administration of two consecutive subcutaneous injections of Imidocarb dipropionate at 5 to 6 mg/kg body weight two weeks apart is currently recommended in the absence of an effective drug and treatment for canine hepatozoonosis associated with *H. canis*. Treatment may take 8 weeks or longer in dogs with heavy infection with good prognosis. A combination of systemic course of doxycycline (10 mg/kg body weight daily for 4 weeks) and imidocarb diproprionate is equally described to be ineffective (Attipa et al., 2018, Baneth, 2011).
	Prevention/control	Control of ticks on dogs and their habitat using ectoparasiticides, and regular mechanical removal of ticks on dogs to avoid oral ingestion of infected ticks during canine grooming. Avoid mating between infected and non-infected dogs to avoid transmission from infected, and dam to puppies. Prevention of dogs from straying, predatory activities on infected animals or ingestion of infected raw from rabbits and related intermediate hosts (Attipa et al., 2018, Baneth, 2011, Johnson et al., 2009).
Canine dirofilariasis	Treatment	Melarsomine dihydrochloride is the only adulticide therapy approved by American Heartworm Society. The first dose is administered deep intramuscularly at 2.5mg/kg body weight into the epaxial lumbar muscle (L3 and L5). After one month of treatment, two same doses given at 24 hours interval are repeated. However, the last two doses are not approved for dogs with caval syndrome. Supportive treatment is also recommended. Administration of doxycycline at 10 mg/kg twice daily (BID) for 28 days before commencement of adulticide therapy is also recommended by the AHS. Surgical removal of adult worms is approved by AHS for dogs with caval syndrome (Bowman and Atkins, 2009), (https://www.heartwormsociety.org/images/pdf/Canine-Guidelines-Summary.pdf).
	Prevention/control	Good preventive measure involves placement of affected dogs or those at risk on a monthly dose of AHS approved larvicidal drugs (Bowman and Atkins, 2009), (https://www.heartwormsociety.org/images/pdf/Canine-Guidelines-Summary.pdf).

Table 1.7: Recommended treatment protocol for canine heartworm (HW) disease. Adapted from American Heart worm Society: (https://www.heartwormsociety.org/images/pdf/Canine-Guidelines-Summary.pdf).

Day	**Treatment protocol**
Day 0	Use positive antigen (Ag) test for HW diagnosis, and microfilaria (MF) test for verification. Confirm negative microfilariae test using Ag test from other manufacturers. Restriction of exercise in affected dogs, ensure more restriction in severely affected dogs. Therapeutic and nursing care including Prednisone at 0.5 mg/kg, given twice daily in the first week, once a day in the second week and every other day in the third and fourth week.
Day 1	Prevention of anaphylaxis in affected dogs via microfilariae preventive treatment and administration of antihistamine and glucocorticoid (if prednisone is not given on day 0)
Day 1-28	Doxycycline at 10 mg/kg twice a day for four weeks, to eliminate *Wolbachia* and potential for heartworm reproduction.
Day 30	Repeat HW preventive treatment
Day 60	Repeat HW preventive treatment via administration of melarsomine at 2.5 mg/kg intramuscularly. Administer prednisone at 0.5 mg/kg for four weeks as carried out on day 0 and ensure exercise restriction for dogs undergoing treatment.
Day 90	Repeat HW preventive treatment via intramuscular injection of a second dose of melarsomine at 2.5 mg/kg
Day 91	Followed by a third dose of melarsomine at 2.5 mg/kg given intramuscularly. Repeat prednisone at 0.5 mg/kg as previously given on day 0 and 60, ensure exercise restriction for a period of 6-8 weeks following the last dose of melarsomine administered.
Day 120	Retest dogs treated for the presence of microfilariae in the blood. Repeat treatment if a positive test is obtained and ensure to test affected dogs after 4 weeks.
Day 271	It is a good practice to reschedule appointments for treated animals not more than six months after the final treatment, antigen and microfilariae screening test should be performed to confirm the effectiveness of the previous treatment protocol.

1.8.8 CVBP diagnosis using metabarcoding next generation sequencing

The application of metabarcoding next generation sequencing (NGS) is currently used in research for diagnosis of canine tick-borne pathogens. Few studies have demonstrated the importance of CVBPs metabarcoding sequencing, including their advantages and disadvantages. Reliable approaches for the control of CVBPs require extensive understanding of canine vectors/pathogen distribution, transmission, and dynamics. Approaches focusing on single specific CVBPs detection may yield insufficient information on the epidemiology of canine vector/pathogen since multiple vectors, pathogens, and hosts may be involved as seen in the emergence of *Coxiella burnetti* (the cause of Q-fever) from *Coxiella-like* endosymbionts (*CLEs*) of ticks (Duron et al., 2015a, Brenner et al., 2021). The life cycle of several vector-borne pathogens and the ability of vectors to transmit pathogens is also affected by the presence of other microbial communities. For example, tick endosymbionts are relevant for continuous nutritional provision, fitness, multiplication, growth, pathogen establishment, and active transmission in ticks (Bonnet and Pollet, 2021). The use of NGS sequencing in CVBPs diagnosis has revealed the complex nature of microbial communities among vectors (especially ticks), indicating the need to apply NGS in the diagnosis of CVBPs.

Several NGS metabarcoding sequencing approaches have been developed to assess tick and canine coinfection with CVBPs including improved detection of multiple pathogens in canine blood (Huggins et al., 2019a) using 16S rRNA bacterial metabarcoding, and ticks (Ravi et al., 2019) using both 16S rRNA bacterial metabarcoding and shotgun metagenomic sequencing approaches to profile tick microbial communities. While Ravi et al. (2019)'s sequencing approach identified pathogens in ticks including tick-borne canine parvovirus, Huggins et al. (2019a) reported the abundance of microbial communities from canine blood. Reports from both studies indicated potential contamination from commercial kits/reagents and environments, and or abundant host reads especially in shotgun metagenomics.

Caution must be applied to ensure appropriate DNA/RNA extraction kits and relevant controls are in place. Reports from such studies provided additional information on common commercial kits/reagents, and environmental contaminants to be considered in NGS metabarcoding (Velasquez-Mejia et al., 2018, Eisenhofer et al., 2019).

However, the use of bacterial 16S rRNA metabarcoding sequencing with host specific blocking primers in recent studies provided an improved and efficient detection of canine vector-borne bacteria with minimal host contamination. The Huggins et al. (2020) approach showed a significant increase in the abundance of CVBPs DNA sequences, and reduction in the abundance of canine host mitochondrial gene reads occurring due to cross reactivity between bacterial primers, tick 16S rRNA, and canine mitochondrial 12S rRNA gene (Huggins et al., 2020). Another protocol for CVBP metabarcoding next generation sequencing focusing on detection of canine protozoan hemiparasites has been described to be more sensitive than conventional PCR. Using primers designed to exclusively amplify the conserved apicomplexan and kinetoplastid parasite 18S rRNA gene. It is recommended for the detection of CVBPs in areas with high prevalence of tick-borne protozoan haemoparasites (Huggins et al., 2019b). The recent description of a multipronged NGS metabarcoding approach to detect a wide range of bacterial, and protozoal haemoparasites in dogs indicates an improvement in CVBPs NGS methodology. The multipronged approach utilizes bacterial 16S and protozoan 18S rRNA gene sequencing to establish a canine blood microbiome (Huggins et al., 2021b). These reports highlight the advancement and the advantage of metabarcoding methods to detect novel CVBD pathogens in dogs over targeted approaches.

1.9 Aim

In tropical and subtropical regions of the world, canine infection with vector-borne pathogens is considered emerging diseases. The presence and prevalence of ticks and CVBPs have been continuously reported in the tropical and temperate regions of the world in the last one-to-two decades. Both urban and rural dogs in Nigeria are

reported to be exposed to tick vectors, making them preferred hosts for canine vector-borne pathogens. In addition, dogs, and other animals co-infected with zoonotic pathogens could serve as reservoir hosts for human infection. Ticks and other vectors may be considered the next human and animal health threat if not properly controlled.

There is no established functional tick and vector-borne disease surveillance scheme in Nigeria. There is a lack of robust research information available on the prevalence of vectors and pathogens they transmit across Nigeria or neighbouring countries. This may affect countries with surveillance scheme on vector borne diseases, as they have little information on the potential vectors and pathogens that could be imported from Nigeria. This study aims to address the scarcity of data on CVBPs, including ticks, in selected parts of the Nigerian communities.

1.10 Objectives

1. To ascertain the prevalence of CVBPs from Nigerian communities using point-of-care (SNAP® 4Dx IDEXX antigen) test kits.
2. To determine the prevalence of hard ticks infesting dogs in Nigeria using conventional light microscopic techniques.
3. Molecular identification and characterization of CVBPs (in canine blood/tick) and hard ticks (from dogs and domestic bird) through the application of DNA extraction, PCR amplification, Sanger sequencing and phylogenetic analysis.
4. To ascertain the risk factors associated with CVBPs and tick infestation in Nigeria following application of a multivariate modelling exercise.
5. To determine metagenomics profiling of selected canine blood and tick samples via application of next generation sequencing technology and bioinformatics analysis.

Chapter 2: Methodology

2.1 Ethical approval

The ethical approval for this study was received from the University of Nottingham School of Veterinary Medicine and Science Ethical Review Committee (Ethical approval number: 2416180702). Samples assessed in this study were collected from Nigerian veterinary clinics following clinical examination of dogs. Due to the low rates of literacy and the plethora of languages in use in Nigeria, verbal consent to participate in the study was obtained from dog owners by the participating veterinarians. Consent obtained from every dog owner was recorded by the veterinarian on the questionnaire forms designed for this study.

2.2 Design of questionnaire for sample and demographic data collection

A sample collection form was designed to obtain demographic data of each animal recruited in this study. Information collected from dogs recruited in this study included client-informed consent, history, date, status, sex, age, breed, level of tick infestation in the dog, number of ticks collected, tick species morphological identification, and whether dogs were positive to tick-borne pathogens via the POC (IDEXX®) test kit.

2.3 Sample size calculation

To arrive at the estimated sample size and determine true prevalence of CVBPs in Nigeria, the online server https://www.openepi.com/SampleSize/SSPropor.htm was used to calculate sample size at 95% confidence level for random sample collection. Two parameters were required to calculate estimated sample size for this study including current Nigerian domestic dog population size and anticipated or accepted prevalence based on previous studies. However, there are no current data on Nigerian domestic dog population apart from the study by Bourn et al. (1994) which estimated 4.5 million dogs in Nigeria. Therefore, we accepted the default option to use one

million as the targeted sample size for this study. In addition, review of previous studies of the prevalence CVBPs in Nigeria using molecular methods showed the highest prevalence of 38% for species of piroplasms (*B. rossi*) (Adamu et al., 2014), 46% for *Ehrlichia* spp. and 41.4% for *Hepatozoon* spp. (Happi et al., 2018). Moreover, taking an arithmetic mean (sum of prevalence for piroplasm, *Ehrlichia*, and *Hepatozoon* spp. divided by three) of all the three highest prevalence mentioned above arrived at an anticipated or accepted CVBPs mean prevalence of 41.8%. Overall, the total sample size was estimated to be 374 dogs (Table 2.1). Sample size calculation in this study was approximated at a 90% confidence interval (Table 2.1). Refer to the flow chart diagram (Figure 2.1) for sample collection, shipment, and laboratory work in Nigeria and the UK.

Table 2.1: showing confidence interval and sample size estimated by taking an arithmetic mean of published prevalence on CVBPs in Nigeria.

Sample Size for Frequency in a Population	
Population size (for finite population correction factor or fpc) (*N*):	1000000
Hypothesized % frequency of outcome factor in the population (*p*):	41.8%+/-5
Confidence limits as % of 100(absolute +/- %) (*d*):	5%
Design effect (for cluster surveys-*DEFF*):	1
Sample Size(*n*) for Various Confidence Levels	
Confidence Level (%)	**Sample Size**
95%	374
80%	160
90%	264
97%	459
99%	646
99.9%	1053
99.99%	1472
Equation: Sample size n = [DEFF*Np(1-p)]/ [$(d^2/Z^2_{1-\alpha/2}$*(N-1)+p*(1-p)]	

Results from OpenEpi, Version 3, open-source calculator—SSPropor.

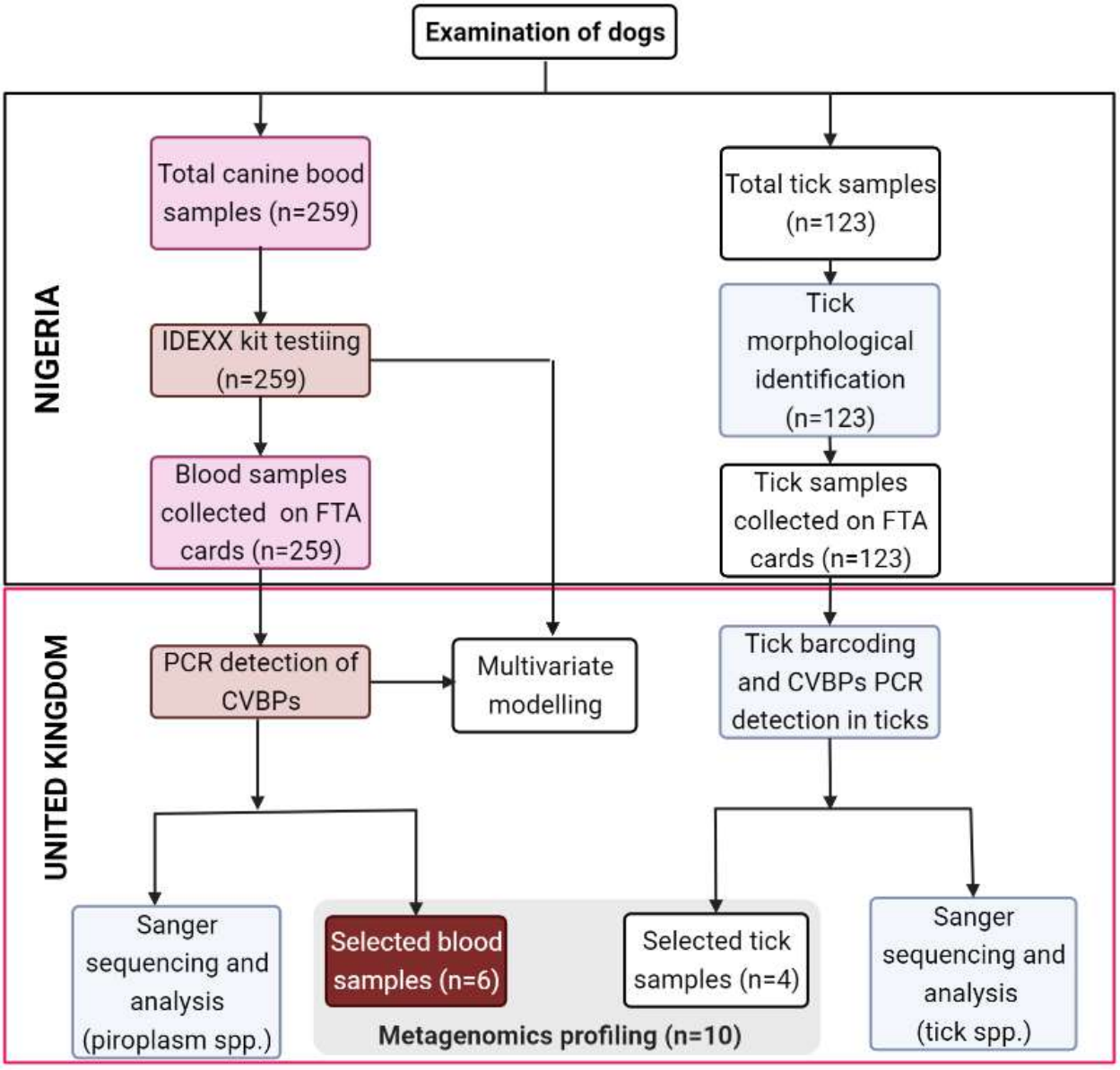


Figure 2.1: A flow chart showing sample collection, preservation, shipment, field and laboratory processing in Nigeria and the United Kingdom.

2.4 Study area, blood sample and tick sample collection

Between June 2018 and November 2019, dogs from four (4) locations in Nigeria cutting across three geopolitical zones were examined by participating veterinarians, 259 canine blood and 111 tick samples were collected from dogs examined. These locations included the federal capital territory (Abuja), Makurdi Benue state (North central), Zaria, Kaduna state (North west), and Ibadan, Oyo state (South west). Sterile vacutainer tubes containing EDTA, needles and syringes provided to participating clinicians were used to collect 2 ml of blood from dogs presented at veterinary clinics that participated in this study, via the cephalic vein following standard veterinary practice procedures. A total of 259 canine blood samples were placed onto Whatman's FTA cards, allowed to air dry at room temperature and placed in plastic bags containing silica gel desiccants. In addition, a minimum of four ticks were collected from a total of 111/259 dogs examined to have tick infestation. Tick samples collected from each dog were temporarily stored in 70% ethanol and then morphologically identified using a standard stereomicroscope as previously described by Walker (2003). Morphological identification, preservation and shipment of tick samples assessed in this study was kindly provided by Dr. Philip Oke, a lecturer/researcher in veterinary entomology at the University of Agriculture Makurdi, Nigeria. Tick samples were ground using a porcelain mortar and pestle, then placed onto Whatman's FTA cards to dry at room temperature and packed in plastic zip bags containing silicon desiccants. Both blood and tick samples collected were transported to the University of Nottingham veterinary laboratories, UK for hard tick molecular barcoding and PCR detection of CVBPs.

In addition, one of the participating veterinarians collected hard tick samples from domestic birds (*Gallus domesticus*) during an ambulatory visit for treatment and management of dogs infested with ticks. Both domestic birds and a dog living within the same household were identified to be infested with hard ticks. Hard ticks rarely infest birds, so these samples were added to the number of ticks collected and have been assessed in this study.

2.5 IDEXX antigen kit testing on canine blood

Positivity for a range of canine tick-borne pathogens from blood was tested using a commercial point of care (POC) test kit (SNAP® 4Dx® test kit by IDEXX Laboratories, Inc Westbrook, ME, USA), according to the manufacturer's instructions. POC test services were kindly provided by participating veterinarians who received prior training at the beginning of this study. This device tests for antibodies against *B. burgdorferi* (s.l.) membrane protein *VIsE* and *Ehrlichia canis* $p^{30}/p^{30\text{-}1}$ outer membrane protein. For this reason, the device can only detect antibodies to *B. burgdorferi* and *E. canis, E. chaffeensis* and *E. ewingii* antibodies derived from *p28* outer surface protein (Stillman et al., 2014). It also tests for antibodies to the *A. phagocytophilum*/*A. platys* major surface protein p^{44}/MSP^2, detecting specific antibodies to these two pathogens (Pantchev et al., 2015). The fourth pathogen tested for was *Dirofilaria immitis* (Stillman et al., 2014).

2.6 DNA extraction, measurement of concentration and quality

Total DNA was isolated from canine tick and blood samples as well as domestic bird tick samples using the Macherey Nagel Nucleospin tissue kit. While the "support protocol for purification of genomic DNA from insects" was used for DNA extraction from tick samples, the "support protocol for genomic and viral DNA from blood samples" was applied to the blood samples. For both tick and blood samples, the pre-lyse sample step of the protocol was modified by the addition of 5 mm stainless Steel beater beads, (QIAGEN® Ltd UK) into each tube containing samples and incubation in a heat block for 3 hours as recommended. The incubated tubes containing beater beads were vortexed after every 15 — 30 minutes to ensure effective lysis, homogenization and high DNA yield before following the rest of the manufacturer's instructions. The quality and concentration of DNA extracted were measured using a Nanodrop 8000 spectrophotometer (Thermo Scientific®) prior to PCR.

2.7 Hard tick identification using polymerase chain reaction (PCR), Gel electrophoresis, DNA purification and Sanger sequencing

Hard ticks were identified using published generic *16S rRNA* gene oligonucleotide primers including 16S+1 and 16S-1, designed to amplify 460 bp of the *16S rRNA* gene from all members of the family *Ixodidae* (Black and Piesman, 1994). For each sample, a 50µl PCR master mix reaction was prepared consisting of the final concentrations of 1X standard Taq (Magnesium-free) reaction buffer (BioLabs®), 200µM dNTP (BioLabs®), 1.5 mM magnesium chloride, 0.2 µM of both forward and reverse primers, 34.5µl of RNase-free water (Sigma-Aldrich, UK), 1.25 units of taq DNA polymerase (BioLabs®), and 5µl of DNA templates. Thermal cycling (Bio-Rad) conditions consisted of 35 cycles of pre/amplification including initial denaturation at 92°C for 1 minute, annealing at 54°C for 30 seconds and extension at 72°C for 90 seconds, followed a final extension at 72°C for 5 minutes (Table 2.2). The positive DNA control (ID: *R. sanguineus* 259) used in this study was kindly donated by Dr Lesley Bell-Sakyi (The Tick Cell Biobank research group, University of Liverpool UK).

2.8 Detection of CVBPs from canine blood and tick samples using PCR

2.8.1 Piroplasm, *Ehrlichia*, *Borrelia*, and *Dirofilaria* species PCR assay

Two published generic Piroplasm qPCR primer pairs were used to assess the prevalence of piroplasms from tick and canine blood samples at the beginning of this study. The first pair of generic Piroplasm *18S rRNA* gene oligonucleotide qPCR primers Piro-A and Piro-B (Table 2.2) were used to amplify 400 bp of the Piroplasm *18S rRNA* gene, following a published protocol (de Marco et al., 2017, Armstrong et al., 1998). While the second pair of generic *Babesia* qPCR primers, Blsu-F and Blsu-R (Table 2.2) amplified 150 bp of the *large subunit ribosomal* DNA gene (*Blsu*) from members of the genus *Babesia sp.* apart from *B. vulpes* n. sp (Qurollo et al., 2017). However, the use of the *Babesia* Blsu-F and Blsu-R primers was discontinued after confirming there was no difference in the number of samples detected from both protocols.

Consequently, the Piroplasm *18S rRNA* gene primers were selected for detection of piroplasms in both tick and canine blood samples. These are generic primers capable of amplifying *18S rRNA* gene from all piroplasms infecting domestic animals. They also offered longer nucleotide sequences (400 bp) following Sanger sequencing.

Two pairs of published PCR primers were used for *Ehrlichia* spp. from tick and canine blood samples including generic *Ehrlichia* GE2F and EHRL3-IP2 primers (Table 2.2) which amplified 100 bp of the *Ehrlichia 16S rRNA* gene in accordance with the published protocol (Breitschwerdt et al., 1998). The *Ehrlichia 16S rRNA* genes were previously published endpoint PCR primers designed to amplify both *Ehrlichia* and *Anaplasma* species and were chosen based on these criteria. While the second pair of primers Ecp30-f and Ecp30-r were published qPCR primers designed to amply 80 bp of the *E. canis p30* gene (Ionita et al., 2013). Both protocols were used to screen tick and canine blood samples collected in this study (Table 2.2).

Generic *Borrelia spp.* qPCR oligonucleotide primers Bor16S3F and Bor16S3R were used to amplify 148 bp of *Borrelia 16S RNA* encoding gene from tick and canine blood samples as previously published (Parola et al., 2011) (Table 2.2).

In addition, a pair of published filariid-specific qPCR primers were used to amplify a 90 bp fragment of filarial species mitochondrial *16S rRNA* gene (Kronefeld et al., 2014), qPCR amplification of filarial species was only carried out on Nigerian canine blood samples to detect the presence of *Dirofilaria* species DNA following IDEXX antigen test (Table 2.2).

For qPCR detection of piroplasm, *Ehrlichia*, *Borrelia* and filarial species, a total volume of 20µl qPCR reaction mix was prepared consisting of 10 µl 2x qPCRBIO SyGreen Mix Lo-ROX (PCR Biosystems, UK), 0.8 µl of both forward and reverse 10µM primers, 3.4 µl of RNase free water (Sigma-Aldrich, UK), and 5 µl of template DNA. All qPCR thermal cycling for pathogens identified were carried out using a Bio-Rad CFX real-time qPCR machine, while results were viewed using the Bio-Rad CFX Maestro software. All qPCR protocols were optimized following temperature gradient qPCR to

identify optimal annealing temperature, standard curve determination to obtained PCR efficiency (95% for all assays), linearity (R^2) and quantification cycle (cq). Following separate gradient and qPCR standard curve optimization for *Piroplasm*, *Ehrlichia*, *Borrelia*, and *Dirofilaria* species using the Bio-Rad CFX real-time qPCR machine, it was observed that the four different PCR protocols were efficient under the same thermal cycling conditions with an annealing temperature of 60°C for 45 s. Both gradient/qPCR standard curve optimization were carried out separately for each pathogen, following critical evaluation and observation that the four thermal cycling conditions were the same, a decision to use a single qPCR thermal cycling condition for the amplification of *Piroplasm*, *Ehrlichia*, *Borrelia*, and *Dirofilaria* species (Table 2.2) was arrived at. All CVBP qPCR thermal cycling conditions consisted of initial denaturation at 98°C for 3 min, followed by 40 cycles of 98°C for 15 s and 60°C for 45 s. qPCR reactions for all pathogens were prepared in triplicates, the presence of an exponential amplification curve distinct from no template control (NTC) was used as a criterion to identify positive samples following each pathogen qPCR screening (Table 2.2).

Pathogen DNA controls used in this study were donated free of charge by different researchers including *Babesia* species (Dr Chris Helps team: Molecular Diagnostic Unit, Langford Vets Bristol UK, and Dr Nicholas Johnson team: APHA, UK), *E. canis* (Dr Lesley Bell-Sakyi and team: The Tick Cell Biobank, University of Liverpool UK), *B. burgdorferi* (s.l.) (Dr Daniel Carter: PHE, UK), and *D. immitis* (Dr Silvana Belo: Global Health and Tropical Medicine University of Lisbon, Portugal).

Table 2.2: Summary of PCR protocols, primers, product size, and references for protocols used during PCR identification of hard ticks and screening of both tick and canine blood samples.

PCR assay			**Thermal cycling conditions (temperature, time, and cycles)**					
	Primers	Size (bp)	Denaturation	Annealing	Extension	Final extension	cycles	References
	Conventional PCR assay							
Hard ticks	16S+1 (5'-CTGCTCAATGATTTTTTAAATTGCTGTGG-3') 16S-1 (5'-CCGGTCTGAACTCAGATCAAGT-3')	460	92°C 1min	54°C 30s	72°C 90s	72°C 5 min	35	(Black and Piesman, 1994)
Ehrlichia	GE2F (5'-GTTAGTGGCATACGGGTGAAT-3') EHRL3-IP2 (5'-TCATCTAATAGCGATAAATC-3')	100	94°C 45s	50°C 30s	72°C 90s	72°C 90s	35	(Breitschwerdt et al., 1998)
	qPCR assay							
Ehrlichia	Ecp30-f (5'-TGGATACTACCATGGCGTTATTGG-3') Ecp30-r (GAGGAGCATCATTTAATACTACAGGAGTT-3')	80	[a]98°C 3min [b]98°C 15s	60°C 45s	[c]55-92°C @0.01s intervals	N/A	40	(Ionita et al., 2013)
Piroplasm	Piro-A (5'-AATACCCAATCCTGACACAGGG-3') Piro-B (5'-TTAAATACGAATGCCCCCAAC-3')	408 – 430	"	"	"	N/A	"	(Armstrong et al., 1998, de Marco et al., 2017)
	Blsu-F- (5'ACCTGTCAAGTTCCTTCACTAAATT-3') Blsu-R 5'-TCTTAACCCAACTCACGTACCA-3')	150	"	"	"	N/A	"	(Qurollo et al., 2017)
Borrelia	Bor16S3F (5'-AGCCTTTAAAGCTTCGCTTGTAG-3') Bor16S3R (5'-GCCTCCCGTAGGAGTCTGG-3')	148	"	"	"	N/A	"	(Parola et al., 2011)

PCR assay			**Thermal cycling conditions (temperature, time, and cycles)**					
	Primers	Size (bp)	Denaturation	Annealing	Extension	Final extension	cycles	References
Dirofilaria	PanFilaF (5'-TGTGCTGCGCTACATCGATG-3') PanFilaR (5'-AAACCGCTCTGTCTCACGAC-3').	90bp	"	"	"	N/A	"	(Kronefeld et al., 2014)
Note: [a]&[b] (initial denaturation and denaturation temperature and time); [c] (melting temperature); N/A (not applicable).								

2.9 Agarose gel electrophoresis and DNA purification of PCR products

For both tick and CVBP (generic *Ehrlichia* and *piroplasms*) PCR products, 1% agarose TAE gel (prepared from tris base, acetic acid, and ethylene diamine tetra acetic acid (EDTA) buffer and agarose powder (Sigma-Aldrich, UK)) with 4 µl of Nancy 520 DNA gel stain (Sigma-Aldrich) was prepared and electrophoresed in a mini gel tank (BIO-RAD®) following the manufacturer's instructions. A 100 bp DNA ladder (BioLabs®) was used to confirm the size of all PCR products following the addition of 5µl (mixed with gel dye) of each PCR positive product recovered. All agarose gel images were visualized and captured on an Imagequant 300 UV camera (DOTmed.com Inc. New York).

PCR products recovered from conventional PCR protocols including tick barcoding and the generic *Ehrlichia 16S rRNA* gene positive PCR products were loaded directly onto 1% agarose gel. However, triplicate wells containing positive qPCR products recovered from Piroplasm *18S rRNA* gene and *Ehrlichia p30* gene qPCR were pooled together and purified (for work convenience) before gel electrophoresis. While tick barcoding positive PCR products recovered were electrophoresed at 100 volts for 60 minutes to view product size of 450 bp, generic *Ehrlichia* conventional PCR products and qPCR products recovered from piroplasm *18S rRNA* gene and *Ehrlichia p30* gene with shorter products were electrophoresed at 80 volts for 60 minutes.

Positive PCR products recovered in this study were purified using the Nucleospin® extract II kit (Macherey-Nagel) "protocol for gel and PCR clean-up". While tick barcoding PCR products were Sanger sequenced using tick *16S rRNA* gene primers (16S+1 and 16S-1), positive (*Babesia* and *Ehrlichia*) PCR products were Sanger sequenced using piroplasms (Piro-A and Piro-B) and *E. canis p30* (Ecp30-f and Ecp30-r) gene primers (Table 2.2). All samples were Sanger sequenced using big dye Terminator v3.1 (Applied Biosystems) by Source Bioscience with data obtained in ABI format.

2.10 DNA Sanger sequence editing, alignment, and Phylogenetic analysis

Following Sanger sequencing, pairwise alignment of all the corresponding forward and reverse nucleotide sequences were carried out to generate consensus nucleotide sequences for each positive sample using Geneious Prime® 2019 v11.0.3+7 (Kearse et al., 2012). The online NCBI nucleotide blast algorithm was searched to confirm the presence of tick (*Rhipicephalus*, *Haemaphysalis*) and CVBPs (*Babesia* and *Ehrlichia*) nucleotides from the consensus sequence generated (Altschul et al., 1990) and compared with published literature on taxonomic classification of ticks (Black and Piesman, 1994, Gray et al., 2013, Barker and Murrell, 2004, Kamani, 2021b, Thompson et al., 2020) and piroplasm (Lack et al., 2012, de Marco et al., 2017). For the construction of both tick (*Rhipicephalus*, *Haemaphysalis*) and *Babesia* species phylogenetic analysis, reference sequences identified were downloaded, assessed for quality, aligned, and trimmed to obtained similar length with corresponding consensus tick and *Babesia* species sequence generated in this study. Maximum likelihood statistical method was selected to calculate the best-fitting nucleic acid substitution models in MEGA 11. Models with the lowest Bayesian information criterion (BIC) scores were selected for the construction of maximum likelihood phylogenetics analysis. Thus, the Tamura-3-parameters (T92) model was selected as the best-fitting substitution model for hard tick phylogenetics (Tamura, 1992), while the Jukes-Cantor (JC) (Jukes and Cantor, 1969) model was selected for Piroplasm nucleotide sequences phylogenetics. Phylogenetic trees were drawn in MEGA 11 software setting the bootstrap values at 1000 replicates (Tamura et al., 2021). Trees drawn using tick nucleotides were rooted to a common house spider (*Parasteatoda tepidariorum,* accession: JN816454), a closely related member of the class Arachnida, while the tree drawn using piroplasm nucleotides was rooted to the genus *Plasmodium vivax*, a closely related member of the phylum Apicomplexa. Initial unrooted trees (not presented here) constructed for both tick and piroplasm species suggested the

common house spider and *Plasmodium vivax* respectively, as the most divergent members of these two groups. The use of house spider and *Plasmodium vivax* as outgroups provided a clear identification of tree roots and the evolutionary pathway.

In addition, phylogenetic trees were constructed using nucleotide rather than amino acid sequences. The use of DNA sequence is common in molecular phylogenetics on ticks and canine vector pathogens. DNA sequence generate more phylogenetic information than amino acid sequences recovered from equivalent proteins. Certain DNA silent or replacement (synonymous) mutations alter DNA sequence without additional alterations in the amino acid sequence. Efficient and reliable application of PCR assays for DNA sample preparation, sequencing and analysis is more likely to provide new and unique evidence following assessment of variable coding and non-coding regions of a genome than amino acid sequence (Brown, 2002).

2.11 Descriptive statistics and multivariate modelling

A Microsoft Excel spreadsheet was used to record data generated in this study, codes were assigned to defined variables and then imported into IBM SPSS statistical software v.26.0 for statistical analysis. Descriptive statistics using cross-tabulation were selected to summarize the frequency of specific CVBPs for the exposure factors recorded, followed by univariate analysis using Chi-square test to assess the association between CVBPs infection in IDEXX antigen test and PCR positive animals and suspected risk factors including age, sex, breed, body weight, season of the year and presence of ticks. The main aim of this analysis was to assess risk factors associated with CVBP prevalence using results from canine blood IDEXX antigen and PCR test, and tick infestation. Two models, one for risk factors associated with CVBP prevalence based on POC test result and one for risk of canine tick infestation were initially assessed (Chapter 3). Similarly, a third model to assess the risk of CVBP prevalence based on PCR test result was also assessed (Chapter 5).

In both cases, a top-down (backward) multivariate modelling strategy where all the predictors were added to first assess which exposure variables were significant or co-founders was then applied. This was followed by a backward stepwise elimination, where only predictors that were significant and/or found to be co-founders ($P< 0.05$ and/or adjusted regression coefficient (R2) of >10%) were retained in the final model. In addition, Cohen's kappa index (κ) was estimated at 95% confidence interval (using IBM SPSS statistical software v.26.0) to assess the agreement between the POC and PCR-based diagnostic test methods as proposed by Landis & Koch. The Cohen's kappa procedure in SPSS was calculated by selecting the analyze menu to set up a crosstabulation, addition of both test results to complete either the row or column, selection of kappa test under statistics, and observed counts under cell display to generate crosstabulation and symmetric measure tables (Kirkwood and Sterne, 2010). The following criteria was used to assess Kappa values to establish agreement between tests used: poor ($\kappa < 0$); light ($\kappa = 0–0.20$); fair ($\kappa = 0.21–0.40$); moderate ($\kappa = 0.41–0.60$); substantial ($\kappa = 0.61–0.80$); and excellent ($\kappa = 0.81–1.00$).

2.12 Methods: Metagenomics profiling of hard ticks and canine blood

2.12.1 Sample preparation, Library Construction, and Illumina Sequencing

A total of 10 DNA samples including 6 dog blood and 4 tick samples initially used for hard tick barcoding and CVBP PCR were selected for NGS, DNA concentration was measured using Qubit 2.0 as described by Breitwieser and Salzberg (2020). Novogene UK LTD performed DNA library preparation and sequencing as follows: The genomic DNA obtained were subjected to ultrasonic vibration (sonication) to obtain random fragments that were end polished (A-tailed) and ligated with Illumina sequencing full-length adaptors. The ligated fragments were further subjected to PCR amplification using P5, while all fragments were indexed with P7 oligos. Purification of PCR products for the final construction of libraries were carried out using AMPure XP system. Distribution of library sizes was confirmed using Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA), and quantified by real-time PCR. Finally, libraries were added to Illumina sequencer using the required concentration and volume and sequencing done in line with the manufacturer's instructions. The Illumina (NovaSeq 6000 PE150 system) sequencing platform was used to generate paired end reads of 150 base pairs.

2.12.2 Data Analysis

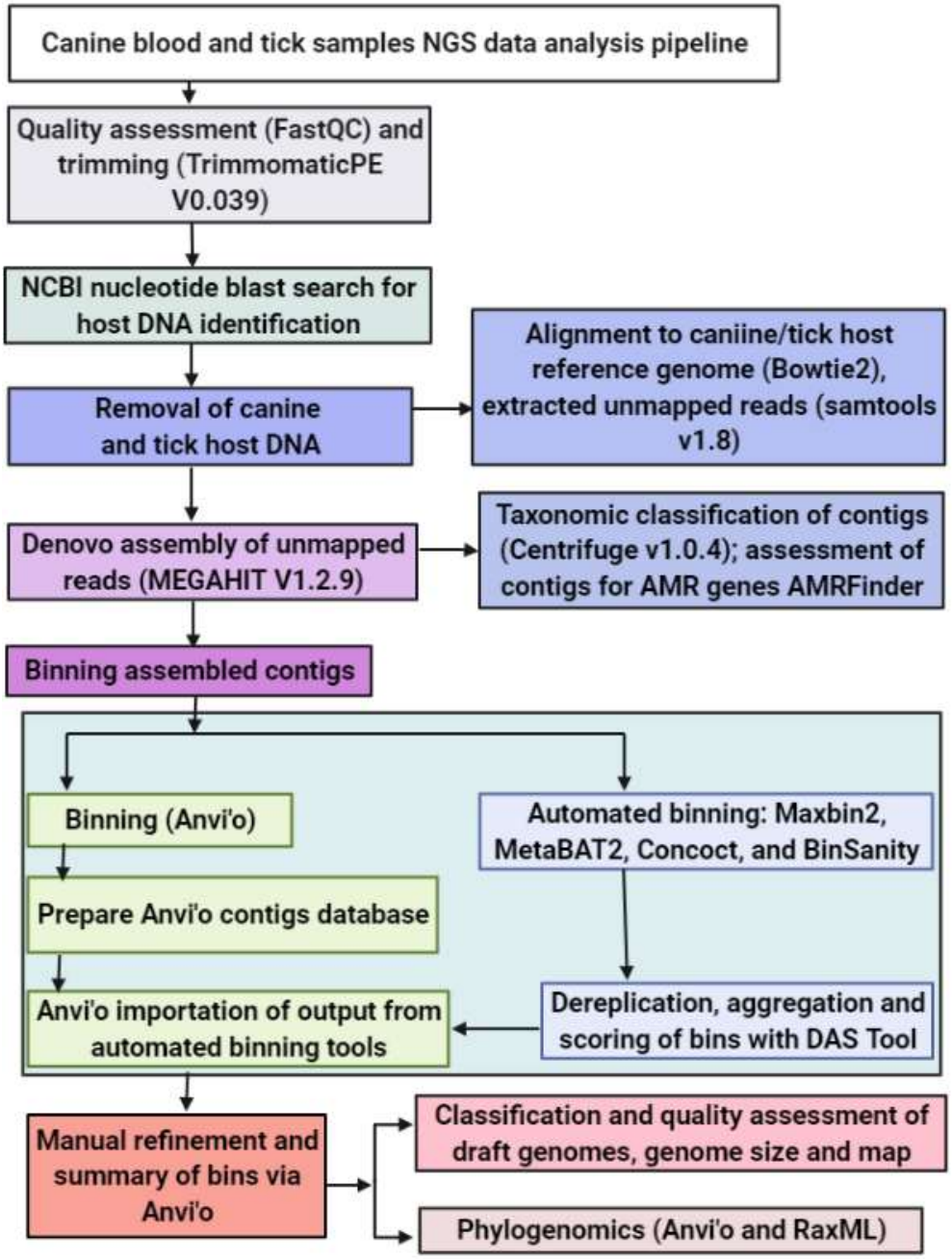


Figure 2.2: A flowchart showing pipeline use to analyze datasets recovered from dog blood and tick samples following NGS, full details regarding the use of these algorithms can be found under appropriate subheadings below.

2.12.3 Sequence quality assessment, removal of canine and tick host DNA

Assessment of sequence quality was carried out using FastQC (Andrews, 210) before and after quality trimming with TrimmomaticPE (Bolger et al., 2014). FastQC reports obtained were summarized into a single MultiQC quality assessment report using MultiQC (Ewels et al., 2016). A summary of raw, trimmed and percentage of reads following quality filtering and trimming is presented in appendix (Table 8.2).

All reads obtained from dog and tick datasets were used as query sequences to search against NCBI blast nucleotide database downloaded using NCBI blast download scripts. GenBank Accession numbers with the highest alignment score, percentage identity, and lowest expected number of chance alignments (E-value) from blast search output were considered as potential host (Camacho et al., 2009).

To remove host reads, paired end reads obtained from each sample were first aligned to either dog or tick DNA depending on sample source using Bowtie2 sequence aligner (Langmead and Salzberg, 2012). NCBI canine genome DNA prebuilt bowtie2 index obtained from igenome browser server (igenome), was used to align Illumina sequence reads obtained from canine blood datasets. Three distinct hard ticks reference genomes belonging to *Haemaphysalis longicornis* (CM023448), *Ixodes scapularis* (NW_021917572), and *Rhipicephalus sanguineus* (NC_051176) were downloaded using the NCBI genome download script. Downloaded reference genomes were concatenated into a single FASTA file in Linux terminal and used to build a bowtie2 index. All hard tick paired end reads were then aligned to the custom-built bowtie2 index. Bowtie alignment yielded SAM files that were first converted to BAM files using samtools view and sort options. Mapped (canine or tick host) and unmapped (non-host) reads were extracted from canine blood and tick dataset BAM files alignments using samtools flag options.

2.12.4 Denovo assembly of unmapped reads, quality assessment of assembly

Unmapped paired end reads extracted following bowtie2 alignment were assembled into contigs using megahit v1.2.9 (Li et al., 2015). Megahit provides an option to assemble multiple paired end reads obtained from one sample to form a single assembly, command line options were also set to retain contigs >1000 bp to ensure efficient memory usage, this automatically established a threshold for taxonomic assignment of assembled contigs using the centrifuge classifier. Quality assessment and statistical data (Appendix 2, Table 8.3) on contigs generated was carried out in command line using Bbmap tools (Wu et al., 2016), and GAAS scripts (Angly et al., 2009).

2.12.5 Taxonomic classification of assembly reads using centrifuge classifier

Centrifuge classifier was used to build a centrifuge custom database to classify the assembled contigs. Bacterial, archaeal, viral, fungi, protozoal, and selected helminths including *Dirofilaria immitis* and *Dirofilaria repens* reference sequences, alongside their taxonomic names and nodes were downloaded from the NCBI database using the centrifuge-download command line script, to create a custom database of about 95 GB in size. Contigs obtained from both canine blood and tick sample datasets were passed as input FASTA files to the centrifuge classifier via command line, generating centrifuge reports and hits output. All centrifuge output files were converted to a Kraken-style report using centrifuge-kreport script (Kim et al., 2016). Individual reports were visualized and compared in R using Pavian (interactive analysis of metagenomics data for microbiomics and pathogen identification) to generate Sankey view and heat map reports (Breitwieser and Salzberg, 2020). Kraken-style reports obtained from all reads were merged into one single report using Kraken tools (Kim et al., 2016).

2.12.6 Binning metagenomics data using Maxbin2, Metabat2, Concoct, Binsanity, DAS_Tool, and Anvio-7

2.12.6.1.1 Merging of assembly contigs, removal of sequence duplicates.

To ensure an efficient result via Anvi'o binning protocol, all contigs obtained from denovo assembly of canine blood and tick sample datasets were merged into single contigs representing canine blood and tick sample datasets before commencing the binning process. The concatenate.py script from variational autoencoders for metagenomics binning (VAMB) tool is an effective and useful script for concatenating contigs from multiple samples (Nissen et al., 2021). This made the binning process more efficient and less time consuming as one do not have to go through individual contig assemblies. Sequence read duplicates were removed using Bbmap tool script dedupe.sh (Bushnell et al., 2017). To ensure contigs naming consistency from bins obtained using other external binning tools and Anvi'o, the reformatted contig files generated to create Anvi'o contigs database (from both canine and tick samples) were also used during binning with external binning tools.

2.12.6.1.2 Binning

Four original genome binning tools (Table 2.3) including MaxBin2 (Wu et al., 2016), MetaBat2 (Kang et al., 2019), Concoct (Alneberg et al., 2014), BinSanity (Graham et al., 2017) and two refining binning tools DAS_Tool (Sieber et al., 2018), and Anvi'o were used to bin contigs generated from canine and tick dataset assemblies (Table 2.3). DAS_Tool and Anvi'o algorithms are genome binning tools that can combine bins generated from different metagenomics external binning tools. The use of different binning tools was to compare performance and output of different binning tools, ensuring reliable and accurate putative genomes are generated.

Table 2.3: External binning and refinement tools used to bin canine and tick datasets prior to Anvi'o refinement (adapted from Yue et al. (2020)).

Genome binning tool	**Consideration**	**Model used**	**binned canine datasets (number of bins)**	**Binned tick datasets (number of bins)**
MaxBin2	k-mer frequencies, coverage single copy genes (SCG)	Uses expectation-maximization model to evaluate the number of bins using SCG,	No (error message)	Yes (25)
MetaBat2	4-mer frequencies, coverage	Uses modified k-medoids to cluster contigs into individual bins.	Yes (7)	Yes (28)
Concoct	k-mer frequencies, coverage	Uses Bayesian method	Yes (85)	Yes (81)
BinSanity	Coverage, tetranucleotide frequencies, percentage GC content	Uses Affinity propagation model	Yes (25)	Yes (95)
DAS_Tool	Utilizes output from external binning tools	Uses contigs names common between original binning tools to refine original bins	No (error message)	Yes (30)

2.12.6.1.3 Binning with an advanced analysis and visualization platform for ‘omics data (Anvi’o)

2.12.6.1.3.1 Preparation of Anvi’o contigs database (db)

The Anvi’o pipeline for metagenomics data analysis published by Eren et al. (2015) was followed throughout this analysis. Before creating the contigs db, contig FASTA files were reformatted, and names simplified using Anvi’o script (anvi-script-reformat-fasta) setting the minimum contig length to 3000 bp. A command line option to generate a report file was also selected to keep track of changes in both reformatted and unformatted files. To obtain external gene calls and annotation function files for creation of Anvi’o contigs db, reformatted contigs were annotated in command line using Prokka (Seemann, 2014). Prokka generated GFF files which served as input into a python script called gff_parser.py. For clarity, gff_parser.py is a published script that is useful in creating genome gene calls and annotation functions text files from the original Prokka annotation GFF files. Open reading frames (ORF) were identified using Prodigal (Hyatt et al., 2010). Following these steps, an Anvi’o contigs db was created using anvi-gen-contigs-database script. Annotation functions were added to the newly created contigs db using anvi-import-functions script, anvi-run-ncbi-cogs script was also executed to ensure additional annotation of genes using functions from the NCBI clusters of orthologous genes (COGs) database (Galperin et al., 2015). Finally, contig db statistics were generated by executing the anvi-display-contigs-stats script. Additional steps in creating the Anvi’o contigs db include anvi-run-hmms to decorate contigs db with hits from hidden Markov models (hmms) which consists of the published bacterial single-copy gene (SCG) collection, the script uses HMMER to search and identify SCG hits from the contigs db. The script anvi-import-taxonomy-for-genes was also used to add classified gene taxonomy to the contigs db, the addition of gene taxonomy to contigs db becomes helpful during interactive interface, manual binning, and refinement of metagenomics data. Although, before importing gene taxonomy,

gene sequences were exported from the contigs db, classified using centrifuge classifier v1.04, then we imported centrifuge output back into Anvi'o contigs db using anvi-import-taxonomy-for-genes script. Exporting gene sequences from contigs db for classification ensures that identification of the gene sequences classified matches with the contigs db created during our analysis.

Finally, the anvi-run-scg-taxonomy script was executed to identify all bacterial single copy genes (SCG) available, then estimated using anvi-estimate-scg-taxonomy. The anvi-estimate-taxonomy script utilizes SCG sequences and genome taxonomic database (GTDB) which consist of over 258, 000 bacterial genomes as of April 2021 (Chaumeil et al., 2019).

2.12.6.1.3.2 Preparation, profiling of individual sample BAM files and merging of individual sample profiles

To ensure that all samples were mapped to contigs with the same naming patterns, a bowtie2 index was created using the reformatted contig fasta file used in building the Anvi'o contigs db. All extracted and unmapped reads used in creating individual sample assemblies were mapped back to the bowtie2 index. The option "–no-unal" was selected in terminal to suppress SAM records for unaligned reads thus, the resulting output files contained only sequences aligned to the bowtie2 index. This yielded individual SAM files (six and four for canine blood and tick sample datasets, respectively) which were sorted and indexed using samtools v1.8.

All individual BAM files were profiled to yield Anvi'o single profile databases. For clarity, the individual sample profile db contains information that is related to the contigs in the contigs db. All profile databases were finally merged into one profile db using anvi-merge script. To be clear, analysis on canine and tick sample datasets were carried out separately. Therefore, anvi-profile databases belonging to six dog blood samples were merged into one profile db, while profile databases from four tick sample datasets were merged into a separate profile database. During anvi-merge, the flag "enforce hierarchical-clustering" was

selected to cluster contigs to ensure successful binning and visualization of clusters during manual binning.

2.12.6.1.3.3 Importing results from external binning tools into Anvi'o, manual refinement, and summary of bins using anvi-interactive

During creation of the Anvi'o environment and installation in the terminal, software packages from external binning tools including MaxBin2, MetaBAT2, BinSanity, Concoct, and DAS_Tool were also installed within the same environment; therefore, it was easier to merge results from these packages. The anvi-cluster-contigs script was executed to cluster contigs saved on the previously merged profile database using the results from external binning tools. However, the external binning tool results refined by DAS_Tool were clustered. The anvi-cluster-contigs script consumes a merged profile database, contigs database, and external binning tool results with a flag called 'driver' indicating the binning algorithm used. After addition of external binning tool results, the script anvi-summarize was executed using a specific collection name (DAS_Tool), to summarize bins created by DAS_Tool before proceeding with the refinement step (Eren et al., 2015).

2.12.6.1.3.4 Manual refinement of putative bins using Anvi'o interactive

Overall, only bins with ≥ 50% completeness and ≤ 10% redundancy were considered for manual refinement via Anvi'o interactive. To select bins that met these criteria, the hypertext markup language (html) file obtained following anv-summarize script on DAS_Tool collection mentioned above (Figure 6.11) was reviewed. Bins belonging to a collection clustered by Anvi'o server were then manually refined by selecting splits or branches from the Anvi'o tree into newly created separate bins, which were then saved on the Anvi'o database. Following manual refinement and saving of new bins via the interactive interface, the anvi-summarize command was repeated via the command line to summarize the

newly created bins and generate a new html file format containing the details for all the newly created bins, as mentioned earlier.

2.12.6.1.4 Quality assessment and classification of genomes recovered from the hard tick datasets

None of the contigs obtained from the six canine blood sample datasets examined in this project yielded putative genomes (confirmed by five binning algorithms used). In contrast, the four tick sample datasets used in this analysis yielded putative genomes. The following quality assessment and genome classification was therefore only applied to the genomes obtained from tick sample datasets.

2.12.6.1.4.1 CheckM Quality assessment

CheckM is an automated tool for evaluating genome completeness and contamination; it makes use of bacterial lineage specific marker genes to place genomes within a reference genome tree (Parks et al., 2015). For this project, the lineage specific workflow was used to assess completeness and contamination of genomes obtained from the five external binning tools and Anvi'o refinement. The lineage specific workflow command consisted of four important steps including placement of putative genomes into a reference genome tree, identification of phylogenetic marker genes from bins, generation of marker gene files for assessment of putative genomes and identification of marker genes to evaluate genome completeness and contamination. A summary containing details on the quality of genomes assessed (Table 6.5).

2.12.6.1.4.2 Classification of genomes using Genome Taxonomic Database Toolkit (GTDB-TK)

GTDB-Tk is an automated and unbiased genome database tool that assigns precise taxonomy classification to multiple archaeal and bacteria genomes. Classification is achieved by passing genomes into Prodigal for gene calls

targeting 120 bacterial and 122 archaeal marker genes. Identified proteins are concatenated, aligned, and trimmed via the execution of a program called HMMER. Placement of genomes into bacterial and archaeal domain specific reference tree is accomplished using pplacer (linear time maximum-likelihood and Bayesian phylogenetic placement of sequences onto a fixed reference tree). In summary, GTDB-Tk make use of reference tree genome placement, average nucleotide identity (ANI, >65% for species assignment) alignment with the reference genome, and relative evolutionary divergence (RED) to successfully classify genomes. In addition, genomes with known species are usually assigned an ANI radius of 95%, while species not known or new appear to have no ANI radius assignment (Chaumeil et al., 2019).

2.12.6.1.5 Construction of genome maps from draft genomes

Results of CheckM genome quality assessment and GTDB-Tk classification were merged, and genomes with high quality assessment via CheckM that were also classified using GTDB-Tk were selected as draft genomes for the four tick sample datasets. Overall, 14 genomes were selected from 10 bacterial genera. All genome maps presented in this study were constructed using circular genome viewer software (CGView) (Figure 6.15 and Figure 6.16), an online interactive microbial genome visualization server (Petkau et al., 2010).

2.12.6.1.6 Estimation of average size of genomes obtained from tick sample datasets

To estimate GC content, number of contigs, and average length of genomes, genome relative abundance and average size (GAAS) tool was used in the command line with default parameters (Angly et al., 2009). Bbmap tool script (Bushnell, 2014) was also used for additional information such as depth of coverage, which could not be generated using GAAS.

2.12.6.1.7 Assessment of contigs for antimicrobial resistance (AMR) gene from assembled contigs

All contigs recovered from canine blood and tick sample datasets assemblies were assessed for the presence of AMR genes using a command line tool called AMRFinder which consist of >4500 high quality AMR reference gene protein database. AMRFinder contains AMR genes protein collections obtained from Comprehensive Antimicrobial Resistance Database (CARD) (Feldgarden et al., 2019).

2.12.6.1.8 Construction of phylogenomic trees

A Phylogenomic tree was first constructed in Anvi'o using translated and concatenated proteins from 14 tick sample draft bacterial genomes and 11 NCBI reference genomes, previously identified as reference genomes for these bacteria via CheckM and GTDB-Tk algorithms. Microbial gene proteins were obtained by first generating Anvi'o contigs databases, followed by execution of anvi-run-hmms script to search and annotate genes present in all contigs database using Anvi'o Hidden Markov Models (HMMs). Finally, a tab-delimited list of the all contigs databases generated was prepared and passed as an input into anvi-get-sequences-for-hmm-hits script, specifying options for Anvi'o algorithm to returns best hits, extract and concatenate amino acid sequences from reference and assembled genomes.

By default, Anvi'o concatenates and aligns all protein sequences using MUSCLE multiple sequence aligner for proteins (Edgar, 2004). The script anvi-gen-phylogenomic-tree was then executed to generate a phylogenomic tree (not shown) in Anvi'o using concatenated, aligned, and trimmed protein sequences as input, and viewed via the anvi-interactive server (Eren et al., 2015). Concatenated and aligned proteins also served as a multiple sequence alignment input file as recommended by Anvi'o developers for construction of an alternative maximum likelihood phylogenomic tree at 1000 bootstraps using RAxML (Kozlov et al., 2019). The RAxML phylogenomic tree (Figure 6.18) generated was viewed

and edited using the interactive tree of life server (iTOL) v5 (Letunic and Bork, 2007).

Chapter 3: Prevalence and risk factors associated with CVBPs based on IDEXX test results

3.1 Background

The term canine vector-borne pathogens (CVBPs) signify a group of pathogens that cause disease in dogs and other domestic animals, transmitted by arthropod vectors including ticks and insects. These pathogens include *Ehrlichia, Anaplasma, Babesia, Theileria Hepatozoon, Borrelia, Bartonella, Rickettsia* and *Dirofilaria* species. The potential zoonotic threat presented by CVBPs such as *Borrelia burgdorferi* (s.l.) (Colunga-Salas et al., 2020), *Ehrlichia* (Springer and Johnson, 2018), and *Anaplasma phagocytophilum* (Nelder et al., 2019), are attributed to the presence of canine populations that usually serve as reservoirs of disease transmission to humans (Irwin, 2014, Piantedosi et al., 2017).

Ehrlichia spp. are pleomorphic intracellular obligatory Gram-negative bacterial causing a severe disease in dogs and cats (McDade, 1990), transmitted *Rhipicephalus sanguineus*, and *Dermacentor variabilis* (Mittal et al., 2017, Montenegro et al., 2017, Harrus et al., 1999). In Nigeria, *Ehrlichia* species prevalence ranging between 7 and 22.9% using morphological, serological, and molecular methods has been recorded (Kamani et al., 2013a, Kamani et al., 2013b, Daramola et al., 2018, Adamu et al., 2014).

Anaplasma species including *A. phagocytophilum* are obligate intracytoplasmic bacteria that cause granulocytic anaplamosis, a febrile illness of dogs and humans (Dumler et al., 2001). While *Ixodes* species are the principal vectors of *A. phagocytophilum* (Beugnet and Marié, 2009, Blanco and Oteo, 2002), *R. sanguineus* is the major tick vector for the transmission of *A. platys* (Almazán et al., 2016). Canine infection with *Anaplasma* species is recorded in different parts of the world including Europe (Germany, England) (Kohn et al., 2008, Bexfield et al., 2005, von Loewenich et al., 2003), Africa (South Africa), (Inokuma et al., 2005), the USA (Ewing et al., 1997, Lester et al., 2005), and

Asia/Russia (Kawahara et al., 2006, Rar et al., 2010, Yang et al., 2013). In Nigeria, recent studies have reported *A. platys* from dogs, camels, and cattle using morphological, serological, and molecular methods. However, *A. phagocytophilum* has not been reported (Lorusso et al., 2016b, Lorusso et al., 2016a, Kamani et al., 2013a, Onyiche et al., 2020, Adamu et al., 2014).

Borrelia burgdorferi (s.l.) complex consists of a group of bacteria transmitted by *Ixodes* tick species causing Lyme disease in dogs, humans, domestic and wild animals. globally (Krupka and Straubinger, 2010, Rudenko et al., 2011, Olsén et al., 1995). Blood transfusion (Wardrop et al., 2005). Rare reports of canine infection with *B. burgdorferi* (s.l.) are available in Africa or Nigeria, as well as North African studies reporting high prevalence of *B. burgdorferi* (s.l.) in *Ixodes ricinus* tick samples from Tunisia (Younsi et al., 2001, Zhioua et al., 1999).

D. immitis, is transmitted by mosquitoes (Michalski et al., 2010, Labarthe et al., 1998), it is the principal aetiology of pulmonary dirofilariasis in humans and dogs globally (Pozgain et al., 2014, Venco et al., 2014, Serrano-Parreño et al., 2017, Kamalu, 1991). Previous studies have demonstrated the presence of *D. immitis* (Ugochukwu et al., 2016, Ogbaje and Danjuma, 2016) microfilariae in samples from Nigerian dogs.

A range of factors including changes in lifestyle including human and animal movement, and population growth are established to be responsible for the emergence and widespread distribution of vectors and vector-borne pathogens across the globe (Medlock et al., 2007, de Marco et al., 2017, Duscher et al., 2015). Factors such as co-infections with more than one pathogen are common in both temperate and tropical countries exacerbates clinical disease and complicates diagnosis and treatment.(Otranto et al., 2009, Spolidorio et al., 2011, Santamaria et al., 2014, Aktas et al., 2015, Alho et al., 2017, Attipa et al., 2017). In addition, the increase in pathogen replication, survival within vectors and subsequent transmission to susceptible hosts are established to be

potentiated by climate change (Gray et al., 2009, Beugnet and Chalvet-Monfray, 2013, Moore et al., 2012).

Studies on CVBP prevalence and risk associated factors have been reported from other parts of the world including Europe (Piantedosi et al., 2017, Angelou et al., 2019), and Africa (Selim et al., 2021a). Reports focusing of prevalence of CVBPs have also been documented in Nigeria. However, there are no studies targeting dogs to provide information on both prevalence and risk factors associated with CVBPs in Nigeria. On the contrary, a previous study that assessed changes in seasonality demonstrated higher prevalence of trypanosomiasis in cattle and goats during the dry season rather than the rainy season (as observed in previous years). Although, this study did not target the canine population, it provided information on the effect of climate and increase in the prevalence of trypanosomiasis in Nigeria (Kalu, Oboegbulem and Uzoukwu, 2001; Majekodunmi et al., 2013), justifying the need for a similar study in dogs. The aim of this study is to assess the prevalence of CVBPs and associated risk factors from dogs presented at veterinary facilities across Nigeria using IDEXX antigen test kits.

3.2 Results

3.2.1 Characteristics of the study population

IDEXX tests were performed on a total of 259 dogs including 223 from Makurdi, 14 from Zaria, 12 from Abuja and 10 from Ibadan. The demographic characteristics of the population are summarized in Table 3.2 (see supplementary data file for more details) and included 63 puppies (< 6 months) and 196 young-adult (> 6 months), 154 males and 105 females, 179 exotic breed and 80 indigenous breed dogs. There were also 172 and 87 dogs weighing ≤ 20 kg and ≥ 21 kg respectively, while 181 dogs were examined during the rainy season (April-October) and 78 dogs were examined during the dry season (November-March). Information on whether dogs were

infested with ticks or not was collected for all dogs presented. A total of 153 dogs were infested with ticks, while 106 dogs from the total population had no tick infestation however, tick samples were only collected from 111 out of the 153 tick infested dogs (Figure 3.1) and (Table 3.2).

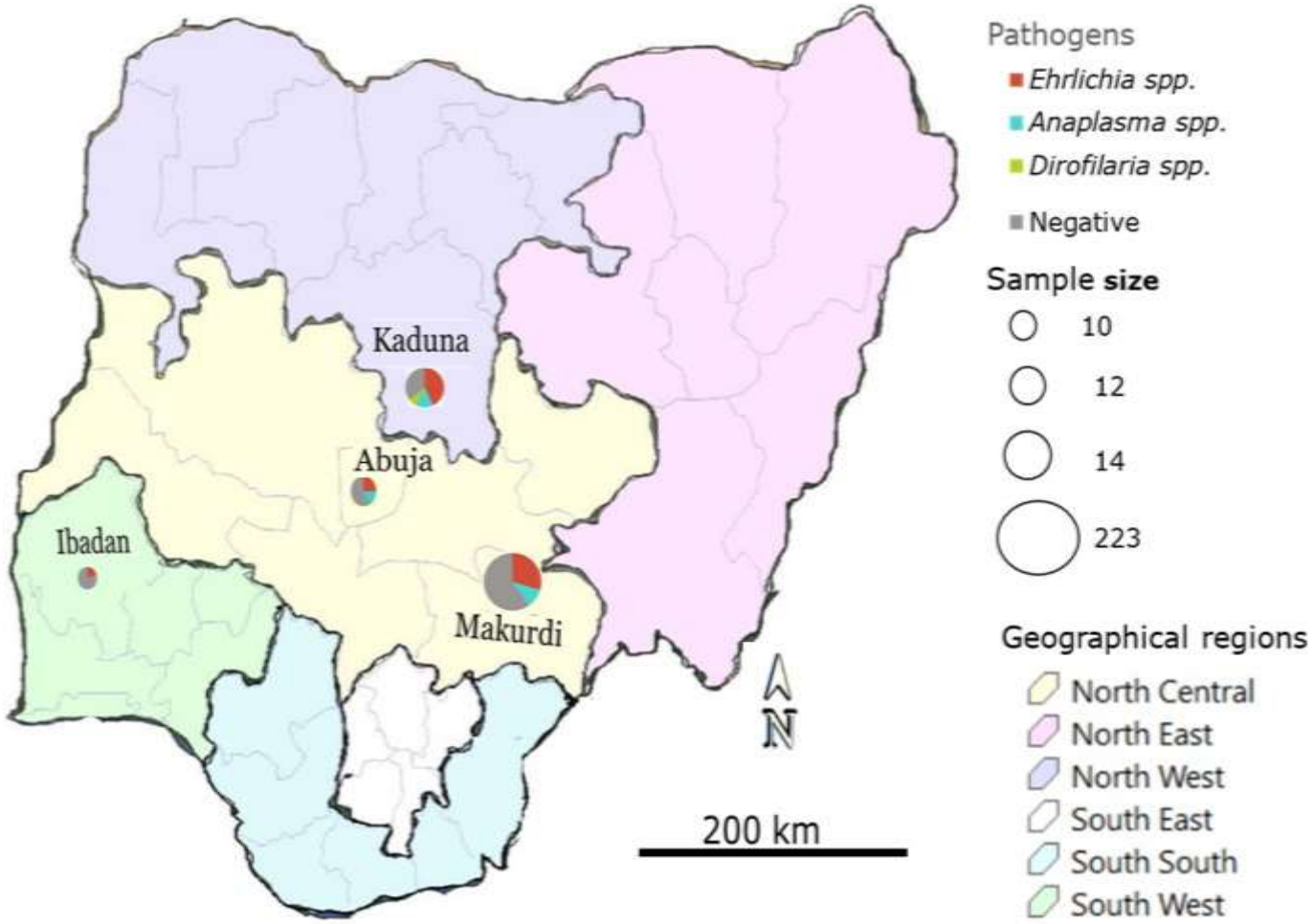


Figure 3.1: Geographical distribution of canine blood samples positive to CVBPs in Nigeria based on the POC test results.

3.2.2 Prevalence of CVBPs among Nigeria dogs based on the POC test results

A total of 40.9% (106/259) of the blood samples collected from Nigerian dogs were positive for CVBPs (Table 3.1). *Ehrlichia* spp. recorded the highest prevalence of 29.7% (77/259), followed by *Anaplasma* spp. with 10.8% (28/259) prevalence. *Dirofilaria* spp. had the least prevalence of 0.4% (1/259), no dog was seropositive to *Borrelia burgdorferi* (s.l.) in this study. In addition, 7.7% (20/259) of the total samples were seropositive for two of the canine tick-borne pathogens (*Ehrlichia* spp. + *Anaplasma* spp.), while the only 0.4% (1/259) canine blood sample that tested positive to *Dirofilaria* antigen was also positive for *Ehrlichia* sp.

3.2.3 CVBPs prevalence and risk factors associated with canine tick infestation in Nigeria following POC test

Not fewer than six exposure variables were considered as risk factors, given that many animals were seropositive for multiple pathogens. This analysis laid more emphasis on predictors of CVBPs in general and not specific CVBP. The summary of univariate analysis (Table 3.2) for CVBP risk factors shows that young and adult dogs were more likely to be seropositive to CVBPs than puppies (95% CI 0.15-0.64; p =0.002), while that of tick infestation shows male dogs were more likely to be infested with ticks than females (Table 3.3). However, the remaining risk factors examined were not statistically significant for the univariate analysis.

When multivariate logistic regression modelling was applied young and adult dogs were more likely to be seropositive to CVBPs than puppies, while dogs infested with ticks had an increased risk of testing seropositive to CVBPs than those without ticks Table 3.4A). In addition, the adjusted model for factors associated with tick infestation showed male dogs were more likely to be infested with ticks (Table 3.4B).

Table 3.1: Prevalence of vector-borne pathogens among Nigerian dogs (n = 259) based on POC test results.

Pathogen	**Positive (n = 259)**	**Prevalence (%)**	**95% CI**
Single pathogen			
Ehrlichia spp.	77	29.7	24.3 – 35.5
Anaplasma spp.	28	10.8	6.9 –14.7
Dirofilaria spp.	1	0.4	0.0 – 1.2
Total	106	40.9	
Multiple pathogens			
Ehrlichia sp. + Anaplasma sp.	20	7.7	
Dirofilaria+ Ehrlichia spp.	1	0.4	
Total	21	8.1	

+: signifies seropositivity results for more than one pathogen, n: sample size, CI: confidence interval.

Table 3.2: Summary of variable factors and univariate analysis of potential predictors associated with CVBPs prevalence in Nigerian dogs (n = 259) using POC test results.

Variable	Level	Total tested (%)	POC test positive (%) for CVBPs	OR 95% CI	P-value
Age	Young and adult	196	74 (37.8%)	Reference	
	Puppy	63	11 (17.5%)	0.35, 0.17 – 0.71	0.004
Sex	Male	154	52 (33.8%)	Reference	
	Female	105	33 (31.4%)	0.90, 0.53 – 1.53	0.694
Breed	Exotic	179	56 (31.3%)	Reference	
	Indigenous	80	29 (36.3%)	1.25, 0.72 – 2.18	0.432
Body weight (kg)	≤20 kg	172	54 (31.4%)	Reference	
	≥21kg	87	31 (35.6%)	1.21, 0.70 – 2.09	0.493
Season of the year	Rainy (April - October.)	181	62 (34.3%)	Reference	
	Dry (November - March)	78	23 (29.5%)	0.80, 0.45 – 1.43	0.454
Presence of ectoparasites	Yes	153	57 (37.3%)	Reference	
	No	106	28 (26.4%)	1.65, 0.96 – 2.84	0.068

POC: Point-of-care test (IDEXX antigen test kit), CI: confidence interval, n: sample size, OR: odd ratio, ≤: less than or equal to, ≥ greater than or equal to, CVBPs: canine tick-borne diseases.

Table 3.3: Summary of variable factors and univariate analysis of potential predictors associated with canine tick infestation in Nigeria (n = 259).

Variable	level	Total examined	Tick prevalence	OR 95% CI	P value
Age	Young and adult	196	117 (59.7%)	Reference	0.720
	puppy	63	36 (57.1%)	0.90, 0.51 – 1.60	
Sex	Male	154	83 (53.9%)	Reference	0.041
	female	105	70 (66.7%)	1.71, 1.02 – 2.86	
Breed	Exotic	179	109 (60.9%)	Reference	0.373
	Indigenous	80	44 (55%)	0.79, 0.46 – 1.34	
Body weight (kg)	≤20 kg	172	106 (61.6%)	Reference	0.240
	≥21kg	87	47 (54.0%)	0.73, 0.43 – 1.23	
Season of the year	Rainy (April - October.)	181	109 (60.2%)	Reference	0.567
	Dry (November - March)	78	44 (56.4%)	0.86, 0.499 – 1.46	
Location	Benue	223	127 (57.0%)	Reference	0. .280
	Kaduna	14	9 (64.3%)	1.36, 0.44 – 4.19	
	Abuja	12	8 (66.7%)	1.51, 0.44 – 5.17	
	Ibadan	10	9 (90.0%)	6.80, 0.85 – 54.62	

CI: confidence interval, n: sample size, OR: odd ratio, ≤: less than or equal to, ≥ greater than or equal to.

Table 3.4(A-B): Summary of multivariate logistic regression analysis of risk factors associated with canine vector borne pathogens (3A) and summary of multivariate logistic regression analysis of risk factors associated with canine tick infestation (3B) in Nigeria.

A	**Variable**	**Adjusted OR**	**95% CI**	**P value**
	Age			
	Young — adult	Reference		
	Puppy	0.3	0.15 — 0.64	0.002*
	Ectoparasites (ticks)			
	Yes	Reference		
	No	1.7	0.99 — 3.03	0.054*
	Location			
	Makurdi	Reference		
	Zaria	2.0	0.63 — 6.62	0.231
	Abuja	0.8	0.22 — 2.66	0.761
	Ibadan	0.3	0.07 — 1.65	0.180

B	Variable	Adjusted OR	95% CI	P value
	Sex			
	Male	Reference		
	Female	1.7	1.02-2.86	0.041*

CI: confidence interval, n: sample size, OR: odd ratio, *p values statistically significant ($p<0.05$).

3.3 Discussion

The aim of this study was to establish the prevalence of CVBPs in Nigeria using a well-established POC test and assess whether there is an association between CVBPs seropositivity and predictors such as age, sex, breed, season of the year, presence of tick infestation and or location. This study was the first to investigate risk factors associated with dogs seropositive to CVBPs in Nigeria.

The results indicate that young and adult dogs infested with ticks were more likely to be seropositive to CVBPs than puppies and dogs without tick infestation, independent of other cofounding factors assessed in this study. In addition, male dogs have an increased risk of tick infestation when compared to female dogs. These findings are in line with previous studies in Taiwan where age and presence of tick infestation were established to increase the risk of infection with canine tick-borne disease (Wu et al., 2009). Previous studies where ectoparasitic treatments have been assessed clearly demonstrate that prevention of ectoparasitic infestation in dogs reduces the prevalence of CVBPs (Otranto et al., 2010a, Pfister and Armstrong, 2016, Panarese et al., 2021).

The Taiwan study attributed risk of tick infestation to increased outdoor activities and lack of ectoparasitic treatment and not dog sex as reported in this study (Wu et al., 2009). It is likely that male dogs have increased outdoor activities due to behavioural differences compared to female, resulting in greater contact with ectoparasites. Previous studies on the assessment of factors influencing the home range size of free-roaming owned domestic dogs in Australia reported that, male dogs have the largest population of free free-roaming dogs (Dürr et al., 2017). In addition, it has been postulated that increased tick infestation in male dogs is physiological as an elevated level of testosterone in male vertebrates is linked to reduced immune status,

predisposing male dogs to increased parasitic infection (Zuk and McKean, 1996).

Over forty percent of the dogs examined tested positive to at least one of the four CVBPs detected using the IDEXX® test kit (except *Dirofilaria,* which was *D. immitis* antigen test), all dogs examined were seronegative to *B. burgdorferi* (s.l.). *Ehrlichia* spp. recorded the highest prevalence (29.7%), followed by *Anaplasma* spp. (10.8%), and *Dirofilaria immitis* (0.4%) as the least prevalent CVBP. Although serological testing was applied in this study, these results affirm previous studies where high *Ehrlichia canis* prevalence of 77.3% (Kamani et al., 2013a), 53% (Adamu et al., 2014), 76.7%, and 22.9% (Daramola et al., 2018, Happi et al., 2018), were reported from naturally infected dogs in Nigeria using PCR. Similarly, high prevalence is reported from neighbouring countries including Algeria (15.38%) (Bessas et al., 2016), and Cameroon (32%) (Ndip et al., 2005). Also, high prevalence of *Ehrlichia* have been reported from other tropical countries of the world sharing similar climate and tick vectors (*R. sanguineus*) with Nigeria including 51.9% in Haiti (Starkey et al., 2016), and 94% in Thailand (Suksawat et al., 2001). *R. sanguineus*, the principal vector for the transmission of *Ehrlichia* spp. has been reported as the most abundant tick population in Nigeria (Kamani et al., 2013a, Kamani, 2021b). *Rhipicephalus* spp. are known to reproduce more in hot climates (Dantas-Torres et al., 2010). Therefore, where dogs receive little or no ectoparasitic treatment, high prevalence of *Ehrlichia* spp. are expected (Angelou et al., 2019). Although, the specific species of *Ehrlichia* present is not known in this study as the POC test used is known to detect several species of *Ehrlichia*. Nonetheless, *Ehrlichia chaffeensis* and *Ehrlichia ewingii* causing canine monocytic ehrlichiosis have been reported in Nigeria (Reye et al., 2012), and the neighbouring country Cameroon where *E. chaffeensis* has been reported in *R. sanguineus* ticks (Ndip et al., 2007). Findings from this study provide additional information on the epidemiology of *Ehrlichia* species from Nigerian dogs.

The relatively low prevalence of *Anaplasma spp*. observed in this study is in line with previous studies that reported a lower prevalence of 6.6% for *Anaplasma spp*. (12/181), compared to *Ehrlichia spp.* prevalence of 12.7% (23/181) in Nigeria (Kamani et al., 2013a), and other parts of the world (Angelou et al., 2019). The serological test used in this study does not distinguish between *A. platys* and *A. phagocytophilum*; however, *A. platys* and *E. canis* are both transmitted by *R. sanguineus*. This may also explain why a high incidence of co-infection of dogs with *Ehrlichia* and *Anaplasma* spp. was observed in this study.

All dogs examined were negative to *B. Burgdorferi* (s.l.) in Nigeria using the commercially POC test kit. To-date, there is no single study on CVBPs that has demonstrated the presence of *B. burgdorferi* (s.l.) in Nigeria, and the principal vector *Ixodes* spp. has not been reported in Nigeria (Kamani et al., 2013a). Although, recent studies from Egypt and Algeria reported *B. burgdorferi* (s.l.), prevalence of 1.8% (9/500) and 5.2% using IDEEX® test kit (Selim et al., 2021a), and IFA test (Azzag et al., 2015), respectively. However, *B. burgdorferi* (s.l.) was not detected via PCR in the same sample from both studies. High prevalence of *B. burgdorferi* (s.l.) have been reported from *Ixodes ricinus* in North African countries including Tunisia (Zhioua et al., 1999), and Morocco (Younsi et al., 2001). *Ixodes ricinus* species are present in North Africa and may spread to other neighbouring countries due to the movement of animals or people. At some point, one may expect *B. burgdorferi* to be reported in Nigeria as the range of tick vectors increase. Previous studies in Nigeria have identified other *Borrelia* spp. responsible for tick-borne relapsing fever (TBRF) syndromes in humans sourced from *Rhipicephalus evertsi* (Reye et al., 2012), and *Ornithodoros* spp. including *B. anserine* (Hovind-Hougen, 1995), and *Candidatus Borrelia kalaharica* (Cutler et al., 2018).

Canine and human borreliosis caused by *B. Burgdorferi* (s.l.), transmitted by *Ixodes* ticks, is usually reported to be more prevalent in temperate climatic

regions, rather than in the tropical parts of the world. This may be why previous studies in India (tropical region) where *Ixodes* spp. were confirmed to be present reported the absence of *B. burgdorferi* (s.l.) in dogs (Wise and Tarlinton, 2012), and humans (Handa et al., 1999). However, the increased population of *Ixodes* ticks called for increased research in this area, with recent molecular studies based on PCR reporting *B. burgdorferi* from goats in India (Patra et al., 2020). There is also a recent report from Egypt where *B. burgdorferi* was reported from canine blood and *R. sanguineus* infesting dogs suggesting that other tick vectors may transmit *B. burgdorferi* (Elhelw et al., 2021) however, this study requires further investigation.

This study indicates that only one dog examined tested positive for *Dirofilaria immitis* (0.4%). There is a single report dating over four decades ago (1975) where a prevalence of 9.2% (34/369) for *D. repens* (microfilaria) was reported in Nigeria using morphological methods (Van Veen and Blotkamp, 1975). Two more studies using morphological surveys for *D. immitis* microfilaria identification from canine blood smears in Nigeria showed a declining prevalence of 3.4 (4/119) and 2.2% (4/186) in 2016 (Ogbaje and Danjuma, 2016, Ugochukwu et al., 2016). However, results from these two studies require additional confirmation, Knott's technique is the recommended test for morphological identification of microfilaria. Wet mount cannot differentiate between pathogenic and non-pathogenic filarial species (Hays et al., 2020), and it is likely that results from these studies may have identified non-pathogenic filarial species.

One would expect a higher prevalence of *D. immitis* in this study given that mosquito species that are major vectors of *D. repens* and *D. immitis* are present in Nigeria (Adeleke et al., 2010). However, the declining prevalence recorded in Nigeria over the last five decades may be attributed to the increased use of ivermectin, a commonly abused antiparasitic agent (macrocyclic lactone) for routine deworming of dogs in Nigeria. Dog owners, and farmers resort to self-treatment of their pets or livestock using ivermectin

without veterinary advice. Inadequate policies/efforts by the government to regulate animal health products and drugs for both pets and livestock is likely to be recorded in Nigeria (author's observation). Prolonged administration of ivermectin can result in "slow kill" (SK) effect on adult filarial worms, alongside the host immune clearance of microfilaraemia (Wolstenholme et al., 2016). There are many studies demonstrating that dogs recently treated with ivermectin, may test negative and/or false negative (where microfilariae are detected) to *D. immitis* antigen tests (Stenzenberger and Gothe, 1999, Drake et al., 2015). Ivermectin is one of the most widely used antiparasitic drugs effective against several species of internal and external parasites including *Dirofilaria* in different animal species (Jin et al., 2013). Irrespective of the route (topical, oral, or subcutaneous in dogs) of administration, the drug has a significant prolonged clinical efficacy and bioavailability in different parts of the body following a single treatment (A. McKellar et al., 2012). Ivermectin is also readily available in West Africa due to the river blindness eradication programme (Basáñez et al., 2006).

The presence of coinfection of dogs with *Ehrlichia* and other CVBPs including *Babesia, Hepatozoon*, and *Anaplasma* species have been reported in Nigeria (Happi et al., 2018). Reports from this study agrees with other studies previously documented in Nigeria and other parts of the world. Although, the aim of this study did not include clinical examination of dogs, underlying coinfection with multiple CVBPs can result in a broad-spectrum of clinical signs or severe illness including anaemia, and death (Otranto et al., 2009, Spolidorio et al., 2011, Santamaria et al., 2014, Aktas et al., 2015, Alho et al., 2017, Attipa et al., 2017). Confounding laboratory diagnosis, and medical management, and lack of a clear understanding of prognosis can be challenging in dogs coinfected with CVBPs (Breitschwerdt and Maggi, 2009, Happi et al., 2018, Meyers et al., 2021). These findings suggest the need for Nigerian veterinarians to be cautious during clinical evaluation of dogs infested with ticks in Nigeria to ensure proper diagnosis and treatment.

Sustained tick control measures may be valuable in preventing coinfection with multiple CVBPs (de Caprariis et al., 2011).

3.4 Conclusion

Canine Lyme disease caused by *B. burgdorferi* is unlikely to be present in Nigeria as demonstrated in this study. However, we confirm the high prevalence of *Anaplasma* and *Ehrlichia* sp. in Nigerian dogs as recorded in other previous studies. This study suggests that puppies are less likely to be seropositive to CVBPs. Young adult dogs with high tick burden are more likely to be seropositive, while male dogs have an increased risk of tick infestation. The prevalence of canine heartworm was unexpectedly low despite the presence of limited previous reports using wet mount and buffy coat techniques and the presence of mosquito vector species in Nigeria (Ugochukwu et al., 2016). This may be due to the common use of ivermectin as a "catch all" antiparasitic medication in humans and animals. Further investigation using a combination of Knott's technique and antigen tests is required to confirm this study. The high prevalence of *Ehrlichia* and *Anaplasma* spp. demonstrates considerable disease burden for the canine population. Although the risk of human infection with *Ehrlichia* and *Anaplasma* spp. is rare, both pathogens are reported to be zoonotic and require adequate control measures to prevent human infection. Routine tick control by dog owners and inclusion of CVBPs as a differential diagnosis by practicing veterinarians during clinical examination is recommended. The report of coinfection with multiple pathogens in this study highlights the need for cautious diagnosis by veterinarians and sustained tick control.

Chapter 4: Molecular characterization of ticks and tick-borne pathogens (TBPs) from ticks

4.1 Background

Ticks are ectoparasitic arthropods that infest and feed on various species of land-dwelling vertebrate animals. Two families of ticks including *Argasidae* and *Ixodidae* infest and transmit pathogens to domestic animals, humans, and birds (Black and Piesman, 1994, Jongejan and Uilenberg, 2004). Tick infestation results in economic losses amounting to millions of dollars in livestock (Jonsson et al., 2001), and cost of prevention, medical treatment, and control for the dogs and their owners where dogs are infected with zoonotic pathogens (El-Bahnasawy et al., 2011). Diverse tick species are responsible for the transmission of a variety of bacterial, protozoal, and viral pathogens to animals and humans globally (Shaw et al., 2001, Chomel, 2011, Biernat et al., 2014, Balling et al., 2014, de Marco et al., 2017, Ogo et al., 2012).

In most parts of the world there is an increasing effort to ensure continuous surveillance of tick populations and pathogens transmitted by ticks. This has led to the identification of exotic tick and pathogen species in different parts of the world including the UK (Jameson and Medlock, 2011), and the United States (CDC, 2021). About ten non-indigenous tick species originating from fifteen different countries have been reported in the UK under the tick surveillance scheme (Hansford et al., 2018). A clear understanding of the changes in the distribution of diverse tick species and tick-borne pathogens can only be achieved through effective tick surveillance programme (Eisen and Paddock, 2021). Previous studies demonstrating the distribution of tick species in Nigerian livestock, covering a town/village, targeting a smaller sample size, or based on morphological methods, where *Rhipicephalus* and *Haemaphysalis* species have been reported (Ofukwu et al., 2008, Lorusso et al., 2013, Kamani et al., 2017, Ibe et al., 2012, Eyo et al., 2014, Ogo et al., 2012), and little information is

available on canine tick infestation. There are gaps in the information available on diverse canine tick species population distribution in various parts of Nigeria. The aim of this study is to identify tick species infesting Nigerian dogs using molecular barcoding methods. Testing for selected range of tick-borne pathogens of dogs to allow comparison of the prevalence of these pathogens in the ticks with the prevalence from the canine blood samples was also carried out.

4.2 Results

4.2.1 Canine and *G. domesticus* hard tick PCR and gel electrophoresis result

Hard tick *16S rRNA* PCR was carried out on a total of 123 representative tick samples (Table 4.1) including 113 adult tick samples collected from 111 dogs and 11 tick samples collected from domestic birds (Figure 4.1). During DNA extraction and PCR, one representative tick sample was taken from each dog, except for one dog (T3) infested with *Haemaphysalis* species where 2 tick samples were processed (T3A and T3B) (Table 4.1). A portion of tick samples was selected to confirm the initial morphological identification, where nucleic acid was extracted from 40/112 domestic dogs and 11/11 bird tick samples purified and Sanger sequenced to yield tick nucleotide sequences of 400 base pairs. While 35/40 of the dog hard tick sequences were identified as *R. sanguineus* (Figure 4.2), 5/40 of these sequences were identified as *H. leachi* (Figure 4.3 and Table 8.1). The NCBI nucleotide blast search results showed that the *R. sanguineus* in this project were matched to over fifteen different *R. sanguineus* (tropical lineage) GenBank entries from various parts of the world (Table 8.1). In addition, all dog tick sequences identified as *H. leachi* matched to *H. leachi* (MN661151) from Chad in Africa described by Thompson et al. (2020).

All hard tick nucleotide sequences obtained from domestic birds showed 94.5 — 96.61 similarity and 98% coverage with a 14,673 bp *Haemaphysalis bancrofti* (NC_041076, Table 8.1) sequence from Australia (Burnard and Shao, 2019); 94.57 — 95.09% similarity and 100% coverage with a 454 bp of *Haemaphysalis doenitzi* submitted from China (Chen et al., 2012). The domestic bird hard ticks were also identified to have percentage identity between 92.37 — 93.53% and 97% coverage with several *Haemaphysalis longicornis* species of 400 — 450 bp long. However, phylogenetic classification placed the bird tick sequences under the *H. longicornis* (Figure 4.4) clade. The molecular identification of ticks carried out in this study was consistent with the initial morphological identification carried out during field sampling therefore, the remainder of tick samples were not sequenced avoiding additional cost.

Table 4.1: Summary of percentages and number of adult ticks collected from Nigerian dogs and local birds, based on morphological and molecular methods of identification, including number of adult ticks sequenced in this project.

Tick genera	Animal host, percentage (%) and number of adult ticks examined		Total percentage and number	
	Canine	Domestic birds *(G. domesticus)*		Number of adult ticks sequenced
Rhipicephalus sanguineus	87 % (107/123)	Not applicable	87% (107/123)	35
Haemaphysalis leachii	4.1% (5/123)	8.9% (11/123)	13.1% (16/123)	16
Total	91.1% (112/123)	8.9% (11/123)	100% (123/123)	51

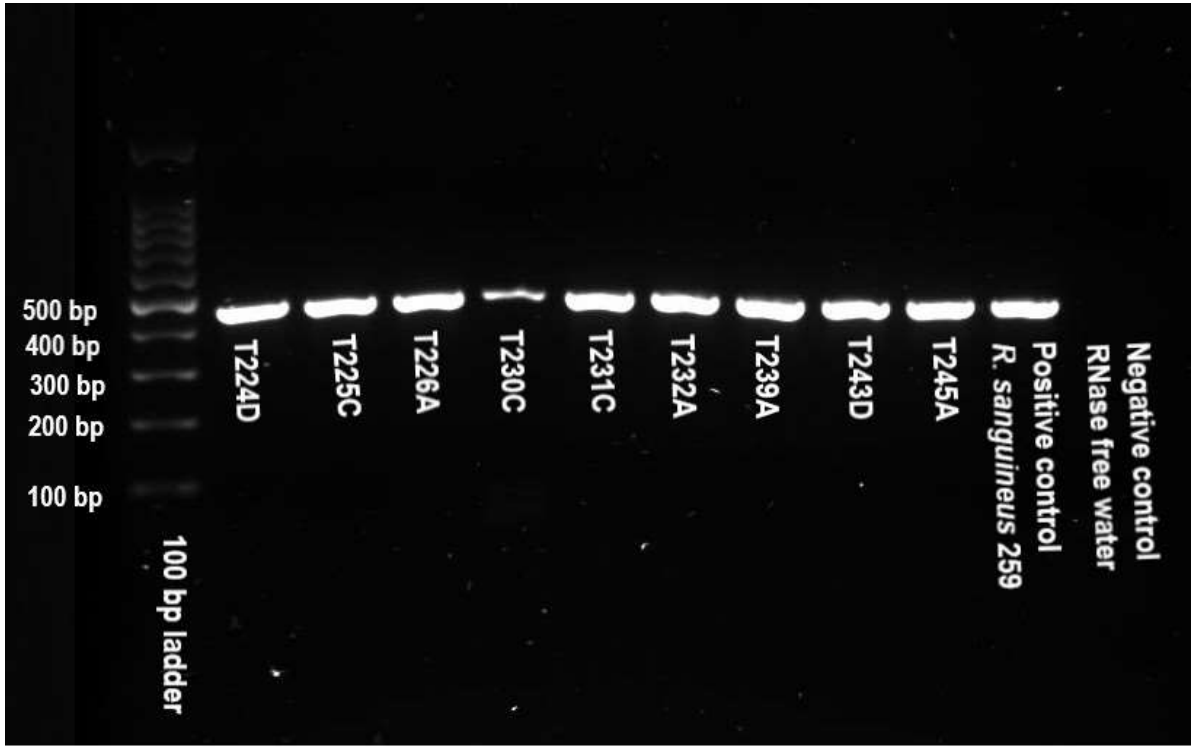


Figure 4.1: An example of 1% agarose gel picture obtained following *16S RNA* gene PCR on Nigerian hard tick samples.

Key: From left to right (L-R), first lane: 100 bp DNA ladder, Lane labeled T224D to T245A (L-R), refers to hard tick samples collected from owned dogs. The last two lanes on the far right represent positive control (*R. sanguineus* DNA (courtesy of Dr Lesley Bell-Sakyi and team, The Tick Cell Biobank, University of Liverpool UK) and negative control (RNase free water), respectively.

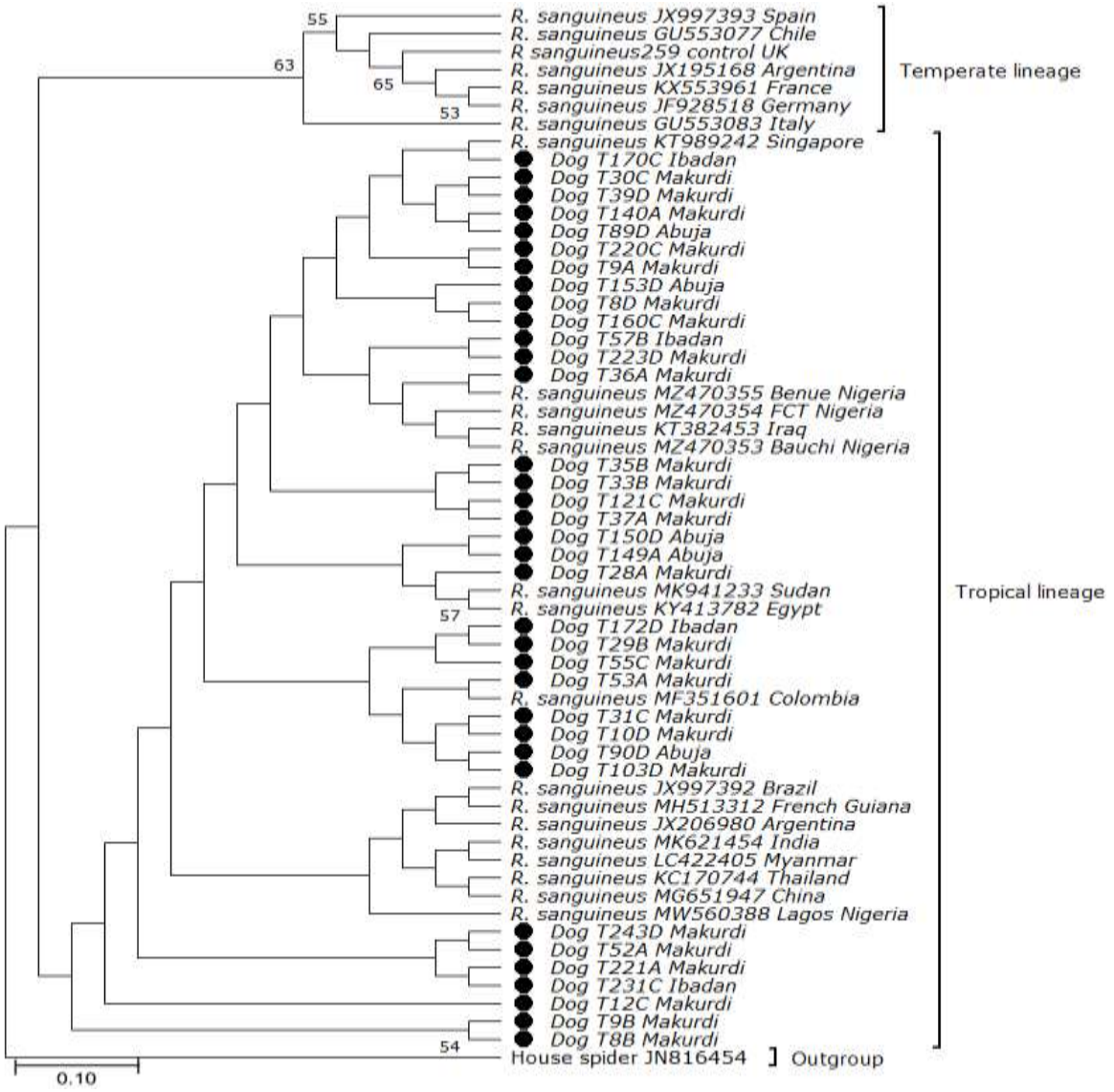

R. sanguineus JX997393 Spain
R. sanguineus GU553077 Chile
R sanguineus259 control UK
R. sanguineus JX195168 Argentina
R. sanguineus KX553961 France
R. sanguineus JF928518 Germany
R. sanguineus GU553083 Italy
Temperate lineage
R. sanguineus KT989242 Singapore
Dog T170C Ibadan
Dog T30C Makurdi
Dog T39D Makurdi
Dog T140A Makurdi
Dog T89D Abuja
Dog T220C Makurdi
Dog T9A Makurdi
Dog T153D Abuja
Dog T8D Makurdi
Dog T160C Makurdi
Dog T57B Ibadan
Dog T223D Makurdi
Dog T36A Makurdi
R. sanguineus MZ470355 Benue Nigeria
R. sanguineus MZ470354 FCT Nigeria
R. sanguineus KT382453 Iraq
R. sanguineus MZ470353 Bauchi Nigeria
Dog T35B Makurdi
Dog T33B Makurdi
Dog T121C Makurdi
Dog T37A Makurdi
Dog T150D Abuja
Dog T149A Abuja
Dog T28A Makurdi
R. sanguineus MK941233 Sudan
R. sanguineus KY413782 Egypt
Dog T172D Ibadan
Dog T29B Makurdi
Dog T55C Makurdi
Dog T53A Makurdi
R. sanguineus MF351601 Colombia
Dog T31C Makurdi
Dog T10D Makurdi
Dog T90D Abuja
Dog T103D Makurdi
R. sanguineus JX997392 Brazil
R. sanguineus MH513312 French Guiana
R. sanguineus JX206980 Argentina
R. sanguineus MK621454 India
R. sanguineus LC422405 Myanmar
R. sanguineus KC170744 Thailand
R. sanguineus MG651947 China
R. sanguineus MW560388 Lagos Nigeria
Dog T243D Makurdi
Dog T52A Makurdi
Dog T221A Makurdi
Dog T231C Ibadan
Dog T12C Makurdi
Dog T9B Makurdi
Dog T8B Makurdi
Tropical lineage
House spider JN816454
Outgroup
55
63
65
53
57
54
0.10

Figure 4.2: Maximum-likelihood phylogenetic tree constructed using *R. sanguineus* nucleotide and reference sequences obtained from GenBank, with a common house spider as an outgroup.

Key: Canine *R. sanguineus* species from this study are specified by black periods and labeled "Dog Tx," "T" and "X" denote individual dogs and sample serial numbers, respectively. The letters A-D represents four tick samples (A-D) collected from each dog. GenBank sequences are labeled using tick genera/species, GenBank accession and country of origin. The phylogenetic tree demonstrated that *R. sanguineus* obtained in this study were closely related to other tropical lineages, and distantly related to temperate lineages. The phylogenetic tree is drawn to display bootstrap support values ≥50%.

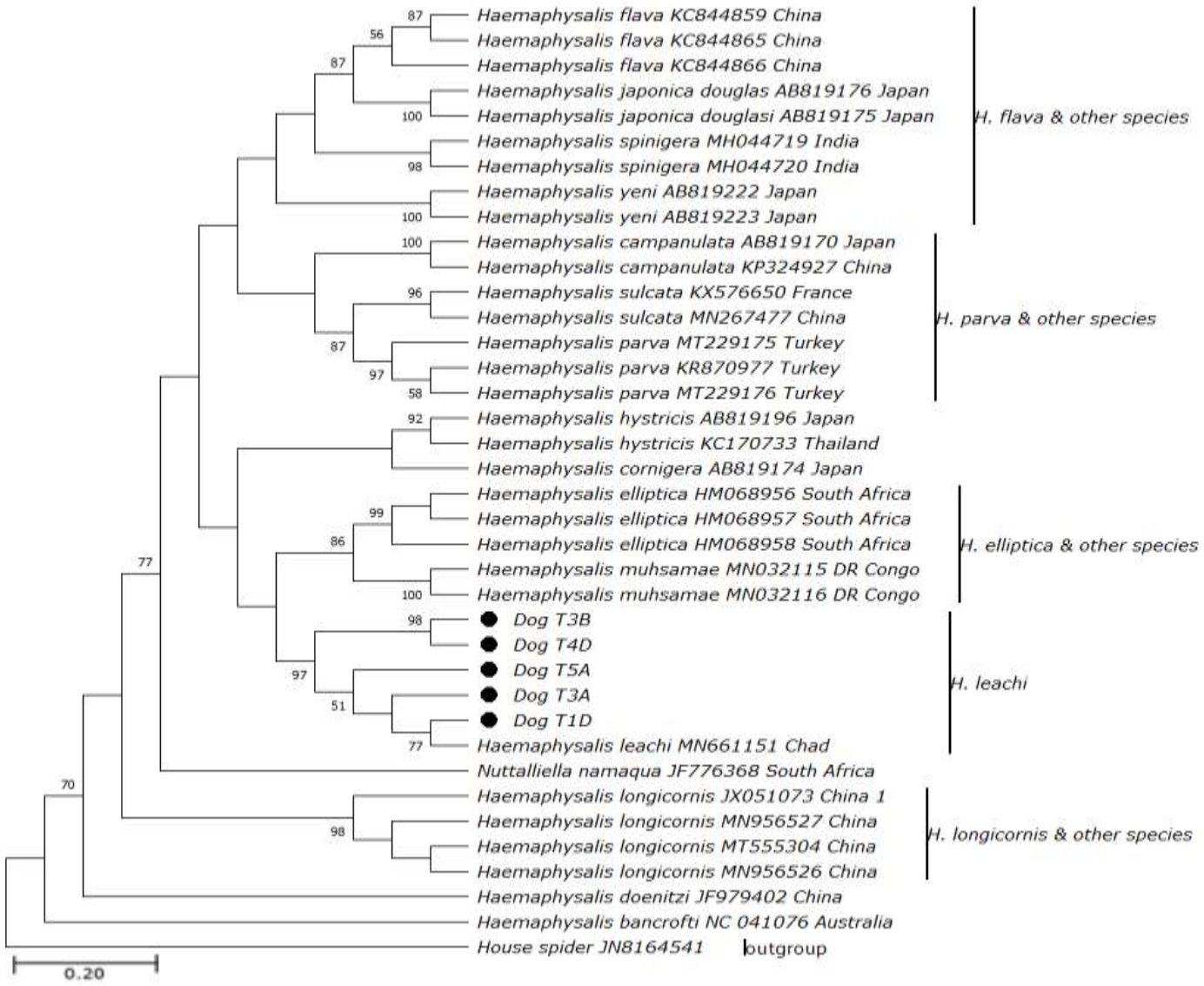

Haemaphysalis flava KC844859 China
Haemaphysalis flava KC844865 China
Haemaphysalis flava KC844866 China
Haemaphysalis japonica douglas AB819176 Japan
Haemaphysalis japonica douglasi AB819175 Japan
Haemaphysalis spinigera MH044719 India
Haemaphysalis spinigera MH044720 India
Haemaphysalis yeni AB819222 Japan
Haemaphysalis yeni AB819223 Japan
H. flava & other species
Haemaphysalis campanulata AB819170 Japan
Haemaphysalis campanulata KP324927 China
Haemaphysalis sulcata KX576650 France
Haemaphysalis sulcata MN267477 China
Haemaphysalis parva MT229175 Turkey
Haemaphysalis parva KR870977 Turkey
Haemaphysalis parva MT229176 Turkey
H. parva & other species
Haemaphysalis hystricis AB819196 Japan
Haemaphysalis hystricis KC170733 Thailand
Haemaphysalis cornigera AB819174 Japan
Haemaphysalis elliptica HM068956 South Africa
Haemaphysalis elliptica HM068957 South Africa
Haemaphysalis elliptica HM068958 South Africa
Haemaphysalis muhsamae MN032115 DR Congo
Haemaphysalis muhsamae MN032116 DR Congo
H. elliptica & other species
Dog T3B
Dog T4D
Dog T5A
Dog T3A
Dog T1D
Haemaphysalis leachi MN661151 Chad
H. leachi
Nuttalliella namaqua JF776368 South Africa
Haemaphysalis longicornis JX051073 China 1
Haemaphysalis longicornis MN956527 China
Haemaphysalis longicornis MT555304 China
Haemaphysalis longicornis MN956526 China
H. longicornis & other species
Haemaphysalis doenitzi JF979402 China
Haemaphysalis bancrofti NC 041076 Australia
House spider JN8164541
butgroup
0.20

Figure 4.3: Maximum-likelihood phylogenetic tree constructed using *Haemaphysalis* species nucleotide and reference sequences obtained from GenBank, with a common house spider as an outgroup.

Key: Canine *Haemaphysalis leachi* species from this study are specified by black periods and labeled "Dog Tx," "T" and "X" denote individual dogs and sample serial numbers, respectively. The alphabets A-D represents one out of the four tick samples (A-D) collected from each dog. GenBank sequences are labeled using tick genera/species, GenBank accession and country of origin. The tree showed that *H. leachi* sequence in this study were closely related to *H. leachi* from Chad in Africa describe by Thompson et al. (2020). The phylogenetic tree is drawn to display bootstrap support values ≥50%.

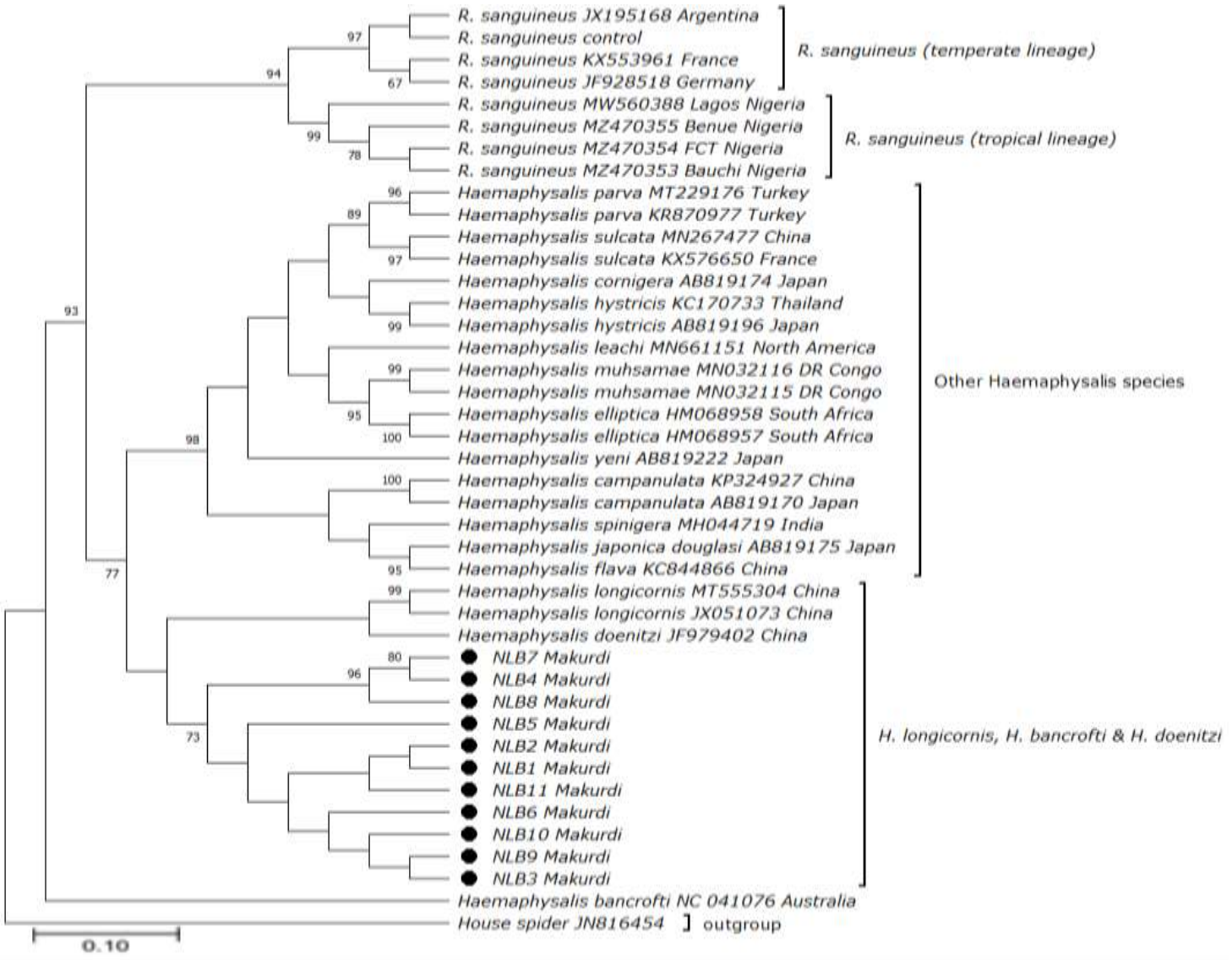

R. sanguineus JX195168 Argentina
R. sanguineus control
R. sanguineus KX553961 France
R. sanguineus JF928518 Germany
R. sanguineus (temperate lineage)
R. sanguineus MW560388 Lagos Nigeria
R. sanguineus MZ470355 Benue Nigeria
R. sanguineus MZ470354 FCT Nigeria
R. sanguineus MZ470353 Bauchi Nigeria
R. sanguineus (tropical lineage)
Haemaphysalis parva MT229176 Turkey
Haemaphysalis parva KR870977 Turkey
Haemaphysalis sulcata MN267477 China
Haemaphysalis sulcata KX576650 France
Haemaphysalis cornigera AB819174 Japan
Haemaphysalis hystricis KC170733 Thailand
Haemaphysalis hystricis AB819196 Japan
Haemaphysalis leachi MN661151 North America
Haemaphysalis muhsamae MN032116 DR Congo
Haemaphysalis muhsamae MN032115 DR Congo
Haemaphysalis elliptica HM068958 South Africa
Haemaphysalis elliptica HM068957 South Africa
Haemaphysalis yeni AB819222 Japan
Haemaphysalis campanulata KP324927 China
Haemaphysalis campanulata AB819170 Japan
Haemaphysalis spinigera MH044719 India
Haemaphysalis japonica douglasi AB819175 Japan
Haemaphysalis flava KC844866 China
Other Haemaphysalis species
Haemaphysalis longicornis MT555304 China
Haemaphysalis longicornis JX051073 China
Haemaphysalis doenitzi JF979402 China
NLB7 Makurdi
NLB4 Makurdi
NLB8 Makurdi
NLB5 Makurdi
NLB2 Makurdi
NLB1 Makurdi
NLB11 Makurdi
NLB6 Makurdi
NLB10 Makurdi
NLB9 Makurdi
NLB3 Makurdi
H. longicornis, H. bancrofti & H. doenitzi
Haemaphysalis bancrofti NC 041076 Australia
House spider JN816454
outgroup
0.10

Figure 4.4:Maximum-likelihood phylogenetic tree constructed using *Haemaphysalis* species nucleotide and reference sequences obtained from GenBank, with a common house spider as an outgroup.

Key: Nigerian domestic chicken (*G. domesticus) Haemaphysalis* nucleotide sequences from this study are specified by black periods and labeled "NLBX," "NLB" and "X" denotes Nigerian local birds and sample serial numbers, respectively. GenBank sequences are labeled using tick genera/species, GenBank accession and country of origin. The phylogenetic tree above showed that *Haemaphysalis* species from domestic chicken in this study were closely related to *H. longicornis* (China), and distantly related to other *Haemaphysalis* species. The phylogenetic tree is drawn to display bootstrap support values ≥50%.

4.2.2 Qualitative PCR screening of Nigerian canine and domestic bird hard ticks for the presence of *Ehrlichia, Borrelia* and Piroplasm DNA.

A total of 122 adult ticks collected from 111 dogs and 11 birds were examined for the presence of Piroplasm, *Ehrlichia* and *Borrelia* DNA. Overall, *Ehrlichia canis* DNA was detected in 63.1% (77/122) of the ticks identified as *R. sanguineus* (s.l.), both tick species identified as *H. leachi* tested negative for the presence of *E. canis* DNA. Sixteen percent (16.4% (20/122)) of ticks tested positive for the presence of Piroplasm DNA. Sanger sequencing of samples positive for piroplasm and NCBI blast search showed these were non-specific PCR products identical to eukaryotic ribosomal DNA, algae (*Poterioochromonas*, Chrysophytes), and another fungal genus (*Brunneoclavispora*, *Wallemia*). Sequences from one tick sample were identified as *Colpodella* (KU159286), a genus from the phylum *Apicomplexa*. With this result further Sanger sequencing was not carried out, as it was observed these primers were non-specific in tick samples and could amplify hard tick environmental contaminants. All 122 adult tick samples screened in this study tested negative for *B. burgdorferi*.

4.3 Discussion

The results of this chapter showed that *R. sanguineus* (the brown dog tick) is the most abundant tick infesting dogs in Nigeria as 87% of dogs examined were infested with *R. sanguineus*, while *Haemaphysalis* species (4.1%) were the second most abundant tick species reported in this study. Both morphological and molecular identification carried out in this study produced the same results, showing that morphological identification methods can be a robust method for tick identification. The 11 domestic bird ticks presented in this study were considered in isolation and were not added to the total canine tick population during calculation of tick species abundance.

Morphological misclassification of tick species may occur where physical damage due to improper tick collection, poor storage condition or collection of ticks at various stages of lifecycle is common. Previous experience is also required to distinguish tick species using morphological methods (Dergousoff and Chilton, 2007). However, in this study it proved reliable when compared to barcoding sequencing. Molecular identification from this study confirmed the identification of 35/40 canine adult hard tick nucleotide sequences as *R. sanguineus*, while the remaining 5/40 canine adult hard tick nucleotide sequences were identified as *Haemaphysalis leachi.*

Other studies identifying canine tick species distribution in Nigeria using morphological methods reported *R. sanguineus* (Ofukwu et al., 2008, Ogo et al., 2012, Lorusso et al., 2013, Kamani et al., 2013a, Adang et al., 2015, Opara M.N et al., 2017, Emmanuel et al., 2017), and *Haemaphysalis leachi* (Ofukwu et al., 2008, Kamani et al., 2013) as the most abundant ticks of dogs in Nigeria. In addition, molecular tick barcoding on a total of 3805 tick samples by Kamani (2021a) reported *R. sanguineus* (n = 3629; 95.4%), and *H. leachi* (n = 155; 4.1 %) as the most abundant canine tick population in Nigeria confirming this study. Although, Kamani (2021a) reported *Rh. (Boophilus) decoloratus* (n = 12; 0.3

%) and *Amblyomma variegatum* (n = 9; 0.2%) as tick species infesting dogs which were not reported in this study.

In addition, phylogenetic analysis showed that the *R. sanguineus* (s.l.) identified in this study were closely related to tropical lineage of *R. sanguineus* (s.l.) from Asia (Singapore, Iraq), South America (Columbia), and Africa (Nigeria, Egypt, Sudan); and distantly related to *R. sanguineus* (s.l.) temperate lineage from Europe (Germany, France, Spain), and South America (Argentina, Chile) as recently reported by Kamani (2021b). Clustering of all Nigerian *R. sanguineus* (s.l.) species identified as tropical lineage into one major clade alongside with sequences submitted from Singapore, Iraq, Colombia, and Africa (Sudan, Nigeria, Egypt) supports a recent report from Nigeria (Kamani, 2021b). This may be due to the widespread of *R. sanguineus* in the tropics, and the presence of minimal genetic sequence variation among *R. sanguineus* (s.l.) tropical lineage in Nigeria and other parts of the world. The geographical similarity between certain parts of Asia (Singapore) and America (Columbia) with a tropical and high mean ambient temperature, and Sub-Saharan Africa including Nigeria is likely to be responsible for the similarity in the distribution of the *R. sanguineus* (s.l.) "tropical lineage" across these regions.

H. leachi infesting Nigerian dogs identified in this study were more related to *H. leachi* (MN661151) from Chad in Africa, described by Thompson et al. (2020). Kamani (2021a) recently demonstrated the presence of *H. leachi* and its role in the transmission of *B. rossi* in Nigeria. In general, both *R. sanguineus* and *H. leachi* are abundant tick species known to have domestic behavior to live in houses with animal hosts, and have been previously reported in Africa (Walker, 2003). Both are also known to be major vectors for the transmission of canine babesiosis (Walker, 2003), and these findings are expected.

The prevalence of hard ticks infesting domestic birds in Nigeria were reported in this study. There are studies using simple morphological observation that reported hard ticks as ectoparasites infesting domestic chicken (*Gallus*

domesticus) (Bala et al., 2011), and captive (Njila et al., 2019) birds in Nigeria. However, to the best of our knowledge, this study is the first to report hard tick infestation in Nigerian domestic chicken (*G. domesticus)* utilizing both morphological and molecular methods. The Nigerian domestic chicken ticks from this study were 96.6% identical to *H. bancrofti* (NC_041076) submitted by Burnard and Shao (2019) from Australia, and 93% identical with several *H. longicornis* species submitted from Asia countries. However, the phylogenetic tree constructed using reference sequences including *H. bancrofti* and *H. longicornis* species demonstrates that the Nigerian bird hard ticks were more likely to be identified as *H. longicornis* from China (JX0501072 and MT555304*)* partly confirming a recent report suggesting that the closest NCBI blast hit is often not the nearest neighbor (Koski and Golding, 2001). *H. bancrofti* is a three-host tick commonly called "the wallaby tick," as it is mostly known to infest marsupials including koalas, kangaroos, possums etc. Occasional infestation of dogs, and other domestic animals (cattle, sheep, horses, pigs) and wild (deer) have been reported in Australia (Laan et al., 2011, Barker and Walker, 2014). Both *H. bancrofti* and *H. longicornis* have common morphological features, and both have been reported in Australia. Although, *Theileria orientalis* was originally known to be transmitted by *H. longicornis*, *H. bancrofti* is established to be the principal vector for the transmission of *T. orientalis* in South Australia. (Barker and Walker, 2014, Stewart et al., 1989).

H. longicornis is a native Asian tick species that has been introduced in several parts of the world, it is currently regarded as exotic tick vector in the US, Europe, UK, and Australia (Beard et al., 2018). *H. longicornis* is known to harbor pathogens such as severe fever with thrombocytopenia syndrome (SFTS) virus (Zhuang et al., 2018), Nairobi sheep disease virus, tick-borne encephalitis virus, lymphocytic choriomeningitis virus, *Borrelia* (including *B. burgdorferi sensu lato*), *Babesia*, *Francisella, Coxiella, Rickettsia, Anaplasma, Ehrlichia* (Zhao et al., 2020). With short sequences of *Haemaphysalis* species generated, coupled with the lack of *Haemaphysalis* sp. reference sequences in the databases, it is

hard to say with certainty that the tick species identified from Nigerian domestic birds could be classified as *Haemaphysalis longicornis*. These findings requires further investigation.

In addition, a total of 122 adult hard tick samples were tested for the presence of tick-borne pathogens such as *E. canis*, *Piroplasm*, and *B. burgdorferi*. Overall, the prevalence of *Ehrlichia* species in *R. sanguineus* was high (63.1%, n=122). The prevalence of *E. canis* recorded from *R. Sanguineus* ticks in this study is higher than a recent study that reported a prevalence of 23.7% (n=258 adult ticks) (Kamani et al., 2013a). This confirms previous studies suggesting *E. canis* as the most common tick-borne pathogen. Of course, *R. Sanguineus* is known as the principal vector for the transmission of *E. canis* globally (Mittal et al., 2017, Montenegro et al., 2017, Harrus et al., 1999) and results from this study are not completely unexpected.

The PCR used for the amplification of Piroplasms *18S rRNA* while proving reliable in the dog blood samples (Chapter five), produced non-specific amplification in the tick samples and is clearly not suitable for use in invertebrates.

None of the tick samples tested in this study were positive for *B. burgdorferi* (s.l.) DNA, partly confirming the results of the previous chapter as to its absence in Nigerian dogs. This is expected as *B. burgdorferi* (s.l.) primarily transmitted by *Ixodes* tick species (Krupka and Straubinger, 2010, Rudenko et al., 2011) not reported in Nigeria. *B. burgdorferi* has been identified from ticks in Northern Africa including Morocco (Sarih et al., 2003) and Tunisia (Zhioua et al., 1999), both studies were performed on *Ixodes* ticks. Continuous surveillance of tick populations for the presence of *B. burgdorferi* (s.l.) and the presence of *Ixodes* spp. is suggested.

4.4 Conclusion

The present study investigated the species identification of hard ticks infesting dogs and birds from Nigeria. It also investigated the prevalence of selected tick-borne pathogens in these ticks. The tropical lineage of *R. sanguineus* (s.l.) and *H. leachi* were the most abundant tick species infesting Nigerian dogs. The presence of a *Haemaphysalis* spp. Closely related to *H. longicornis* infesting birds in Nigeria was demonstrated in this study. High prevalence of *E. canis* in *R. sanguineus* ticks collected from Nigerian dogs was reported, demonstrating that *E. canis* is a major concern in Nigerian dogs.

Chapter 5 : Molecular characterization of CVBPs and associated risk factors from Nigerian dogs

5.1 Background

CVBPs include viruses, bacteria, and parasites including protozoa and helminths that infect canines. The most common CVBPs include *Ehrlichia*, *Anaplasma*, *Babesia*, *Hepatozoon*, *Borrelia*, *Bartonella*, and *Rickettsia* and *Dirofilaria* species. They are usually transmitted by ticks and insects (Irwin, 2014). Numerous factors including continuous movement of people, animals, or goods through different means of transport have been established to be responsible for the emergence of ticks and CVBPs in different parts of the globe (Medlock et al., 2007, de Marco et al., 2017). Infection of dogs with more than one species of CVBPs are common and may present with varying non-specific clinical signs (Otranto et al., 2009, Spolidorio et al., 2011, Santamaria et al., 2014, Aktas et al., 2015, Alho et al., 2017, Attipa et al., 2017). Certain species of CVBPs such as *Borrelia burgdorferi* (s.l.), *Ehrlichia*, *Anaplasma phagocytophilum* are zoonotic and potential threats to human public health (Irwin, 2014). Effective control of CVBPs is therefore beneficial to both the animal and human population.

There are few reports on the prevalence of CVBPs in Nigeria focusing on one or two pathogens and regions in Nigeria (Van Veen and Blotkamp, 1975, Ugochukwu et al., 2016, Daramola et al., 2018, Adamu et al., 2014). To date, no study has assessed risk factors associated with dogs positive to CVBP via PCR diagnostics. The aim of this study is to detect multiple CVBPs using polymerase chain reaction (PCR) and evaluate the risk factors associated with dogs positive to CVBP using PCR.

5.2 Results

5.2.1 Prevalence of CVBPs among Nigerian dog study population based on PCR screening test

A total of 259 canine blood samples were screened for various CVBPs using PCR. Dog breed identification was determined by participating veterinarians; 179 dogs were an exotic breed, while 80 of the dogs examined were classified as indigenous breeds. In general, 57.9% (150/259) of the canine blood samples screened were positive for at least one canine vector-borne pathogen. *Ehrlichia* spp. recorded the highest prevalence of 40.5% (105/259), followed by *Babesia* spp. with a prevalence of 17.4% (45/259) via qPCR. Moreover, 7.3% (19/259) of the total samples tested positive for both *Ehrlichia* and *Babesia* via qPCR (Figure 5.1). Neither *Dirofilaria* and nor *B. burgdorferi* DNA was detected from canine blood samples tested via qPCR (Table 5.1). The following criteria was used to establish if a sample was positive or not during qPCR. First, all the triplicate wells on the qPCR plate must have had the same amplification curve, melting curve and temperature. Second, the samples identified to be positive from that plate must be confirmed by Sanger sequencing, and NCBI nucleotide blast search must confirm the presence of such pathogens. For the *Babesia* and *Ehrlichia* species, certain nucleotide sequences retrieved were noticeably short with an inadequate quality and could not be identified at species level or included in the phylogenetic trees. Such results were simply termed to be *Babesia* or *Ehrlichia* species positive.

In addition, reports from CVBPs prevalence and Cohen's Kappa index values (95% confidence interval) calculated using results from POC test and PCR-based test on canine blood samples collected from Nigerian dogs demonstrated a poor agreement ($\kappa < 0$) between POC and PCR-based test used in this study (Table 5.4).

5.2.2 CVBPs prevalence based on qPCR results and associated risk factors

The model to assess risk factors was built using information on all dogs that tested positive for all CVBPs (piroplasm *18S* gene or *Ehrlichia p30* gene primers qPCR results or both) rather than a single pathogen. Only results obtained from the *E. canis P30* primers were used in the risk assessment model, as the *Ehrlichia* generic PCR primers were less sensitive. The summary of multivariate analysis for CVBPs risk factors shows that exotic dogs were more likely to test positive to CVBPs via qPCR than indigenous dog breeds (95% CI 0.28-0.81; p=0.007). In addition, dogs examined during the rainy season were more likely to test positive to CVBPs via qPCR than those examined during the dry season (95% CI 0.29 - 0.87; p =0.014) (Table 5.3).

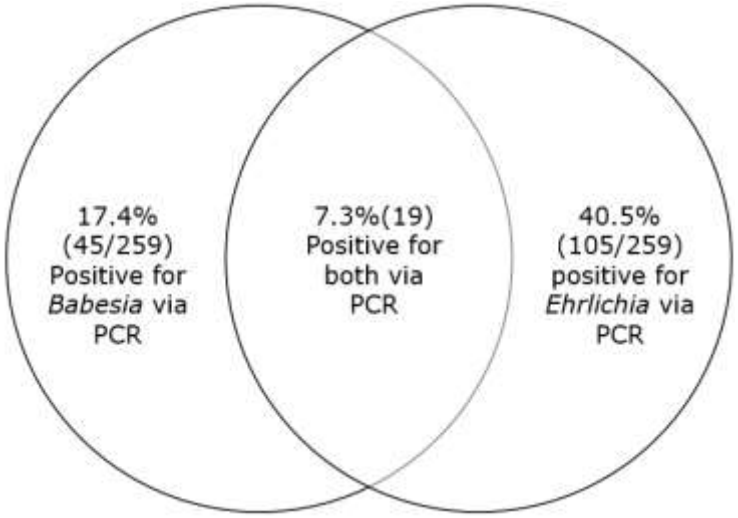


Figure 5.1: Percentages and number of canine blood samples that were positive to both *Babesia* and *Ehrlichia* via PCR screening test.

Table 5.1: Prevalence of CVBPs among Nigerian dogs (n = 259) based of qPCR screening test.

Pathogen	qPCR-positive (%)	95% CI	qPCR-negative (%)
Single infection			
Babesia spp.	45 (17.4%)	12.7 – 22.4	214 (82.6%)
Ehrlichia spp.	105 (40.5%)	34.7 – 46.7	154 (59.5%)
Total	150 (57.9%)		109 (42.1%)
Mixed infection			
Babesia spp. + *Ehrlichia* spp.	19 (7.3%)		

+: signifies qPCR positive results for more than one pathogen, n: sample size. Note, the term 'infection' used here only describe positivity to qPCR test and not actual infection.

Table 5.2: Summary of variable factors and univariate analysis of potential predictors associated with CVBPs prevalence in Nigerian dogs based on qPCR screening test (n = 259).

Variable	level	Total tested (%)	qPCR positive (%) for CVBPs	OR 95% CI	P value
Age	Young and adult	196	96 (49%)	Reference	
	puppy	63	34 (54%)	0.82, 0.46 — 1.45	0.491
Sex	Male	154	77 (50%)	Reference	
	female	105	53 (50.5%)	1.02, 0.62 — 1.67	0.940
Breed	Exotic	179	100 (55.9%)	Reference	
	Indigenous	80	30 (37.5%)	0.47, 0.28 — 0.81	0.007
Body weight (kg)	≤20 kg	172	84 (48.8%)	Reference	
	≥21kg	87	46 (52.9%)	1.18, 0.70 — 1.97	0.540
Season of the year	Rainy (April - October.)	181	100 (55.2%)	Reference	
	Dry (November - March)	78	30 (38.5%)	0.51, 0.29 — 0.87	0.014
Presence of ectoparasites	Yes	153	82 (53.6%)	Reference	
	No	106	48 (45.3%)	1.4, 0.85 — 2.29	0.189
Location	Benue	223	109 (48.9%)		
	Kaduna	14	5 (35.7%)		
	FCT	12	8 (66.7%)		
	Ibadan	10	8 (80%)	0.58, 0.19 — 1.79	0.134

CI: confidence interval, n: sample size, OR: odds ratio, ≤: less than or equal to, ≥ greater than or equal to, CVBPs: canine tick-borne diseases. The model to assess risk factors was built using information on all dogs that tested positive for all CVBPs (*Babesia* or *Ehrlichia* or both) rather than a singular pathogen.

Table 5.3: Summary of adjusted model on multivariate logistic regression analysis of risk factors associated with CVBPs detected using qPCR.

Variable	Adjusted OR	95% CI	P value
Breed			
Exotic	Reference		
Indigenous	0.5	0.27 – 0.82	0.008*
Season			
Rainy (April – October)	Reference		
Dry (November – March)	0.5	0.28 – 0.86	0.013*
Age			
Young and adult	Reference		
Puppy	1.4	0.76 – 2.49	0.297
Sex			
Male	Reference		
Female	0.9	0.53 – 1.51	0.688
Ectoparasites (ticks)			
Yes	Reference		
No	1.4	0.81 – 2.29	0.236

CI: confidence interval, n: sample size, OR: odd ratio, *p values statistically significant ($p<0.05$).

5.2.3 Piroplasm species PCR results

The piroplasm *18S* gene qPCR test identified 17.4% (45/259) as positive. Although, all the 45 PCR products were Sanger sequenced, only 8/45 Sanger sequences including six *B. vogeli,* and two *B. rossi* (Figure 5.2) were of adequate quality. The quality of the remaining positive samples was not adequate for species identification using phylogeny construction, they were only identified at genus level as *Babesia* spp. using the NCBI nucleotide blast search algorithm. The *Babesia* species phylogenetic tree constructed was rooted to the genus *Plasmodium vivax*, a closely related member of the phylum Apicomplexa. The initial unrooted trees (not presented here) suggested *Plasmodium vivax* as the most divergent member of the group.

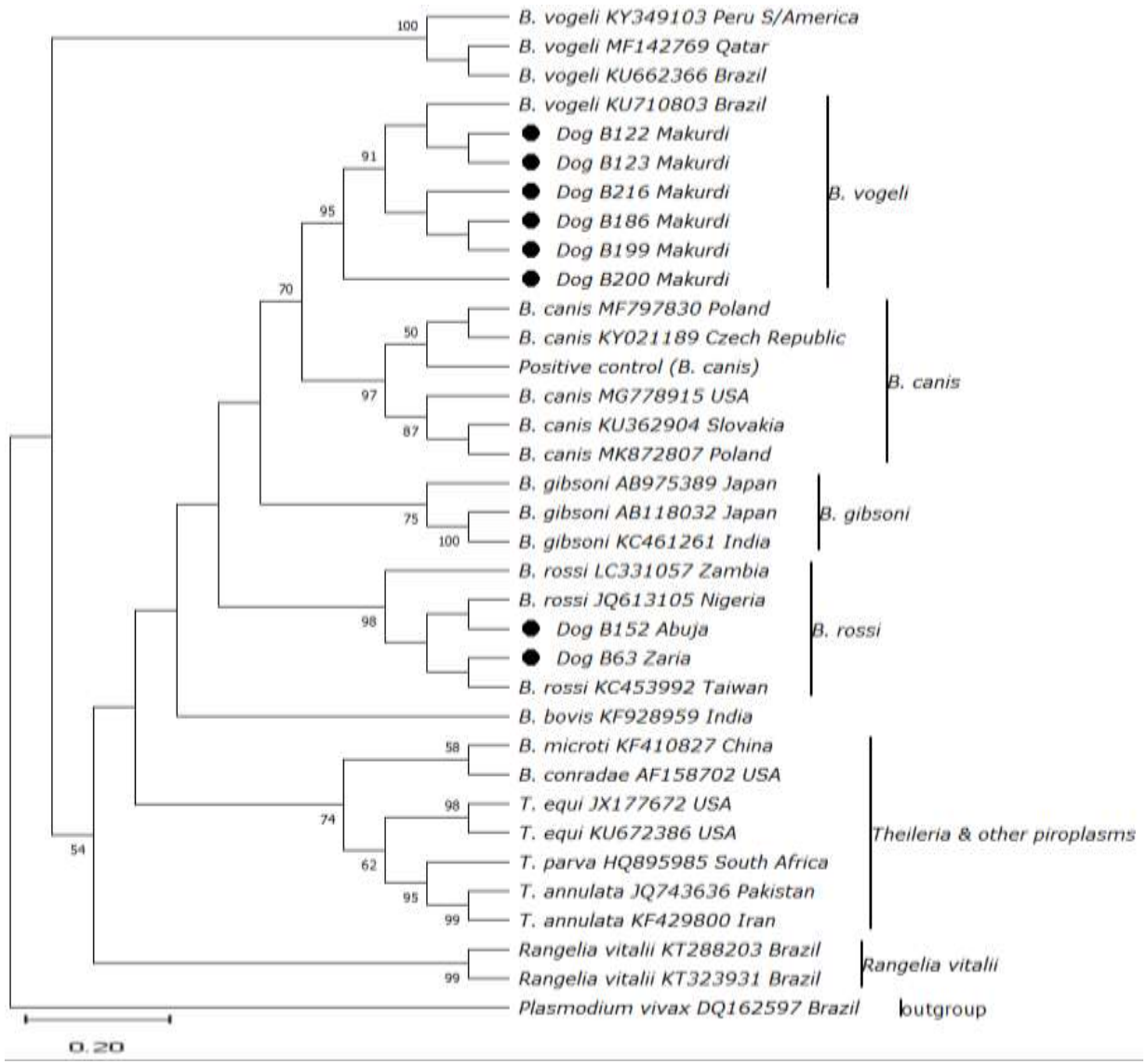

B. vogeli KY349103 Peru S/America
B. vogeli MF142769 Qatar
B. vogeli KU662366 Brazil
B. vogeli KU710803 Brazil
Dog B122 Makurdi
Dog B123 Makurdi
Dog B216 Makurdi
Dog B186 Makurdi
Dog B199 Makurdi
Dog B200 Makurdi
B. vogeli
B. canis MF797830 Poland
B. canis KY021189 Czech Republic
Positive control (B. canis)
B. canis MG778915 USA
B. canis KU362904 Slovakia
B. canis MK872807 Poland
B. canis
B. gibsoni AB975389 Japan
B. gibsoni AB118032 Japan
B. gibsoni KC461261 India
B. gibsoni
B. rossi LC331057 Zambia
B. rossi JQ613105 Nigeria
Dog B152 Abuja
Dog B63 Zaria
B. rossi KC453992 Taiwan
B. rossi
B. bovis KF928959 India
B. microti KF410827 China
B. conradae AF158702 USA
T. equi JX177672 USA
T. equi KU672386 USA
T. parva HQ895985 South Africa
T. annulata JQ743636 Pakistan
T. annulata KF429800 Iran
Theileria & other piroplasms
Rangelia vitalii KT288203 Brazil
Rangelia vitalii KT323931 Brazil
Rangelia vitalii
Plasmodium vivax DQ162597 Brazil
outgroup
100
91
95
70
50
97
87
75
100
98
58
98
74
54
62
95
99
99
0.20

Figure 5.2: Piroplasm maximum-likelihood phylogenetic tree constructed using nucleotide sequences generated from this study and GenBank reference sequences with *Plasmodium vivax* as an outgroup.

Key: Nigerian Piroplasm species nucleotide sequences are named dog "Bxxx," "B" and "xxx" represents blood sample and individual sample serial number, respectively. GenBank sequences are labeled using tick genera/species, GenBank accession and country of origin. The tree showed piroplasm species obtained in this study were closely related to *B. vogeli* and *B. rossi* from Nigeria and other parts of the world. The phylogenetic tree is drawn to display bootstrap support values ≥50%.

5.2.4 *Ehrlichia* species PCR results

In addition, 40.5% (105/259) of the total samples were positive for *E. canis* using the second pair of *E. canis p30* gene qPCR primers, while 1.9% (5/259) of the total samples tested positive via both *Ehrlichia* PCR protocols (Figure 5.3). Three percent (8/259) of the total canine blood samples screened were positive for *Ehrlichia* species using the first pair of *Ehrlichia 16S rRNA* gene generic conventional PCR primers. Six of the positive samples (6/8) recovered from *Ehrlichia 16S rRNA* generic conventional PCR (confirmed via gel electrophoresis) showed 100% identity with *Ehrlichia* spp., while 2/8 (B174 and B259) were identified as *Anaplasma platys* following Sanger sequencing, and NCBI nucleotide blast search. Both B174 and B259 were positive via the *E. canis p30* gene qPCR, though only B259 was positive for both *Ehrlichia* and *Anaplasma* species using the IDEXX® serological test kit.

DNA nucleotide sequence obtained using *Ehrlichia 16S rRNA* and *p30* gene sets of primers were of inadequate quality and between 50 to 100 bp long. Therefore, species classification of sequence using phylogenetic analysis was not possible and species identification was based on the use of the online NCBI nucleotide blast search.

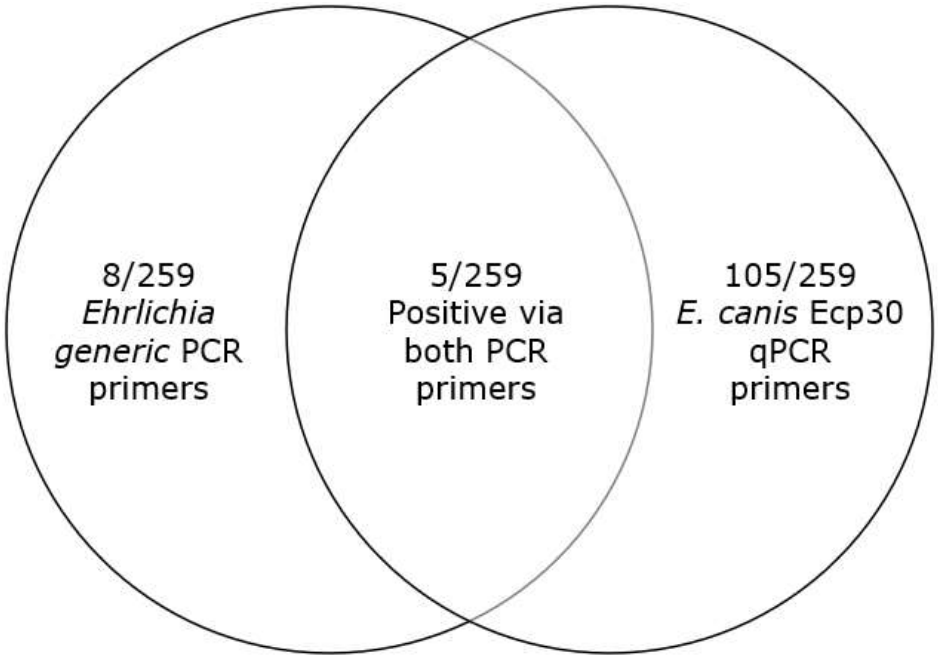


Figure 5.3: Venn diagram showing blood samples positive for *Ehrlichia* species using *Ehrlichia* GE2F/EHRL3 and Ecp30-f/Ecp30-r pair of PCR primers.

5.2.5 Borrelia and *Dirofilaria* species PCR results

A total of 259 canine blood samples were screened for the presence of *Borrelia* and *Dirofilaria* species DNA using generic *Borrelia* and filaroid-specific published *16S rRNA* gene qPCR primers. All samples tested were neither positive for *Borrelia* nor *Dirofilaria* species. The qPCR protocols used were assessed and optimized to be one of the most efficient qPCR protocols used in this study. Moreover, these are optimized and published qPCR protocols that have been used in previous studies. Positive controls for each qPCR protocol were assessed and identified to be positive. Although poor sample quality and concentration were the greatest challenges faced in this study, we confirmed the presence of canine DNA from all samples extracted using the canine GAPDH gene PCR (Tarlinton et al., 2013). Also, these same samples served as DNA templates for the Piroplasm *18S rRNA* and *Ehrlichia 16S rRNA* gene PCR protocols where positive results were obtained. Therefore, these were recorded as true negative results.

Table 5.4: Percentage prevalence of CVBPs and Cohen's Kappa index values (95% confidence interval) calculated using results from POC and PCR-based test on Nigerian canine blood samples.

CVBP species	**POC test: % (n)**		**PCR-based test: % (n)**		**K (CI)**
	Positive	negative	Positive	PCR +ve from POC -ve	
Ehrlichia canis	29.7 (77)	70.3 (182)	40.5 (105)	28.6 (74)	-0.00 (-0.12 — 0 .11
Anaplasma	10.8 (28)	89.2 (231)	Np	Np	Np
Dirofilaria	0.4 (1)	99.6 (258)	0 (0)	0 (0)	Np
Babesia	Np	Np	17.4 (45)	Np	Np

Note: point-of-care test (POC: IDEXX antigen test); % (n) represents percentage prevalence and/or negative and number of samples in parenthesis; K (CI) represents Cohen's kappa index value and 95% confidence interval in parenthesis; PCR +ve from POC -ve represents percentage and number of samples that were negative on the POC test but later identified to be positive via PCR-based test; Np represents calculations not presented where pathogens were only detected via one diagnostic assay.

5.3 Discussion

The aim of this study was to confirm the preliminary findings study on CVBP prevalence (Chapter 3) using the POC test kits, with the addition of an extra pathogen (*Babesia*) known to be present at a high prevalence in Nigeria that is not included in the POC test kit. We also applied findings from CVBP prevalence based on qPCR in multivariate modelling, with an attempt to determine the risk factors for dogs testing positive to CVBPs via qPCR.

The results of the risk factor assessment based on qPCR prevalence showed that exotic dog breeds were significantly at risk of testing positive to CVBPs via qPCR than indigenous dog breeds. Dogs were more likely to test positive to CVBPs pathogens via qPCR during the rainy season than the dry season of the year, confirming that season of the year is a risk factor for CVBPs infection. The qPCR adjusted model represents current infection with CVBPs in dogs examined in this study. Although, exotic dog breeds were over presented in this study, it is likely that findings from this study support the popular conception by Nigerian veterinarians that the presence of clinical disease and parasitaemia detectable by PCR is less likely to be reported in Nigerian indigenous dog breeds. Factors such as dog breed, immunity, and co-infection etc. can determine the host response to CVBP infection. It is likely Nigerian indigenous dogs have immunity due to previous exposure to CVBP infection and maintain protective level of antibody post infection. While exotic dog breeds (which tend to be valued and confined) without sufficient immunity and/or lack of prior exposure to CVBPs lack protective immune response, leading to the presence of clinical disease or positive PCR test (Boulanger and Wikel, 2021) recorded in this study. There may be the presence of genetic factors responsible for inherited tolerance to CVBP infection in Nigerian dogs as reported in cattle (Latre de Late et al., 2021, Wragg et al., 2022).

Results from this study showed that *Ehrlichia spp*. is the most prevalent CVBP (40.5%) from the Nigerian canine population, particularly in Benue state, where 86% of samples examined in this study were collected. The prevalence for *Ehrlichia* in this study is higher than previous reports using molecular techniques in Nigeria, due to the assessment of more samples in this study. Previous studies demonstrated prevalence of 11% and 12.7% from a total of 100 and 180 sample sizes respectively (North central: Plateau and Kwara states, South-south: Rivers state, and North west: Kaduna state) (Kamani et al., 2013b), 7% from a sample size of 100 dogs (North central: Plateau sate) (Adamu et al., 2014), and 22.9% from a sample size of 205 dogs (South West: Ogun state) (Daramola et al., 2018). These also suggest differences in geographical locations and a more robust study with a larger sample size is required to provide detailed information on CVBPs in Nigeria.

Moreover, co-infection with *Ehrlichia* and *Babesia canis* was found in 7.3% of samples. Canine co-infection with *Babesia* and *Ehrlichia* species is common and has been reported in previous studies (Inpankaew et al., 2016). Infection with multiple CVBPs can also result in non-specific clinical signs and severe disease in the affected animal (Otranto et al., 2010b). CVBP infection poses health challenges such as anaemia in infected dogs (Rojas et al., 2014), and confronts clinicians with difficulty regarding diagnosis and medical management (Otranto et al., 2009). Although, this study did not focus on clinical disease manifestation and management of dogs with co-infection, these findings showed that CVBPs in Nigeria may include more than one pathogen including *Babesia*, *Ehrlichia* and *Anaplasma* species.

The prevalence of *Anaplasma platys* was extremely low in this study (0.8%), previous studies in Nigeria have reported relatively higher prevalence from canine blood using microscopy: 12.5% (n = 8) (Happi and Antia, 2012), and PCR: 4% (n = 100) (Adamu et al., 2014), and 6.6% (n = 180) (Kamani et al., 2013a). The preliminary screening for CVBPs in this study using IDEXX test kit reported 10.8% (28/259) prevalence of *Anaplasma* species supporting previous

studies in Nigeria. It is likely that the low prevalence of this pathogen may be attributed to the generic PCR protocol inefficiency, the generic PCR protocol used for screening both *Ehrlichia* and *Anaplasma* spp. was less sensitive.

Recent studies using molecular techniques have established the presence of *B. vogeli* and *B. rossi* from Nigerian dogs (Kamani et al., 2013a, Sasaki et al., 2007, Kamani et al., 2010, Adamu et al., 2014), as reported in this study. *B. gibsoni* was not reported in this study supporting the study by Kamani et al. (2019a) that *B. gibsoni* has not been reported from Nigeria using molecular techniques. The authors have no clear explanation for the absence of *B. gibsoni* in Nigeria, *B. gibsoni* has been reported in Australia (Jefferies et al., 2003), Europe (Hartelt et al., 2007), America (Birkenheuer et al., 2005), and Asia (Miyama et al., 2005), where tick species such as *Haemaphysalis* species (including *H. longicornis*) and *R. sanguineus* present in Nigeria have been reported as known vectors (Kamani et al., 2019a). Additional studies using a larger tick and canine blood sample size to establish the presence or absence of *B. gibsoni* in Nigeria is required.

qPCR results from a total of 259 blood samples targeting the *Borrelia 16S* gene showed that all Nigerian canine blood samples tested negative for the presence of *Borrelia 16S rRNA* gene DNA, confirming the POC test results from the previous chapter. The primary vectors known to transmit *B. burgdorferi* (s.l.) (*Ixodes spp.)* are also not reported in Nigeria (Krupka and Straubinger, 2010, Rudenko et al., 2011), and this result is not entirely unexpected. Little information is available on the prevalence of *B. burgdorferi* (s.l.) from canine blood samples in Africa, apart from the seroprevalence reports of 1.8% (9/500) in Egypt (Selim et al., 2021a), and 5.2% (11/213) in Algeria (Azzag et al., 2015). Most of the previous studies reporting *B. burgdorferi* via molecular techniques in Africa have been carried out on tick species known to be associated with dogs and not canine blood samples. These studies reported prevalence of 30.5% (Zhioua et al., 1999), and 34% (Younsi et al., 2001), from *Ixodes ricinus* in Tunisia. Other *Borrelia* species such as those responsible for tick-borne relapsing

fever syndromes in people from *Rhipicephalus evertsi* (Reye et al., 2012), and *Candidatus Borrelia kalaharica* from *Ornithodoros savignyi* (Cutler et al., 2018), have been reported in Nigeria. *B. burgdorferi (s.l.)* appears not to be present in the Nigerian canine population as demonstrated via serological and PCR methods used in this study.

In the same way, qPCR screening of a total of 259 blood samples for the presence of *Dirofilaria* using filariid-specific qPCR primers indicated the absence of *Dirofilaria* species in Nigerian dogs. To date, there are three separate reports four decades apart on *Dirofilaria* prevalence in Nigeria using morphological methods including a 9.2% (34/369) *D. repens* microfilariae (Van Veen and Blotkamp, 1975), 3.4% (4/119) *D. immitis* microfilariae (Ugochukwu et al., 2016), and 2.2% (4/186) *D. immitis* microfilariae (Ogbaje and Danjuma, 2016), prevalence. The published filaroid specific qPCR primers used for the detection of filarial species in this study were designed using consensus sequence obtained from alignment of animal and human parasitic filarial nematodes complete mitochondrial reference genomes including *D. immitis, D. repens, Onchocerca volvulus, Onchocerca flexuosa, Wuchereria bancrofti, Brugia malayi, Setaria digitata*, and *Setaria tundra*. This assay is recommended to be highly efficient for the detection of *Dirofilaria* and other filarial species (Kronefeld et al., 2014) as confirmed on *D. immitis* control DNA samples used in this study. One would expect a higher prevalence in this study since the PCR diagnostic tool is more sensitive than morphological methods used in previous studies. The absence of *Dirofilaria* species could be due to lack of decent sample quality, as most clinicians were less generous with sample collection on FTA card. Low DNA concentrations were recorded from all samples following DNA extraction however, other blood borne pathogens were amplified from these specimens. It is more likely that the low prevalence of *Dirofilaria* species recorded in Nigeria is due to the routine use of ivermectin as a broad spectrum antiparasitic drug (Jin et al., 2013, Stenzenberger and Gothe, 1999, McKellar and Gokbulut, 2012). The PCR test is dependent on the detection of circulating microfilaraemia.

Routine use of ivermectin can also results in a "slow kill" (SK) effect on adult filarial worms, alongside clearance of microfilaraemia leading to a negative PCR test (Wolstenholme et al., 2016).

It is relevant to state that the agreement between POC and PCR-based test was demonstrated to be poor ($\kappa < 0$) following Cohen's kappa index for *E. canis* prevalence. There was little concordance between these two methods regarding the number of samples identified to be positive for *E canis*. The lack of agreement between the POC and PCR assay recorded in this study is similar to another recent study (Lara et al., 2020), as more samples negative on the POC test were positive via PCR. The poor agreement may suggest the possibility for false positive and/or negative results following CVBDs diagnosis with one test.

IDEXX test kits are commercially available, affordable, and widely used for field or clinical diagnosis of dogs exposed to CVBP infection in the field or veterinary clinics. The absence of concordance between the two tests is due to the ability of the POC test kit to detect antibodies against *E. canis* from convalescent dogs without active infection, while the PCR assay used in this study only detected dogs with acute or active infection. This has been demonstrated in previous studies on detection of antibodies against *Ehrlichia, Anaplasma, B. burgdorferi* as well as antigen to *D. immitis* which suggested that a negative POC test results should be interpreted with caution as 28.6 % (74/182) of canine blood negative via IDEXX test kit were reported to be positive for *E. canis* via PCR-based test (Chandrashekar et al., 2010) as reported in this study.

5.4 Conclusion

Taken together with the POC results from the earlier chapter, this study suggests that to date canine Lyme disease (*B. burgdorferi* (s.l.)) is absent in Nigeria. This is expected as the disease is regarded as one of temperate regions and the tick vector is not thought to be present in Nigeria. The low prevalence of canine *Dirofilaria* spp. with only one animal reactive on the POC test kit and all negative

via PCR is surprising, given previous reports of *Dirofilaria* in Nigeria. It is likely the widespread use of Ivermectin an antiparasitic with SK effect on canine heart worm has drastically reduced the prevalence of this pathogen in Nigerian canine population. The anticipated prevalence of 41.8% was used to estimate sample size in this study after taking mean average of all CVBPs prevalence studies in Nigeria. The estimated total sample size to determine the prevalence of CVBPs at 95% confidence interval was 374. A total of 259 samples were collected at 90% confidence interval, showing that the total sample size assessed in this study was adequate to detect the expected prevalence.

This report showed that the cause of canine ehrlichiosis (*Ehrlichia* spp.), and babesisosis (*Babesia* spp.) remains the most prevalent pathogens infecting Nigerian dogs. This study confirmed the absence of *B. canis*, *B. gibsoni*, and *B. vulpes* n. sp in Nigerian dogs as previously reported (Obeta et al., 2020, Kamani et al., 2019a). This is likely due to the absence of tick vectors including *D. reticularis* (*B. canis*) and *I. ricinus (B. vulpes* n. sp) reported to transmit these pathogens in Europe, America, and Asia (Mierzejewska et al., 2018, Birkenheuer et al., 2020, Barash et al., 2019, Checa et al., 2018, Sang et al., 2021).

These findings suggest that exotic dog breeds are more likely to test positive to CVBPs via PCR and that active infections are more common during the rainy season. This contrasts with the POC results for *Ehrlichia* and *Anaplasma*, which indicated a higher risk in older dogs owing to cumulative antibody levels over time, with no effect of season or breed. Seropositivity reflects a prior exposure to CVBPs with older animals having more chance to be exposed. Higher infection with CVBPs in the rainy season is to be expected, as the rainy season is characterized by high tick abundance.

The finding that the risk for active infection is lower in native breed dogs is however intriguing. Nigerian vets and dog owners hold the belief that these animals are less susceptible to disease when compared with imported exotic dog breeds and this molecular study provides the first evidence to show that this

belief may have some basis. Factors such as dog breed, immunity, and co-infection etc. can determine the host response to CVBP infection. As genetic resistance to both ticks and tick-borne pathogens is also known to occur in African cattle (Mattioli et al., 2000, Wragg et al., 2022) and goats (Chiejina and Behnke, 2011), adequate understanding of this phenomenon requires further investigation. Comparison of results from POC and PCR-based test in this study using Cohen's Kappa index demonstrated that a combination of both the POC and PCR-based test is more likely to offer accurate and reliable results for effective diagnosis of and treatment of CVBDs (specifically *E. canis*).

Chapter 6: Metagenomics profiling of ticks and canine blood from Nigeria

6.1 Background

Ticks are external parasitic arthropods that spend part or the whole of their life cycle feeding mostly on blood from vertebrate animals, transmitting emerging and zoonotic bacterial, protozoa and viral pathogens between vertebrate hosts worldwide (Shaw et al., 2001, Chomel, 2011, Biernat et al., 2014, Balling et al., 2015, de Marco et al., 2017, Ogo et al., 2012). *Ehrlichia*, *Anaplasma*, *Babesia*, *Theileria, Hepatozoon*, *Borrelia*, *Bartonella*, and *Rickettsia,* tick-borne encephalitis virus (TBEV) are some infectious microbes transmitted by ticks to animals and humans (Shaw et al., 2001, Chomel, 2011, Biernat et al., 2014, Balling et al., 2015, de Marco et al., 2017, Ogo et al., 2012, Irwin, 2014).

Tick endosymbionts are intracellular bacteria known to live in mutual association within ticks and are transmitted from adult female ticks to newly emerging larvae through eggs. These endosymbionts include *Coxiella-like, Francisella-like, Rickettsia-like,* and *Wolbachia spp.* For example, DNA of a *Coxiella-like* endosymbiont that shares the same genera with the cause of Q-fever (*Coxiella burnetii)* has been reported from various tick species including *Haemaphysalis*, *Rhipicephalus, Ixodes*, and *Amblyomma* species across the globe (Ben-Yosef et al., 2020, Kobayashi et al., 2021, Tsementzi et al., 2018). In addition, co-infection of *Ixodes, Haemaphysalis*, and *Dermacentor* tick genera with *Coxiella-like, Francisella-like, Rickettsia-like* species have been reported (Spitalska et al., 2018).

Ticks have known restricted blood feeding habits, endosymbionts are responsible for daily delivery of nutrients essential for their survival, reproduction, and transmission of pathogens. It is established that ticks depend on endosymbionts for survival, and reduced ability of ticks to survive, reproduce and transmit pathogens has been observed in ticks following elimination of endosymbionts

with antimicrobial agents (Zhong et al., 2007, Li et al., 2018, Guizzo et al., 2017). Studies on the treatment of the *Coxiella-like* endosymbiont of *R. sanguineus* with antibiotics demonstrated a prolonged feeding period, reduced weight, and number of eggs post completion of bloodmeal (Ben-Yosef et al., 2020). Similarly, the frequency of vertical transmission of bacterial endosymbionts in ticks is reported to be determined by its importance for tick host fitness and reproduction (Ahantarig et al., 2013). The presence and interaction of tick bacterial endosymbionts is also essential for the increase in the number or virulence of pathogens that can be transmitted by ticks, as demonstrated in recent studies on *Dermacentor andersoni* (Gall et al., 2016).

The global interest and knowledge on the distribution of microbial communities of animal hosts and arthropods including ticks of veterinary and medical importance is rapidly increasing. The dynamic nature of ticks requires a close evaluation as microbial organisms have continuously been transmitted vertically via eggs, while different vertebrate animals continue to serve as hosts to various stages in the life cycle of ticks, altering tick microbiota (Varela-Stokes et al., 2017). The use of next generation sequencing to define tick microbiota can provide information regarding factors responsible for the survival and spread of pathogens in ticks. Adequate knowledge of canine and tick microbiota prevalence and interaction is relevant in the development of unique prevention and control measures to target.

Most of the diagnoses of animal ticks and tick-borne pathogens in Nigeria are achieved using morphological identification, culture, thin blood smears, and haematological examination. These methods may be successful for certain pathogens whose morphological and clinical presentation is well established. However, it is difficult to establish diagnosis for ticks and tick-borne pathogens that are exotic or not culturable.

There are few studies on ticks and tick-borne pathogens using serology and PCR to provide information on the prevalence of tick-borne pathogens in dogs, and

only one recent study on hard tick metagenomics by Agwunobi et al. (2021) is available in Nigeria. There is insufficient data on tick surveillance, including genomic surveillance of ticks and tick-borne pathogens using NGS, there is also little information on the prevalence of tick bacterial endosymbionts. This study presents information on the metagenomics profiling of canine blood and ticks, including a *Coxiella-like* endosymbiont putative genome. The report provides background information justifying the need for a comprehensive tick surveillance programme in Nigeria.

6.2 Results

6.2.1 Sample collection

The study examined a total of six canine blood samples collected from young to adult dogs including 4/6 males, 2/6 females, 5/6 tick infested and 1/6 non-tick infested dog, 5/6 exotic, and 1/6 indigenous breeds. In addition, four tick samples including 3/4 canine hard ticks and 1/4 bird tick with unreported data (labelled as NLB6D) were assessed. Overall, metagenomics sequencing and analysis was applied to a total of ten samples in this study (Table 6.1).

Table 6.1: Showing demographic details of canine blood and tick samples used in metagenomics sequencing.

Sample ID	season	Dog age or stage of ticks	Dog sex	Dog/bird breed	Tick infestation	Tick species and morphology	Location
Canine blood samples							
B35	rainy	YA	female	Indigenous	Yes	*R. sanguineus*	BN-Mkd
B144	rainy	YA	male	Exotic	No	N/A	BN-Mkd
B153	rainy	YA	female	Exotic	Yes	*R. Sanguineus*	FCT-Abj
B168	rainy	YA	male	Exotic	Yes	*R. Sanguineus*	BN-Mkd
B194	dry	YA	male	Exotic	Yes	*R. Sanguineus*	BN-Mkd
B210	rainy	YA	male	Exotic	Yes	*R. Sanguineus*	BN-Mkd
Hard tick samples							
T5A	rainy	adult	N/A	Indigenous	N/A	*Haemaphysalis sp.*	BN-Mkd
T223D	rainy	adult	N/A	Exotic	N/A	*R. Sanguineus*	BN-Mkd
T231C	rainy	adult	N/A	Exotic	N/A	*R. Sanguineus*	Oyo-Ibd
NLB6D	N/A	adult	N/A	*Gallus domesticus*	N/A	N/A	BN-Mkd

KEY: YA means young to adult, N/A: Not applicable, BN-Mkd: Makurdi, Benue state, FCT-Abj: Federal capital territory Abuja, Oyo-Ibd: Ibadan Oyo state.

6.2.2 Quality assessment of NGS sequence after trimming

Forty-two (42) paired end reads were sequenced from ten samples (appendix: Table 8.2), 26/42 paired end reads were sequenced from six canine blood samples (appendix: Table 8.2), while 16 paired end reads were sequenced from four hard tick samples. Overall, a total of 800309700 raw reads were obtained from 42 datasets generated, 98.8% (790847797) of the raw reads passed quality assessment and trimming (Table 8.2). Similarly, all paired end reads generated from canine blood samples had ≥ 83% unique reads and ≤ 20% duplicate reads. While all paired end reads obtained from hard tick sample datasets had ≥ 67% and unique and ≤ 33% duplicates reads. Hard tick sample datasets from the Nigerian local bird (NLB6D) accounted for most of the duplicate reads recorded from tick sample datasets (Figure 6.1). Only 1% of 42 datasets contained over-represented sequences (Figure 6.2). All sequence reads had a mean Phred Score of 33 (Figure 6.3). A total of 17/42 reads had ≥ 50% GC content, 18/42 had slightly abnormal GC content of ≥ 40%, while 7/42 of reads had an unusual GC content (Figure 6.4). Sequence length distribution from canine and tick sample datasets were within a range of 147 — 150 bp (Figure 6.5). In general, the result of sequence read quality assessment showed that these sequences were of sufficient quality to be used in downstream analysis.

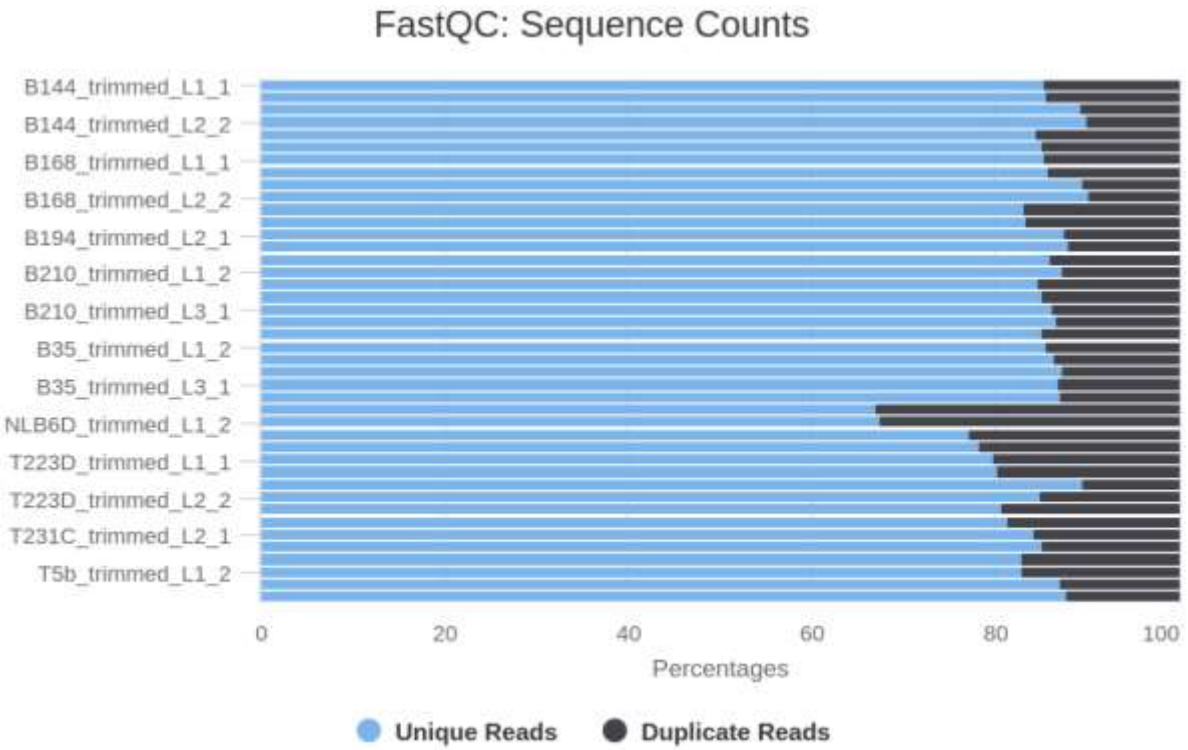


Figure 6.1: Percentage of unique and duplicate reads from selected reads following quality filtering and trimming. Datasets generated from 10 samples are shown on the y-axis, the chart has been trimmed to only show the number of unique and duplicate reads.

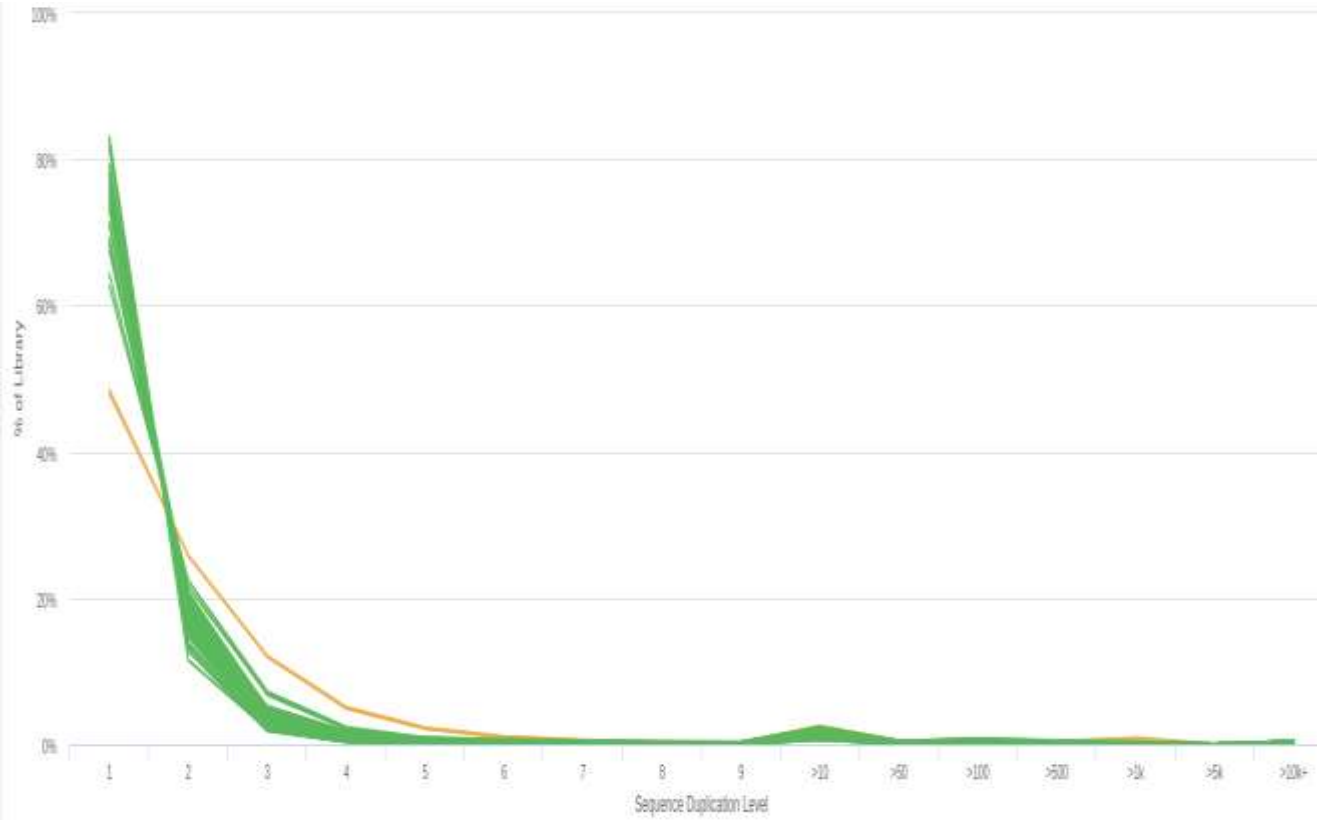


Figure 6.2: Relative level of duplication for individual sequence reads. Green lines indicate sequence reads without duplicates, while the yellow lines indicate sequence reads with duplicates.

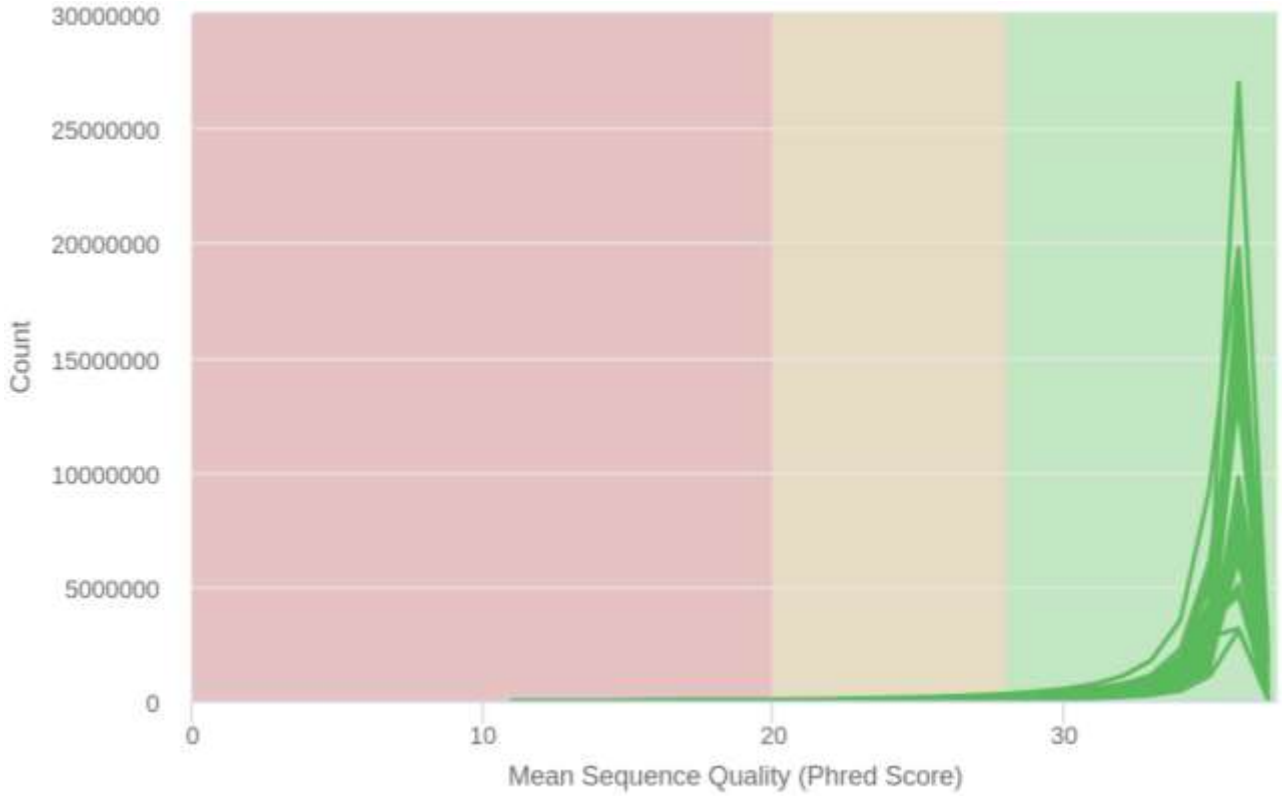


Figure 6.3: A chart showing number of sequences reads (y-axis) and average quality scores (x-axis), majority of reads have quality scores ≥34.

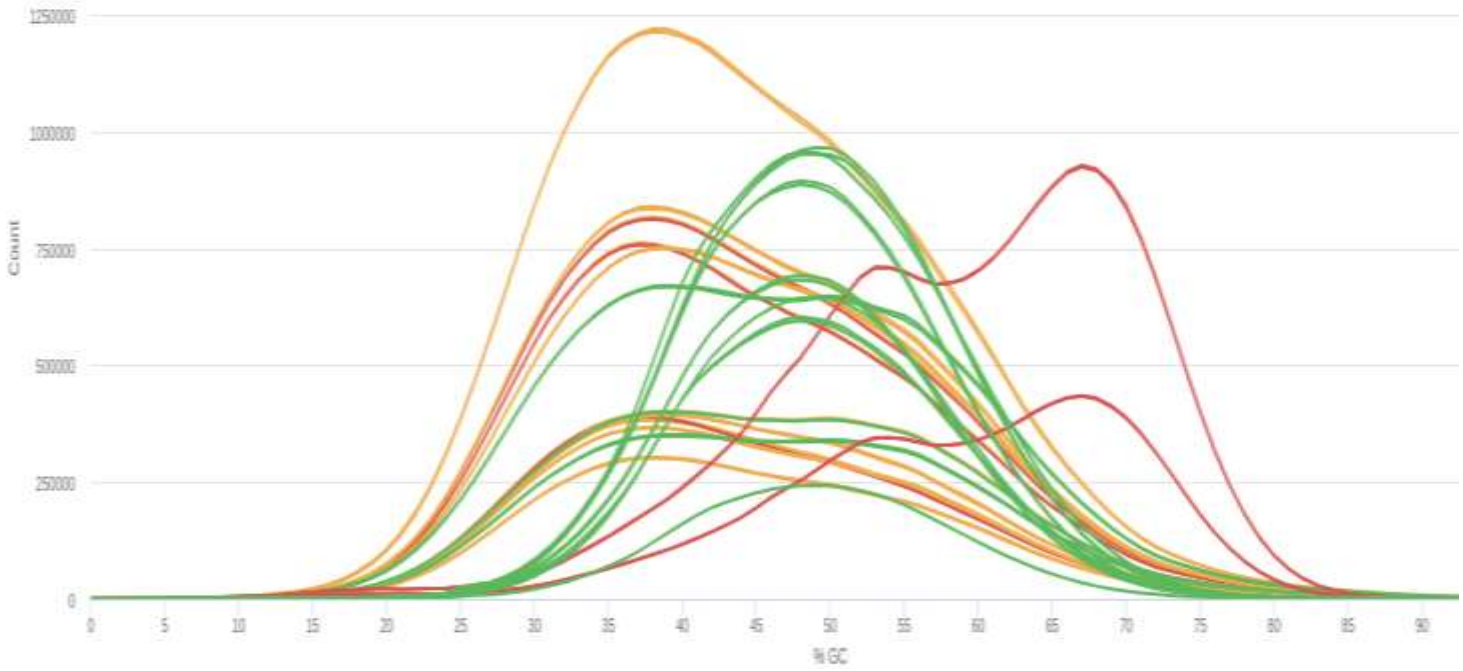


Figure 6.4: Average GC content for sequence reads generated from canine and tick samples.

Key: Reads with normal random library (**green**) have approximate normal distribution (50%). **Yellow** chart lines signify sequence reads with slightly abnormal GC content, while the **red** lines signify reads with an unusual GC content.

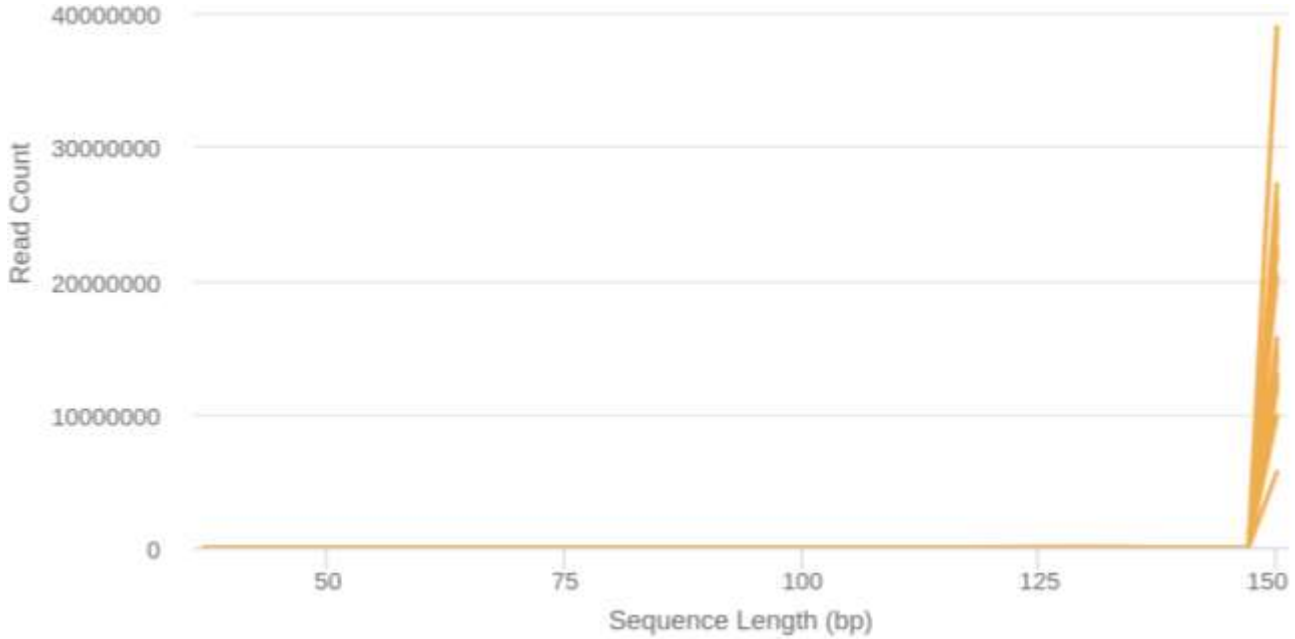


Figure 6.5: Distribution of sequence reads length generated from canine and tick sample datasets showing sequence reads (**yellow** chart, x-axis indicates) generated have average length between 145 — 150 bp.

6.2.3 Removal of canine and tick host DNA

Overall, the alignment statistics generated using Samtools showed that >96% of the total reads obtained from the canine blood sequence reads aligned properly with the canine reference genome used during bowtie2 alignment. Therefore, <4% of the total canine blood sequence reads were extracted as unmapped reads for downstream analysis including denovo assembly, taxonomic classification, and binning (Table 6.2). Conversely, <23% of the total sequence reads from the hard tick datasets mapped to the hard tick reference genomes, while >77% of the reads were extracted as unmapped reads for downstream analysis (Table 6.2).

The longest and shortest contig lengths from the canine blood dataset assemblies were 9.92 Mb kb and 5.88 Mb, respectively, while the longest and shortest contigs (Table 8.3) N50 (minimum contig length required to cover 50% of the genome) were 2824 and 827, respectively. Similarly, the hard tick sample datasets had longest and shortest contig lengths of 633.58 Mb and 78.58 Mb, respectively, whereas the longest and shortest contigs N50 for the hard tick datasets were 124 kb and 6 kb, respectively (Table 8.4). This clearly shows why more sequence reads were assigned taxonomic identification during centrifuge classification, and draft genomes were also generated from the tick sample datasets.

Table 6.2: Showing percentage of mapped and unmapped reads generated from alignment of canine and tick datasets to reference canine and tick host genome using Bowtie2 and Samtools during removal host DNA.

Sample name (host)	QC-passed reads	reads mapped to canine/tick host	Unmapped reads	Mapped (%)	Unmapped (%)
Canine blood sample datasets					
B1444 (canine)	76337856	73867808	2470048	96.76	3.24
B153 (canine)	77835922	75455371	2380551	96.94	3.06
B168 (canine)	47787546	46404856	1382690	97.11	2.89
B194 (canine)	75741334	73162447	2578887	96.60	3.40
B210 (canine)	43428330	42080423	1347907	96.90	3.10
B35 (canine)	103920630	99869449	4051181	96.10	3.90
Total reads	425051618	410840354	14211264	96.66	3.34
Tick sample datasets					
NLB6D (tick)	74990450	7167036	67823414	9.56	90.44
T223D (tick)	11340574	4667733	6672841	41.16	58.84
T231C (tick)	71178434	37810978	33367456	53.12	46.88
T5B (tick)	72579038	2838192	69740846	3.91	96.09
Total reads	230088496	52483939	177604557	22.81	77.19

6.2.4 Taxonomic classification

Note that during assessment of centrifuge classification report, taxa assigned ≥10 sequence reads were considered as "major microbe in the taxonomic profile". In addition, identification of microbial classification at specific species level were termed "specific microbial ID in metagenome" if >50 sequence reads were assigned to the species rank, and "uncertain" if <50 sequence reads were assigned to the species rank (Table 6.3 and Table 6.4).

Next generation sequencing and taxonomic classification of sequence reads obtained from canine blood datasets identified several operational taxonomic units (OTU) that were assigned four distinct microbial domains (Figure 6.6) including Archaea (0.6%), Bacteria (29.0%), Eukaryota (2.1%), and viruses (0.5%J). *Euryarchaeota* (0.4%) was the only phylum identified from Archaea domain, while reads assigned to the bacteria domain were classified under six distinct phyla including *Bacteroidetes* (2.2%), Cyanobacteria (0.7%), *Deinococcus-Thermus* (0.3%), *Firmicutes* (3.6%), *Proteobacteri*a (9.3%), and *Tenericutes* (0.4%). *Euglenozoa* (0.3%) and *Ascomycota* (1.6%) were the two distinct microbial phyla identified under Eukaryota domain, whereas microbes classified under the domain viruses were identified under four distinct kingdoms including *Bamfordvirae* (0.2%), *Orthornavirae* (0.01%), *Pararnavirae* (0.01%), and *Shotokuvirae* (0.06%). *Leishmania major (Apicomplexa)* was the only canine pathogen genera detected from all canine blood samples assessed in this study. Other specific microbial taxa identified included *Mycobacterium* (*Actnobacteria*), Amsca *Moorei entomopoxvirus* (virus of moths)*, Edwardsiella, Pseudomonas* (Proteobacteria), and *Clostridium* (*Firmicutes*). Mosquito-borne *Plasmodium* (*Euglenozoa*) species known to infect humans was also reported from Nigerian canine blood (Table 6.3 and Figure 6.6). Table 6.3 shows that most of the pathogens tested for during field sampling with the IDEXX test kit and during qPCR were not detected from canine blood sample datasets. Overall, microbial taxa identified here were present in all the six canine blood datasets assessed. In addition, the high number of certain bacteria taxa identified from canine blood datasets including the phylum *Actinobacteria* which consist of the family

Streptomycetaceae (3.9%) etc. may be due to environmental contaminants from the FTA cards used in this study.

Similarly, we identified OTU assigned to fourteen key phyla following taxonomic classification of four hard tick sample datasets cutting across four distinct microbial domains (Table 6.4 and Figure 6.7) including Bacteria (20.02%), Eukaryota (0.97%), Archaea (0.50%) and viruses (0.23%). While the family *Haloarculaceae* was the only family of microbes identified from the Archaeal phyla called *Euryarchaeota*, two distinct phyla were identified under the domain viruses including *Peploviricota* consisting of the family Herpesviridae, and *Nucleocytoviricota* consisting of two families namely *Ascoviridae* and *Baculoviridae* (Table 6.4). Unlike the canine blood datasets, three distinct phyla of veterinary and public health importance were identified from the *Eukaryota* microbial domain including Ascomycota containing the family Saccharomycetaceae (genus *Candida*), Euglenozoa containing the family *Leishmaniinae*, and Apicomplexa where the family *Plasmodiidae* and *Theileriidae* were identified (Table 6.4). In addition, major microbes identified from hard tick datasets taxonomic profile were within bacterial domain including *Proteobacteria* (10.39%), *Actinobacteria* (3.51%), *Firmicutes* (2.13%), *Bacteroidetes* (1.63%), *Tenericutes* (0.13%), *Spirochaetes* (0.09%), *Fusobacteria* (0.08%), and *Chlamydiae* (0.02%) (Table 6.4). Although, most of the microbes identified under Proteobacteria are not tick-borne pathogens, major microbial families of veterinary importance identified in hard tick datasets under the phyla *Proteobacteria* include *Anaplasmataceae (Ehrlichia), Wolbachieae (Wolbachia), Rickettsiaceae (Rickettsia, Orientia), Yersiniaceae (Yersinia), Pasteurellaceae (Haemophilus parainfluenzae), Coxiellaceae,* and *Piscirickettsiaceae (Piscirickettsia)* etc. (Table 6.4). Four distinct bacteria families were identified under the phylum *Actinobacteria* including soil, water and plant or environmental contaminants (*Streptomycetaceae, Nocardiaceae, Microbacteriaceae*), and *Mycobacteriaceae* consisting of *Mycobacterium* spp. (Table 6.4 and Figure 6.7). *Firmicutes*, a phylum containing gram-positive bacteria with tough cell wall, was another phylum containing pathogenic microbial families identified

in this study. Although the *Bacillaceae, Staphylococcaceae, Listeriaceae, Streptococcaceae,* and *Clostridium* (Table 6.4) are pathogens, they may also be regarded as opportunistic microbes. Their relative abundance from tick sample datasets may be due to environmental contamination. Consequently, four families of soil, water and environmental bacteria contaminants including *Cytophagaceae, Flavobacteriaceae, Weeksellaceae*, and *Prevotellaceae* were identified in taxonomic profile under the phylum *Bacteroidetes* (Table 6.4 and Figure 6.7). The family *Mycoplasmataceae* containing pathogenic microbes including *Mycoplasma spp.* under *Tenericutes* was reported from hard tick sample datasets. Taxonomic profile under the phyla *Chlamydiae* and *Spirochaetes* showed the prevalence of pathogenic microbial families including *Chlamydiaceae,* and *Spirochaetaceae*, respectively. Finally, the phylum Fusobacteria (containing the family *Fusobacteriaceae* and *Coxiellaceae*), a bacteria endosymbiont was reported from hard tick sample datasets (Table 6.4 and Figure 6.7). Overall, the microbial genera identified were present in all four tick sample datasets identified, except for the genus *Mycoplasma* identified from only two hard tick samples (T231C and T5B). Out of all the tick microbial pathogens identified following NGS and the previous POC and qPCR tests, there was only one sample in which *Ehrlichia* was identified (T223D). The dog from which this tick sample was collected from tested positive for *Ehrlichia canis* during field screening with the POC test kit.

Table 6.3: Microbes of veterinary and public health importance across six canine blood sample datasets classified using Centrifuge.

Rank	Major microbe in taxonomic profile	B35	B144	B153	B168	B194	B210	Abundance (%)
D	*Bacteria*	*	*	*	*	*	*	29.04
P	*Actinobacteria*	*	*	*	*	*	*	8.78
F	*Streptomycetaceae*	*	*	*	*	*	*	3.90
G	*Streptomyces*	*	*	*	*	*	*	3.68
P	*Firmicutes*	*	*	*	*	*	*	3.57
G	*Clostridium*	*	*	*	*	*	*	0.51
S	*Clostridium botulinum*	*	*	*	*	*	*	0.13
P	*Tenericutes*	*	*	*	*	*	*	0.36
P	*Proteobacteria*	*	*	*	*	*	*	9.28
C	*Gamma proteobacteria*	*	*	*	*	*	*	4.11
F	*Enterobacteriaceae*	*	*	*	*	*	*	0.56
G	*Klebsiella*	*	*	*	*	*	*	0.14
G	*Escherichia*	*	*	*	*	*	*	0.10
S	*Escherichia coli*	*	*	*	*	*	*	0.08
G	*Enterobacter*	*	*	*	*	*		0.06
S	*Enterobacter cloacae*	*	*	*	*	*		0.06
G	*Edwardsiella*	*	*	*	*	*		0.10
F	*Pseudomonadaceae*	*	*	*	*	*	*	0.83
G	*Pseudomonas*	*	*	*	*	*	*	0.80

Rank	Major microbe in taxonomic profile	B35	B144	B153	B168	B194	B210	Abundance (%)
P	*Bacteroidetes*	*	*	*	*	*	*	2.20
C	*Flavobacteria*	*	*	*	*	*	*	1.33
O	*Flavobacteriales*	*	*	*	*	*	*	1.33
F	*Flavobacteriaceae*	*	*	*	*	*	*	0.83
G	*Flavobacterium*	*	*	*	*	*	*	0.24
P	*Spirochaetes*	*	*	*	*	*	*	0.26
P	*Fusobacteria*	*	*	*	*	*	*	0.24
G	*Fusobacterium*	*	*	*	*	*	*	0.20
S	*Fusobacterium varium*	*	*	*	*	*	*	0.07
D	*Eukaryota*	*	*	*	*	*	*	2.10
K	*Fungi*	*	*	*	*	*	*	1.58
P	*Ascomycota*	*	*	*	*	*	*	1.56
P	*Euglenozoa*	*	*	*	*	*	*	0.31
S1	*Leishmania major strain Friedlin*	*	*	*	*	*	*	0.31
P	*Apicomplexa*	*	*	*	*	*	*	0.19
G	*Plasmodium*	*	*	*	*	*	*	0.18
S	*Plasmodium yoelii*	*	*	*	*	*	*	0.10
S1	*Plasmodium falciparum*	*	*	*	*	*	*	0.05
D	*Archaea*	*	*	*	*	*	*	0.58
P	*Euryarchaeota*	*	*	*	*	*	*	0.42
G	*Methanosarcina*	*	*	*	*	*	*	0.06
P	*Candidatus Lokiarchaeota*	*	*		*	*		0.02

Rank	Major microbe in taxonomic profile	B35	B144	B153	B168	B194	B210	Abundance (%)
G	*Candidatus Prometheoarchaeum*	*	*	*	*	*	*	0.02
S	*Candidatus Prometheoarchaeum syntrophicum*	*	*	*	*	*	*	0.02
D	*Viruses*	*	*	*	*	*	*	0.53
K	*Bamfordvirae*	*	*	*	*	*	*	0.20
P	*Nucleocytoviricota*	*	*	*	*	*	*	0.19
S	*Amsacta moorei entomopoxvirus*	*	*	*	*	*	*	0.15

Key: Table rows shown in green colour indicates major phyla in taxonomic profile, IDEXX test positive blood sample (✓), qPCR positive tick sample (⊙), qPCR positive blood sample (**✚**), NGS positive sample (**∗**). Blood and tick samples (B35, B144, B168, and B194) that tested positive using IDEXX test and qPCR were negative following NGS, exception of B210 which tested positive for piroplasm via qPCR and NGS. Major bacteria, archaeal, eukaryote (fungal and protozoal), and viral phyla (P), family (F), genera (G), and species (S) in taxonomic profile

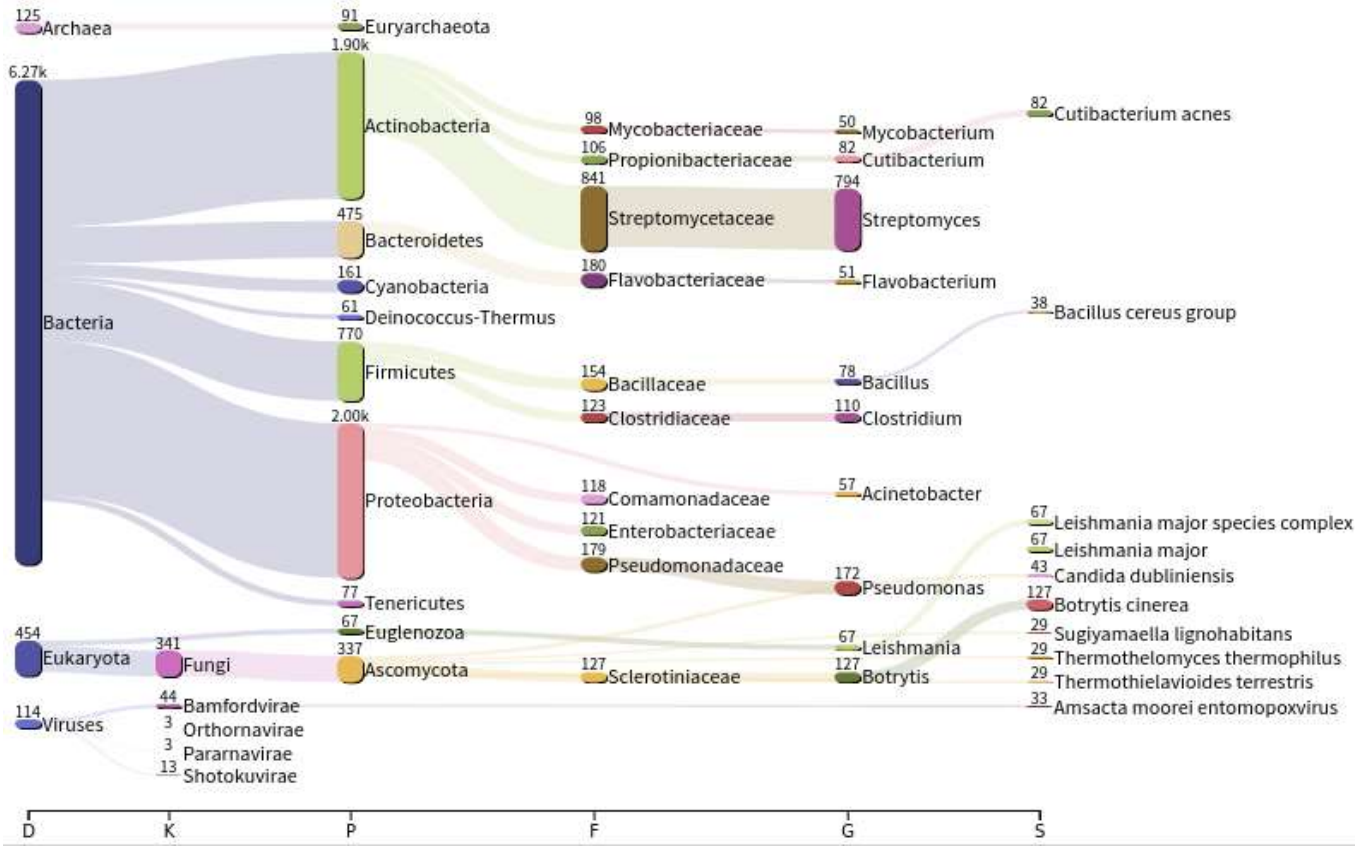


Figure 6.6: A Sankey view diagram drawn in R using Pavian showing microbial taxonomy ranks assignment to sequence reads generated from six canine blood sample datasets. The Sankey view is adjusted to display a minimum of 15 microbial taxonomy at each taxonomic rank: D=domain, K=kingdom, P=phylum, F=family, G=genus and S=species.

Table 6.4: Microbes across four hard tick sample datasets classified using Centrifuge.

Rank	***Major microbe in taxonomic profile***	NLB6D	T223D	T231C	T5B	Abundance (%)
D	*Bacteria*	*	*	*	*	20.02
P	*Proteobacteria*	*	*	*	*	10.39
F	*Anaplasmataceae*	*	*	*	*	0.02
F	*Wolbachieae*	*	*	*	*	0.01
G	*Wolbachia*	*	*	*	*	0.01
G	*Ehrlichia*	*	✓*		*	0.01
F	*Rickettsiaceae*	*	*	*	*	0.02
G	*Rickettsia*	*	*	*	*	0.02
G	*Orientia*	*	*	*	*	0.01
S	*Orientia tsutsugamushi*	*	*	*	*	0.01
F	*Pseudomonadaceae*	*	*	*	*	0.67
G	*Pseudomonas*	*	*	*	*	0.65
F	*Enterobacteriaceae*	*	*	*	*	0.42
G	*Klebsiella*	*	*	*	*	0.07
G	*Enterobacter*	*	*	*	*	0.06
S	*Enterobacter cloacae*	*	*	*	*	0.04
G	*Escherichia*	*	*	*	*	0.05
S	*Escherichia coli*	*	*	*	*	0.04
G	*Salmonella*	*	*	*	*	0.04
S	*Salmonella enterica*	*	*	*	*	0.03
F	*Yersiniaceae*	*	*	*	*	0.14
G	*Serratia*	*	*	*	*	0.08
S	*Serratia marcescens*	*	*	*	*	0.02
G	*Yersinia*	*	*	*	*	0.05
S	*Y. pseudotuberculosis, Y. enterocolitica*					
F	*Morganellaceae*	*	*	*	*	0.09
G	*Proteus*	*	*	*	*	0.03
G	*Providencia*	*	*	*	*	0.03
S	*Providencia rettgeri*	*	*	*	*	0.01

Rank	*Major microbe in taxonomic profile*	NLB6D	T223D	T231C	T5B	Abundance (%)
F	*Erwiniaceae*	*	*	*	*	0.08
G	*Pantoea*	*	*	*	*	0.04
G	*Erwinia*	*	*	*	*	0.02
G	*Buchnera*	*	*	*	*	0.01
F	*Vibrionaceae*	*	*	*	*	0.19
G	*Vibrio*	*	*	*	*	0.17
F	*Legionellaceae*	*	*	*	*	0.09
G	*Legionella*	*	*	*	*	0.08
S	*Legionella pneumophila*	*	*	*	*	0.02
F	*Pasteurellaceae*	*	*	*	*	0.10
G	*Haemophilus*	*	*	*	*	0.03
S	*Haemophilus influenzae*	*	*	*	*	0.01
S	*Haemophilus parainfluenzae*	*	*	*	*	0.01
F	*Hafniaceae*	*	*	*	*	0.03
G	*Edwardsiella*	*	*	*	*	0.02
F	*Francisellaceae*	*	*	*	*	0.03
G	*Francisella*	*	*	*	*	0.03
F	*Piscirickettsiaceae*	*	*	*	*	0.03
G	*Piscirickettsia*	*	*	*	*	0.01
S	*Piscirickettsia salmonis*	*	*	*	*	0.01
F	*Burkholderiaceae*	*	*	*	*	0.93
G	*Ralstonia*	*	*	*	*	0.34
F	*Comamonadaceae*	*	*	*	*	0.86
G	*Comamonas*	*	*	*	*	0.27
F	*Alcaligenaceae*	*	*	*	*	0.17
G	*Achromobacter*	*	*	*	*	0.08
S	*Achromobacter xylosoxidans*	*	*	*	*	0.03
G	*Bordetella*	*	*	*	*	0.05
F	*Campylobacteraceae*	*	*	*	*	0.16
G	*Campylobacter*	*	*	*	*	0.09
S	*Campylobacter concisus*	*	*	*	*	0.01
F	*Helicobacteraceae*	*	*	*	*	0.04

Rank	*Major microbe in taxonomic profile*	NLB6D	T223D	T231C	T5B	Abundance (%)
G	*Helicobacter*	*	*	*	*	0.04
S	*Helicobacter pylori*	*	*	*	*	0.02
F	*Coxiellaceae*		*	*	*	0.07
G	*Coxiella*		*	*	*	0.06
S	*Candidatus Coxiella mudrowiae*		*	*	*	0.06
D	*Eukaryota*	*	*	*	*	0.97
F	*Streptomycetaceae*	*	*	*	*	1.04
G	*Streptomyces*	*	*	*	*	1.00
F	*Mycobacteriaceae*	*	*	*	*	0.35
G	*Mycolicibacterium*	*	*	*	*	0.16
G	*Mycobacterium*	*	*	*	*	0.15
S	*Mycobacterium simiae*	*	*	*	*	0.03
G	*Mycobacteroides*	*	*	*	*	0.02
F	*Nocardiaceae*	*	*	*	*	0.19
G	*Rhodococcus*	*	*	*	*	0.11
F	*Microbacteriaceae*	*	*	*	*	0.29
G	*Microbacterium*	*	*	*	*	0.12
P	*Firmicutes*	*	*	*	*	2.13
F	*Bacillaceae*	*	*	*	*	0.57
G	*Bacillus*	*	*	*	*	0.32
F	*Staphylococcaceae*	*	*	*	*	0.15
G	*Staphylococcus*	*	*	*	*	0.12
S	*Staphylococcus aureus*	*	*	*	*	0.02
F	*Listeriaceae*	*	*	*	*	0.03
G	*Listeria*	*	*	*	*	0.03
S	*Listeria monocytogenes*	*	*	*	*	0.01
F	*Streptococcaceae*	*	*	*	*	0.15
G	*Streptococcus*	*	*	*	*	0.12
S	*Streptococcus suis*	*	*	*	*	0.01
F	*Clostridiaceae*	*	*	*	*	0.20
G	*Clostridium*	*	*	*	*	0.18

Rank	*Major microbe in taxonomic profile*	NLB6D	T223D	T231C	T5B	Abundance (%)
S	*Clostridium botulinum*	*	*	*	*	0.03
P	*Tenericutes*	*	*	*	*	0.13
F	*Mycoplasmataceae*			*	*	0.08
G	*Mycoplasma*			*	*	0.04
G	*Mycoplasmopsis*			*	*	0.03
F	*Spiroplasmataceae*	*	*	*	*	0.03
G	*Spiroplasma*	*	*	*	*	0.03
P	*Bacteroidetes*	*	*	*	*	1.63
F	*Cytophagaceae*	*	*	*	*	0.53
G	*Spirosoma*	*	*	*	*	0.49
S	*Spirosoma aerolatum*	*	*	*	*	0.41
F	*Flavobacteriaceae*	*	*	*	*	0.38
G	*Flavobacterium*	*	*	*	*	0.10
F	*Weeksellaceae*	*	*	*	*	0.18
F	*Chryseobacterium group*	*	*	*	*	0.12
G	*Chryseobacterium*	*	*	*	*	0.10
F	*Prevotellaceae*	*	*	*	*	0.03
G	*Prevotella*	*	*	*	*	0.03
P	*Chlamydiae*	*	*	*	*	0.02
F	*Chlamydiaceae*	*	*	*	*	0.01
G	*Chlamydia*	*	*	*	*	0.01
P	*Spirochaetes*	*	*	*	*	0.09
F	*Spirochaetaceae*	*	*	*	*	0.04
G	*Treponema*	*	*	*	*	0.03
F	*Borreliaceae*	*	*	*	*	0.01
G	*Borreliella*	*	*	*	*	0.01
F	*Leptospiraceae*	*	*	*	*	0.02
G	*Leptospira*	*	*	*	*	0.02
P	*Fusobacteria*	*	*	*	*	0.08
F	*Fusobacteriaceae*	*	*	*	*	0.05
G	*Fusobacterium*	*	*	*	*	0.05

Rank	*Major microbe in taxonomic profile*	NLB6D	T223D	T231C	T5B	Abundance (%)
P	*Ascomycota*	*	*	*	*	0.69
F	*Candida/Lodderomyces*	*	*	*	*	0.17
G	*Candida*	*	*	*	*	0.17
S	*Candida dubliniensis*	*	*	*	*	0.17
P	*Euglenozoa*	*	*	*	*	0.15
F	*Trypanosomatidae*	*	*	*	*	0.15
F1	*Leishmaniinae*	*	*	*	*	0.15
G	*Leishmania*	*	*	*	*	0.15
S	*Leishmania major*	*	*	*	*	0.15
P	*Apicomplexa*	*	*	*	*	0.11
F	*Plasmodiidae*	*	*	*	*	0.09
G	*Plasmodium*	*	*	*	*	0.09
S	*Plasmodium yoelii*	*	*	*	*	0.03
S	*Plasmodium vinckei*	*	*	*	*	0.02
S	*Plasmodium falciparum 3D7*	*	*	*	*	0.03
F	*Theileriidae*	*	*	*	*	0.02
G	*Theileria*	*	*	*	*	0.02
S	*Theileria orientalis strain Shintoku*	*	*	*	*	0.02
D	*Archaea*	*	*	*	*	0.50
P	*Euryarchaeota*	*	*	*	*	0.36
F	*Haloarculaceae*	*	*	*	*	0.03

Rank	*Major microbe in taxonomic profile*	NLB6D	T223D	T231C	T5B	Abundance (%)
D	*Viruses*	∗	∗	∗	∗	0.23
P	*Peploviricota*	∗	∗	∗	∗	0.02
F	*Herpesviridae*	∗	∗	∗	∗	0.01
P	*Nucleocytoviricota*	∗	∗	∗	∗	0.04
F	*Ascoviridae*	∗	∗	∗	∗	0.01
G	*Ascovirus*	∗	∗	∗	∗	0.01
F	*Baculoviridae*	∗	∗	∗	∗	0.03
G	*Alphabaculovirus*	∗	∗	∗	∗	0.02
G	*Betabaculovirus*	∗		∗	∗	0.01

Key: IDEXX test positive blood sample (✓), qPCR positive tick sample (⊙), qPCR positive blood sample (✚), NGS positive sample (∗). Blood and tick samples (B35, B144, B168, and B194) that tested positive using IDEXX test and qPCR were negative following NGS, exception of B210 which tested positive for piroplasm via qPCR and NGS. Major phyla in taxonomic profile are highlighted in green. Major bacteria, archaeal, eukaryote (fungal and protozoal), and viral phyla (P), family (F), genera (G), and species (S) in taxonomic profile

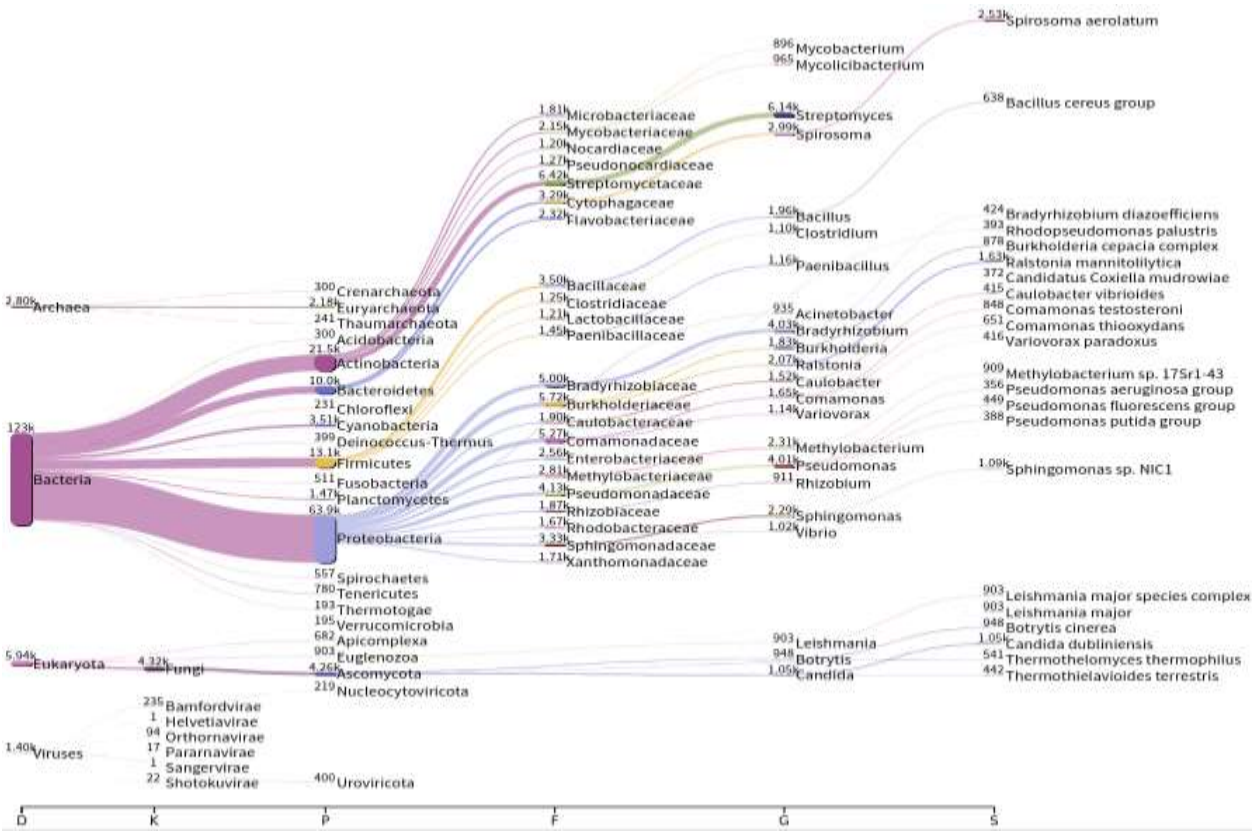


Figure 6.7: A Sankey view diagram drawn in R using Pavian showing microbial taxonomy ranks assignment to sequence reads generated from four hard tick sample datasets. Note that we have adjusted Sankey view to display a minimum of 15 microbial taxonomy at each level. The bar below represents taxonomic ranks D=domain, K=kingdom, P=phylum, F=family, G=genus and S=species.

6.2.5 Binning

6.2.5.1 Automated binning of six canine sample datasets using MaxBin2, MetaBAT2, Concoct, BinSanity, and DAS_Tool

Assembled contigs obtained from the canine blood sample datasets (Table 8.3) were not binned using the automated binning algorithms used in this study. Error messages showed that contigs obtained from canine blood sample datasets contained no bacterial single copy genes or any other genome, thus bins were only generated using BinSanity, Concoct, MetaBat2 and Mycc. Similar error messages were received when output from external binning tools that successfully binned canine blood datasets were added as input to be refined using DAS Tool.

BinSanity, Concoct, Mycc, and MetaBAT2 binning algorithm use information on kmer frequencies, coverage, contigs length, tetranucleotide counts, and GC content, while the model of binning using MaxBin2, and DAS Tool uses data on bacterial single copy genes in addition to other information used by the former (Yue et al., 2020). Additional steps were taken to assess the quality of bins obtained from canine blood sample dataset using CheckM and anvi-interactive server, where output from automated binning tools were directly clustered to Anvi'o contigs database obtained from canine blood datasets as collections. All bins generated from the six canine blood sample datasets were assessed to have 0.00% low completion and redundancy. As a rule of thumb, only bins with >50 completeness and <10 redundancy can be chosen as putative genomes for refinement, thus genomes were not generated from the canine blood datasets. Results for the binning of the canine blood sample datasets are therefore not presented in this report.

6.2.5.2 Automated binning of four tick sample datasets using MaxBin2, MetaBAT2, Concoct, BinSanity, and DAS_Tool

On the other hand, 188.57 Mb nucleotides consisting of 33,505 contigs recovered from the four tick sample datasets following quality control, removal of tick species host DNA and denovo assembly (Table 8.4) were

successfully binned using five different binning algorithms. Out of the four automated binning algorithms used Maxbin2 formed 28 bins, and 12/28 bins formed by Maxbin2 were assessed to have >50 completeness. MetaBAT2 formed 39 bins, 9/39 bins were assessed to have >50% completeness. Similarly, BinSanity yielded 52 bins, 8/52 bins have >50% completeness. Again, 75 bins were clustered using Concoct, 10/75 bins generated using Concoct have >50% completeness.

Following dereplication and aggregation of the output of the bins generated by the four binning algorithms used in this project using DAS_Tool, a total of 14 high quality bins were obtained. In summary, DAS_Tool generated 14 bins, 10/14 bins recorded completeness between 90 and 100%, 4/14 bins had percentage completeness between 60 and 88%, while percentage redundancy for all bins generated using DAS Tool were between 0 and 5.9% (Figure 6.8). Moreover, MetaBAT2 and Concoct had a similar performance generating more bins with >90% completeness, followed by BinSanity, while MaxBin2 had the least number of bins with >90% percentage completeness (Figure 6.8 and Figure 6.9).

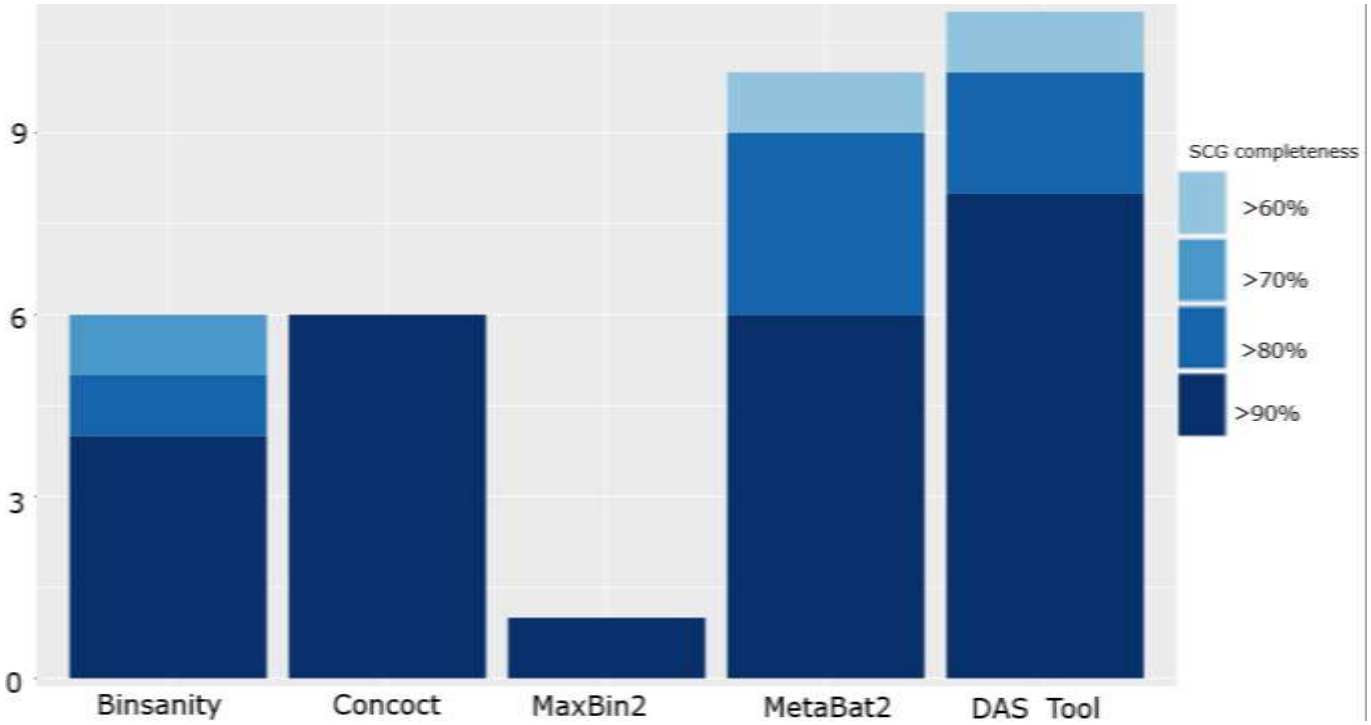


Figure 6.8: Quality refined bins generated from tick sample datasets by DAS_Tool, using output from MaxBin2, MetaBAT2, BinSanity and Concoct as input.

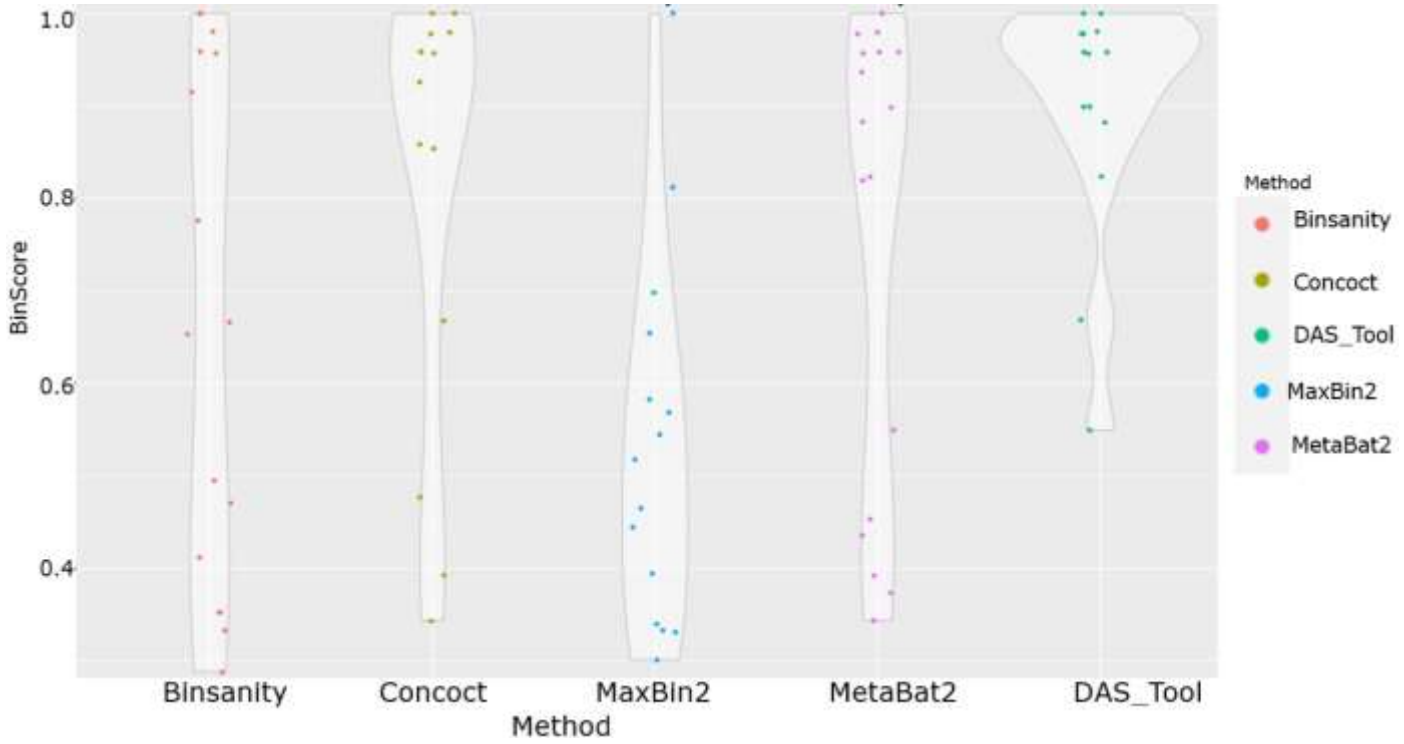


Figure 6.9: Score distribution of bins per automated binning tool following binning of tick sample datasets.

6.2.5.3 Binning and refinement of tick sample datasets output generated from automated binning tools using Anvi'o interactive

Contigs from the four tick sample datasets were binned and refined using four external binning tools and DAS_Tool. Although the outputs obtained from all external binning tools were clustered into Anvi'o contigs database, result presented in this analysis originated from DAS_Tool, as all binning output from other external tools were refined via DAS_Tool. Following the execution of anvi-refine and anv-summarize scripts on bin collections from DAS_Tool, 85 individual bins were generated, only 22/85 bins generated had >50% percentage completion and <10% redundancy, respectively (Table 6.5). The Anvi'o binning algorithm assigned taxonomic ranks to bins including the following bacteria families: *Burkholderiaceae* (12/22), *Microbacteriaceae* (4/22), *Spirosomaceae* (2/22), *Weeksellaceae* (2/22), and *Coxiellaceae* (2/22) (Table 6.5).

Assessment of percentage recruitment of genomes from datasets binned in this project showed that >90% of bins were recruited from domestic bird hard tick (NLB6D) datasets (identified as *Haemaphysalis longicornis* using NCBI blast nucleotide search, Figure 6.10). Canine hard tick (T5B) sample datasets (identified as *Rhipicephalus sanguineus*) also contributed to all bins generated in this study (Figure 6.10). Although *Haemaphysalis longicornis* contributed to over 90% of the bins identified to have over 50% completeness, the two *Coxiella* genomes generated were recruited mostly from the canine hard tick sample datasets T223D, T231C and T5B (Figure 6.10).

Figure 6.11 below is an Anvi'o interactive dendrogram drawn using the initial Bin_19 classified as *Candidatus Coxiella mudrowiae*, showing clades which were identified as potential contaminants. These contaminants were removed following a manual refinement to generate Bin_19_1 (Figure 6.12) with approximately 1% percentage contamination. Figure 6.13 is an example of two genomes recruited from the two tick sample datasets that were classified as *Candidatus Coxiella* spp. (Bin_19_1 on the left and refined_1_1_1 on the right).

Table 6.5: Characteristics of bins generated from DAS_Tool and refined via Anvi'o algorithm, showing length, number of contigs, N50, GC content, percentage completion/redundancy, and taxonomic assignment.

Bin ID	Length	Number of contigs	N50	GC content	Percentage Completion (%)	Percentage Redundancy (%)	Genus
Bin_Bin_20	3470073	194	24358	67	87	0	*Aquabacterium*
Bin_Bin_61_sub_3	5025429	369	18367	62	92	1	*Comamonas*
Bin_Bin_74	6562745	75	154748	65	100	4	*Curvibacter*
Bin_Bin_8_sub_2_1	3188713	49	148945	69	99	0	*Microbacterium*
Bin_MAXBIN__004_1_1	3586992	72	86040	70	100	3	*Microbacterium*
Bin_MAXBIN__010_sub_1	10362751	819	35003	51	52	6	*Spirosoma*
Bin_METABAT__21	5525608	348	24510	70	94	4	*Pseudacidovorax*
Bin_METABAT__23_1_1	3901081	63	92291	36	51	0	*Chryseobacterium*
Bin_contigs_Bin_19_1	1435946	16	365940	38	51	1	*Coxiella*
Bin_contigs_Bin_3_1	4688589	41	268910	66	99	3	*Ralstonia*
Refined_1_1_1_1	2135860	92	146978	38	51	6	*Coxiella*
Refined_2_1_1	4724951	48	268910	66	99	3	*Ralstonia*
Refined_3_1_4_1	3901081	63	92291	36	51	0	*Chryseobacterium*
Refined_3_2_1	8592014	444	43768	50	58	1	*Spirosoma*
Refined_4_1_1_1	3506891	68	86040	70	100	3	*Microbacterium*
Refined_4_2_1_1	2560183	29	149501	70	94	0	*Microbacterium*
Refined_5_1	4230630	78	51607	66	93	1	*Curvibacter*
Refined_6_1_1	5140831	182	36088	70	96	1	*Methylobacterium*
Refined_6_2_1_1_1	3677667	204	24410	70	65	1	*Pseudacidovorax*
Refined_6_3	3123372	192	22084	66	82	15	*Pelomonas*
Refined_6_4	6006508	330	27504	70	52	13	*Pelomonas*
Refined_7_1_1_1	3749158	271	18058	62	87	0	*Comamonas*

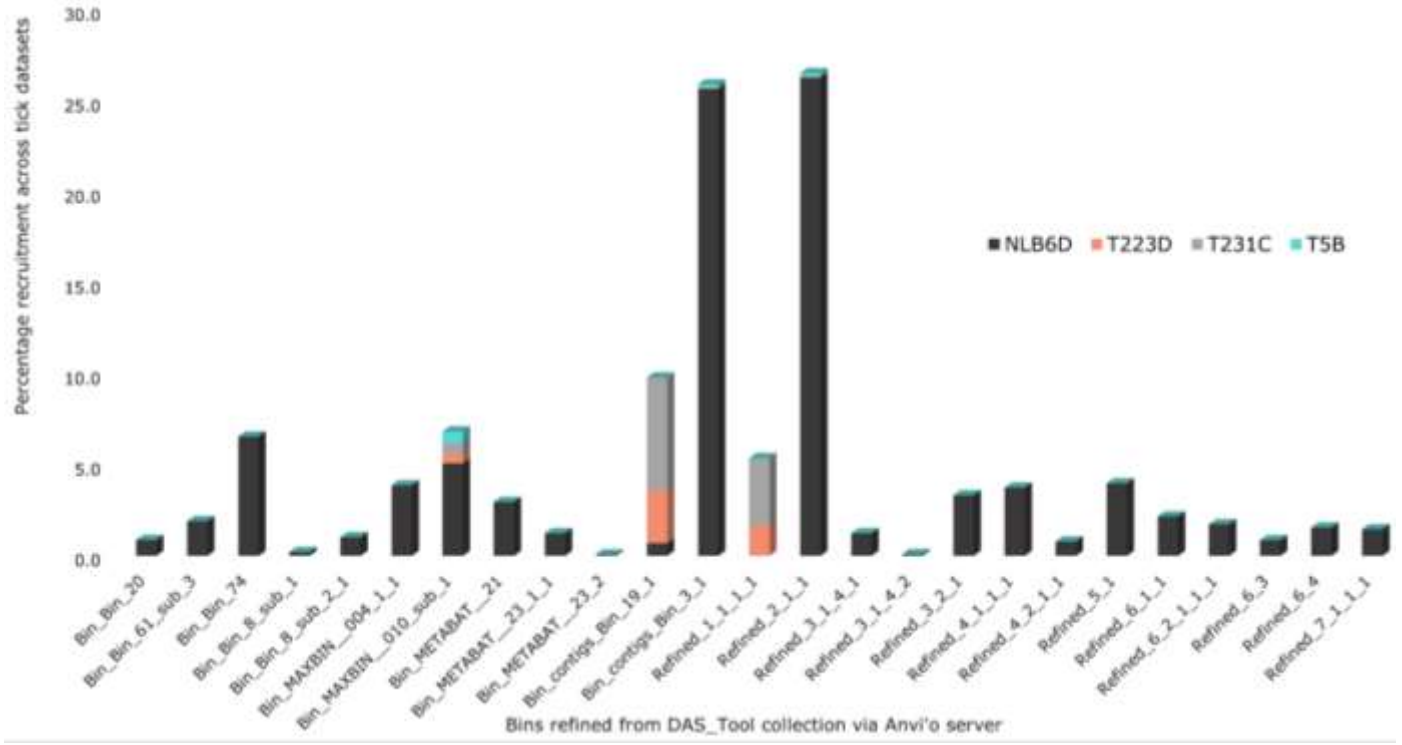


Figure 6.10: Percentage recruitment of bins across four hard tick sample datasets.

Key: The y-axis represents percentage recruitment across samples, while x-axis represents identity assigned to bins during analysis. The two *Candidatus coxiella mudrowiae* genomes can be seen in the middle of the x-axis.

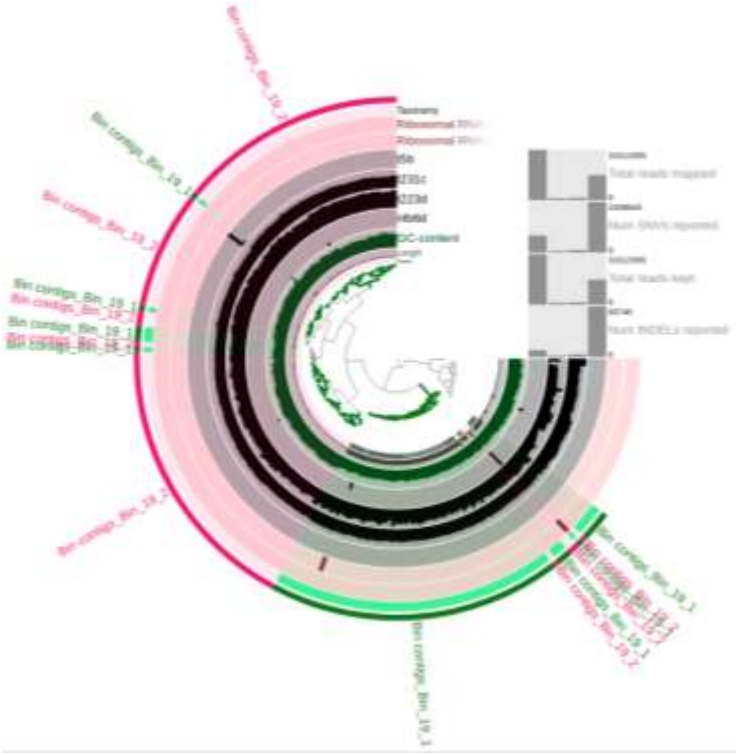


Figure 6.11: Anvi'o bin refinement process using DAS_Tool Bin_19 from hard tick sample datasets.

Key: The green rings from the centre of the genome represent GC content, while the dark 2 rings represent relative abundance of the draft genome from T223D and T231C hard tick sample datasets. The red and green outer rings represent new refined bins formed from the initial Bin_19.

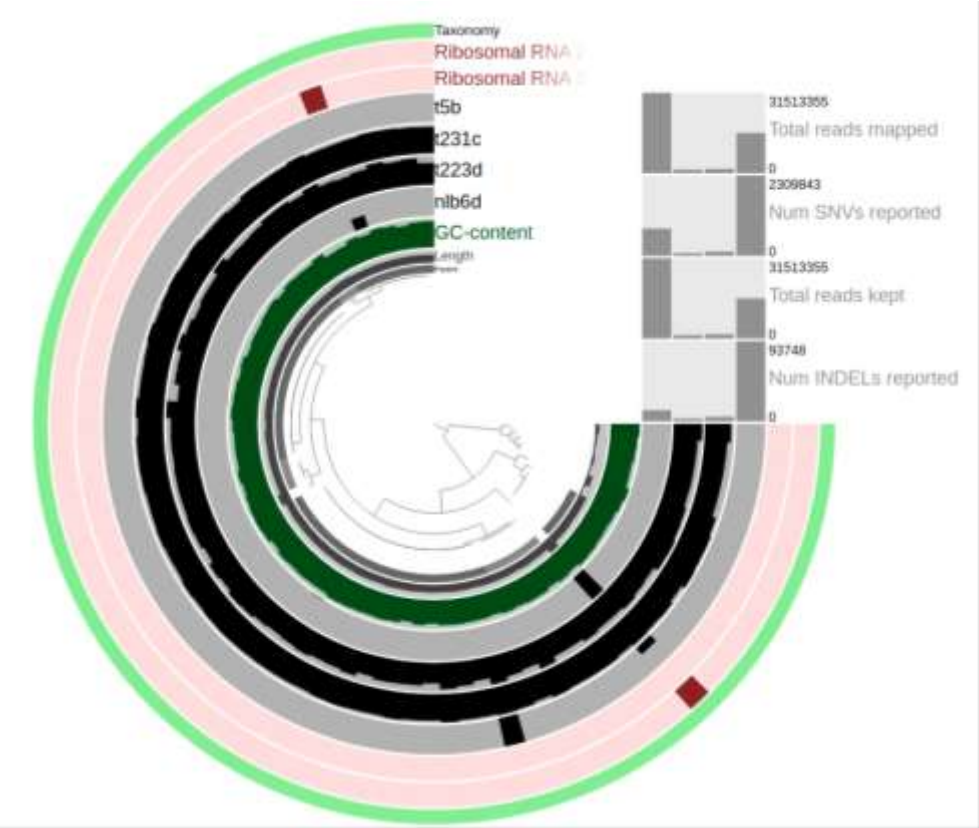


Figure 6.12: Showing a refined bin recruited majorly from two canine hard tick sample datasets (dark rings), the outermost green ring signifies the uniqueness of the refined bins as all splits in this bin belong to one microbial genus called *Candidatus coxiella mudrowiae*.

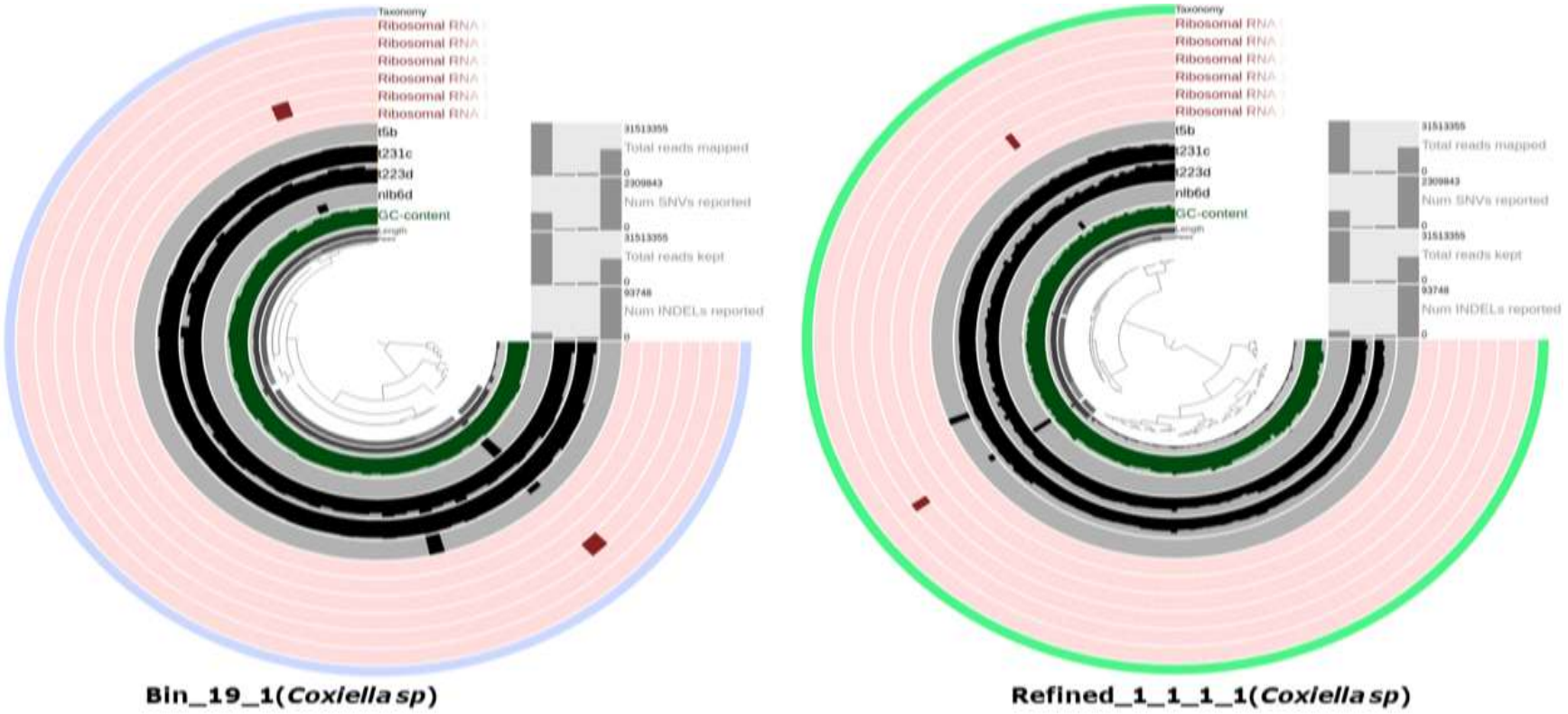


Figure 6.13: An example of Anvi'o interactive server dendrograms showing the recruitment of two bins classified as *Candidatus coxiella mudrowiae* genomes from T223D and T231C datasets.

6.2.5.4 Assessment and classification of draft genomes

Quality assessment of all the genomes classified from Anvi'o refinement and DAS_Tool collection were confirmed by running CheckM lineage workflow on all bins summarized. Percentage GC count (Figure 6.14: A), and completeness/redundancy (Figure 6.14: B) of draft genomes following CheckM workflow analysis were similar with previous results generated using the Anvi'o algorithm. Thus, 22/22 genomes summarized via the Anvi'o algorithm passed the quality assessment using CheckM. However, not all genomes (>50% completeness or <10 redundancies) were classified using GTDB-Tk. GTDB-Tk assigned taxonomy to 14/22 putative genomes initially identified via the Anvi'o algorithm. It is important to note that the taxonomic assignment of genomes by GTDB-Tk classifier was similar to that of Anvi'o binning process, this may serve as a confirmation of the results initially generated via Anvi'o server. Additionally, we compared average nucleotide identity (ANI) and multiple sequence alignment (MSA) between 14 draft genomes and corresponding reference genomes. The percentage ANI and MSA between draft and reference genomes were >95% and 71 — 96%, respectively (Figure 6.14:C). For this report, only genomes that met quality check criteria via CheckM and were successfully classified using GTDB-TK are presented under CheckM genome quality assessment report (Figure 6.14: A).

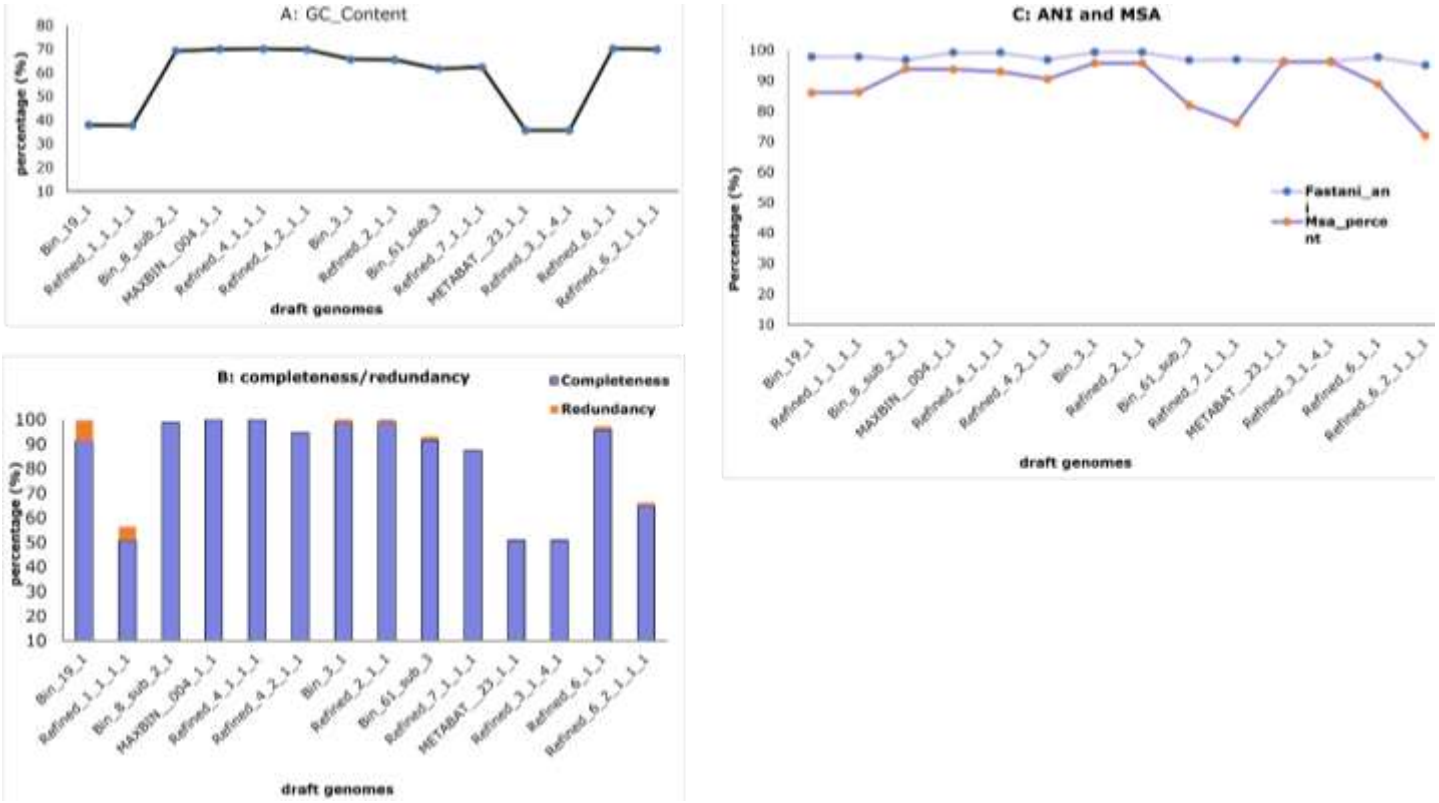


Figure 6.14: Shows percentage GC count (A), completeness and redundancy (B) of genomes generated from hard tick sample datasets, genome quality assessment was carried out using CheckM workflow. The graph on your right (C) shows percentage average nucleotide identity (ANI) and multiple sequence alignment (MSA) between draft genomes and reference genomes identified via GTDB-Tk classifier.

6.2.5.5 Genome size and statistical estimation using GAAS and Bbmap tools script

Estimation of genome statistics on the draft genomes were carried out on all the draft genomes however, results presented in this present here are summary reports of the genomes identified as *Coxiella* and *Microbacterium* species. The *Coxiella* species draft genomes have the same GC content of 38% as the reference genome *Candidatus Coxiella mudrowiae*. The *Candidatus Coxiella mudrowiae* reference genome used in this study is about 279kb longer than the Bin_19_1 draft genome and 420kb shorter than the Refined_1_1_1_1 draft genome obtained from this study (Table 6.6).

Table 6.6: Comparison between draft and reference genomes generated from canine hard tick sample datasets.

Statistical parameters	Bin ID		Reference genome
***Coxiella species* genomes**			
	Bin_19_1	Refined_1_1_1_1	*Coxiella mudrowiae*
Number of sequences	16	92	1
Number of sequences >1kb	16	92	1
Number of sequences >10kb	11	18	1
Number of Ns	Not applicable	Not applicable	181
Number of nucleotides (counting Ns)	1,435,946bp	2,135,860 bp	1,715,759 bp
GC-content not counting Ns (%)	38.1	37.8	38
GC-content (%)	38.1	37.8	38
N50	365940	146978	1715759
L50	2	4	1
N90	46027	5734	1715759
L90	7	43	1
***Microbacterium* species genomes**			
	MAXBIN_004_1	Bin_8_sub_2_1	*Microbacterium* spp.
Number of sequences	72	50	1
Number of sequences >1kb	72	50	1
Number of sequences >10kb	54	41	1
Number of Ns	0	0	0
Number of nucleotides (counting Ns)	3,586,992 bp	3,188,713 bp	3,424,892 bp
GC-content not counting Ns (%)	70.1	69.4	69.9
GC-content (%)	70.1	69.4	69.9
N50	86040	148945	3424892
L50	12	9	1
N90	26735	26929	3424892
L90	40	28	1

6.2.5.6 Construction of genome maps using genomes generated from tick sample datasets and corresponding NCBI reference genomes

Out of the fourteen draft genomes recovered from the tick sample datasets cutting across five bacteria families, genome maps were constructed on selected genera including genomes identified as *Candidatus Coxiella mudrowiae* and *Microbacterium* species (Figure 6.15 and Figure 6.16).

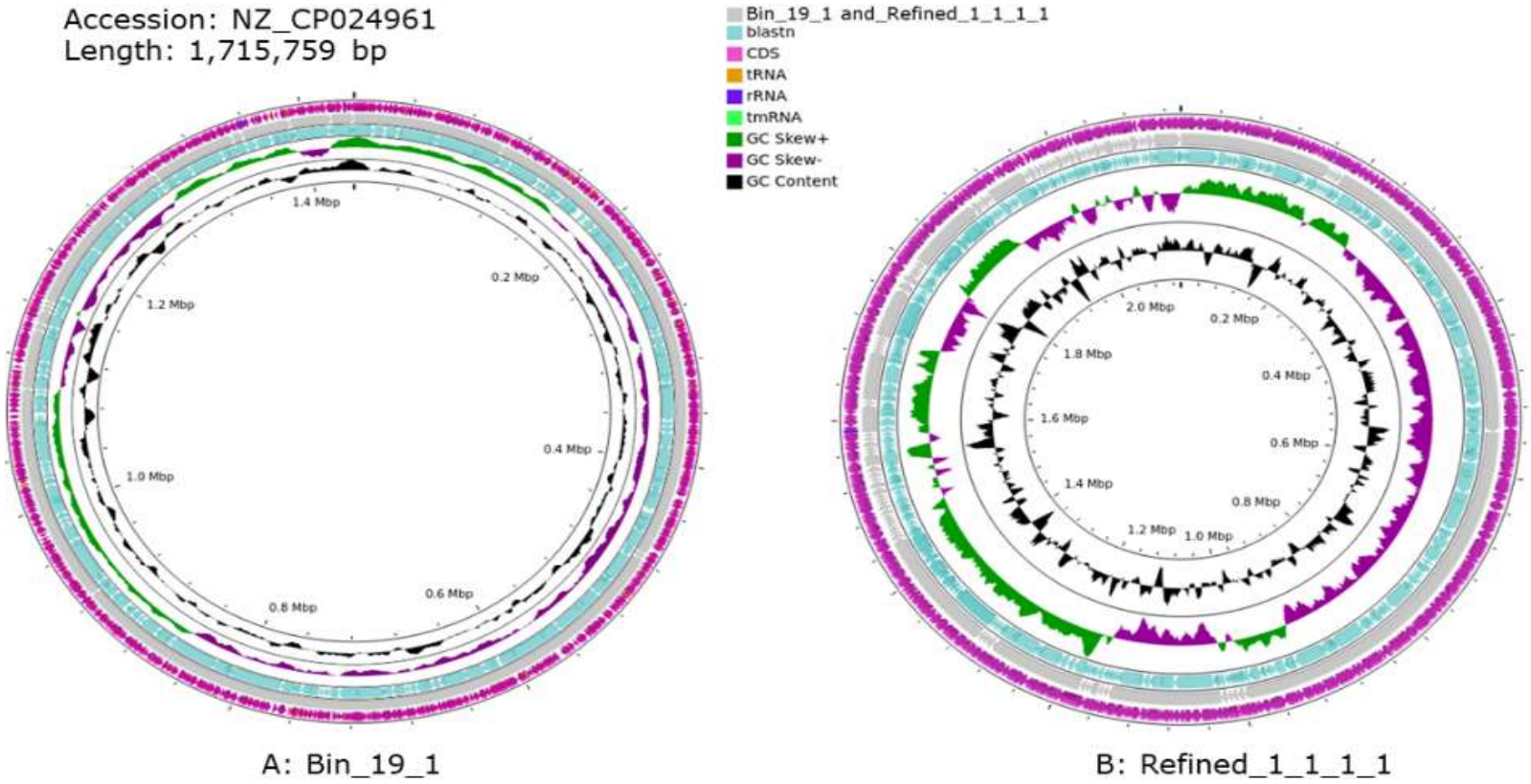


Figure 6.15: *Candidatus coxiella mudrowiae* genome maps drawn using draft and NCBI reference genome.

Key: The outer to the innermost ring shows **purple** rings (coding sequences annotated using Prokka algorithm), gray ring (draft genomes), **blue** rings (NCBI blast search alignment using *C. mudrowiae*), and GC contents. Maps were adjusted to display both forward and reverse stands for all parameters.

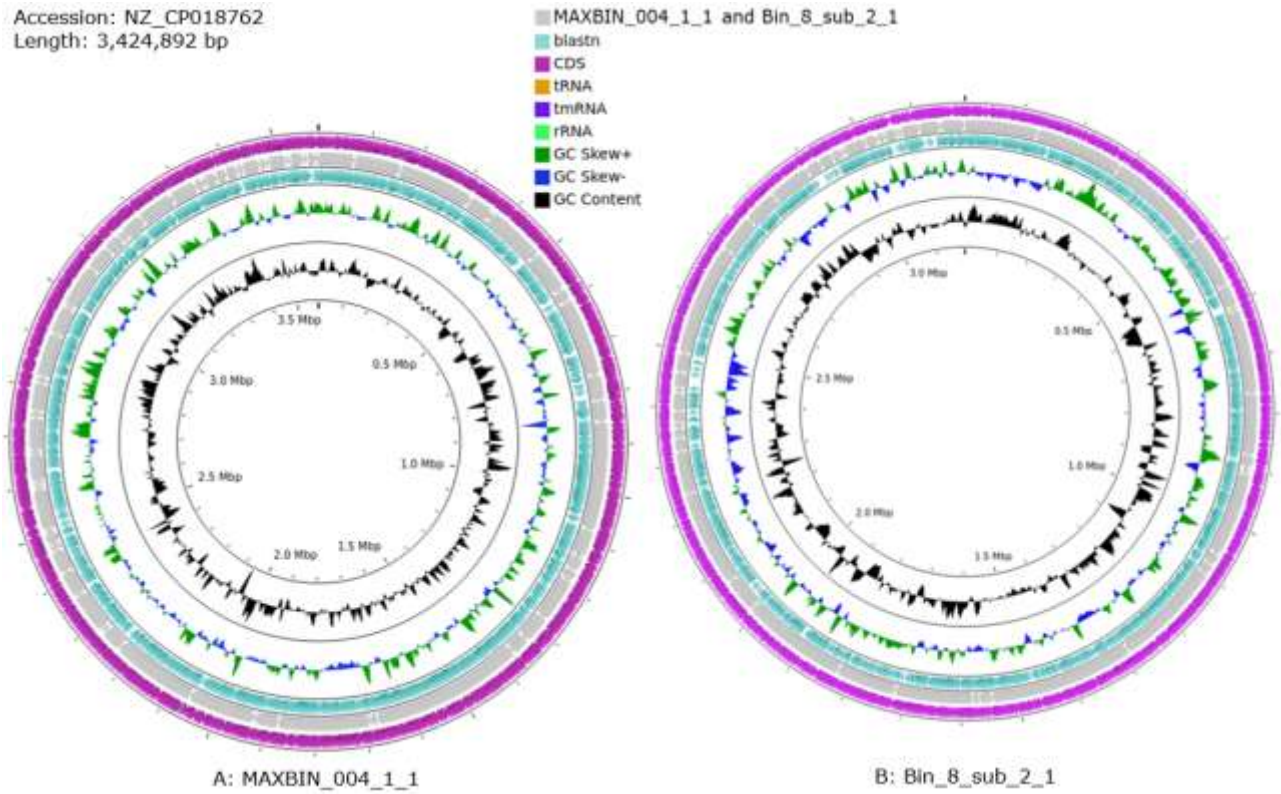


Figure 6.16: *Microbacterium aurum* genome maps drawn using draft and NCBI reference genome.

Key: The outer to the innermost ring shows **purple** rings (coding sequences annotated using Prokka), gray ring (draft genomes), **blue** rings (NCBI blast search alignment with *M. aurum*), and GC contents. Maps were adjusted to display both forward and reverse stands for all parameters.

6.2.5.7 Assessment of antimicrobial resistance genes (AMR) from canine blood and hard tick sample datasets

The assembled contigs (Table 8.3 and Table 8.4) obtained from six canine blood and four hard tick sample datasets were assessed for the presence of AMR genes using NCBI AMRFinder tool however, AMR genes were not detected from the six canine blood sample datasets contigs. Conversely, AMR and stress genes were detected from 12 contigs belonging to T5B and NLB6D datasets assemblies cutting across six antimicrobial classes. These include Lincosamide (2), Sulphonamide (2), Quaternary ammonium (1), Beta-lactam (6), Streptomycin (1), and Macrolide (1). The stress gene detected here was associated with the antimicrobial agent quaternary ammonium from the domestic bird (NLB6D) tick datasets contigs (Figure 6.17). Overall, AMR genes identified have approximately 100% identity and coverage with the reference gene sequences identified using NCBI AMRFinder algorithm (Table 6.7 and Figure 6.17).

Table 6.7: Showing number of contigs and AMR genes detected from two tick dataset assemblies.

No of contigs with AMR genes	Gene symbol	Antimicrobial class	Element type
NLB6D (1)	lnu(A)	Lincosamide	AMR
NLB6D (2)	sul1/sul2	Sulphonamide	AMR
NLB6D (1)	qacEdelta1	Quaternary ammonium	STRESS
NLB6D (1) and T5B (6)	blaBJP/blaTEM-116/blaOXA-443	Beta-lactam	AMR
NLB6D (1)	Aph (3'')-Ib	Streptomycin	AMR
NLB6D (1)	erm (C)	Macrolide	AMR

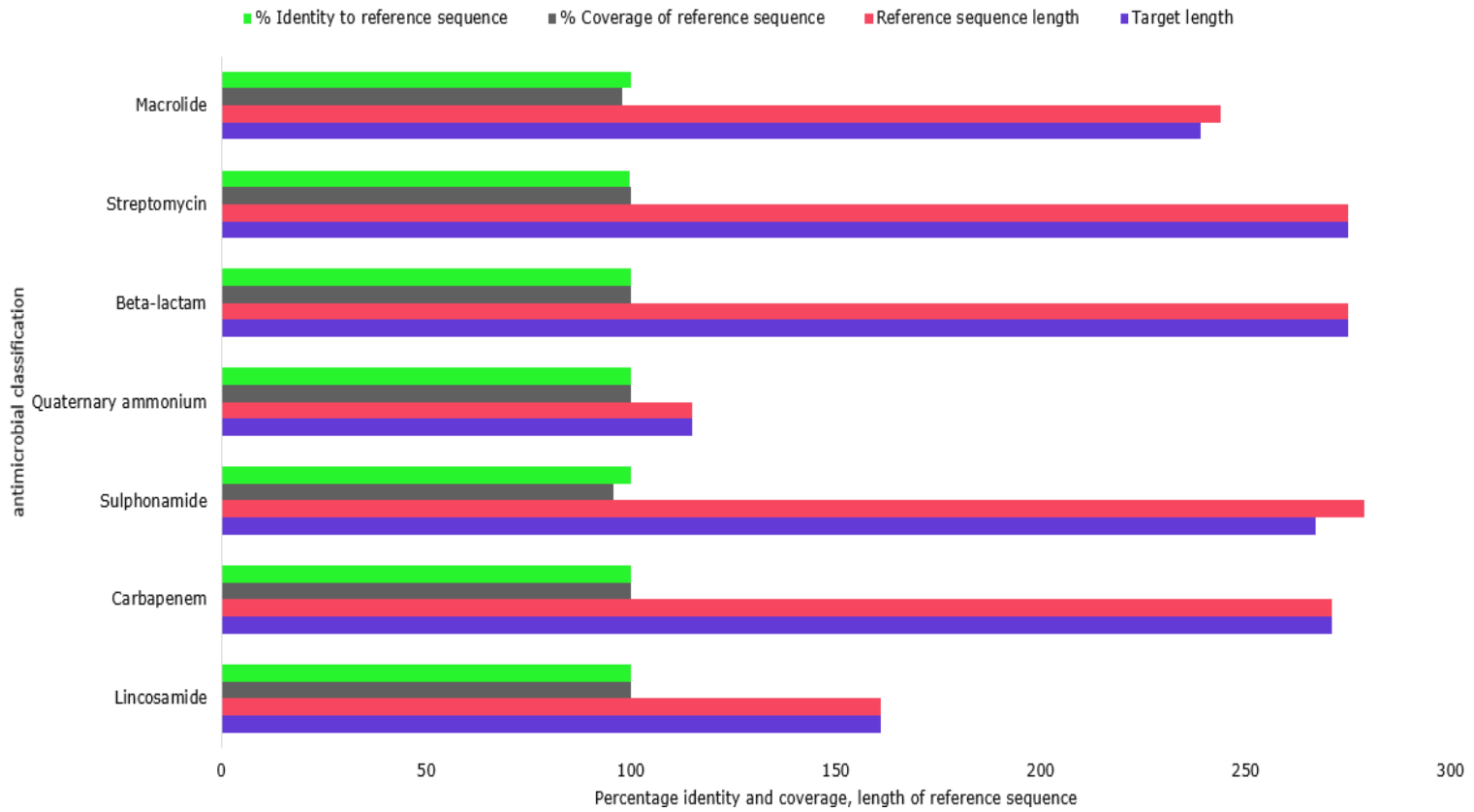


Figure 6.17: Shows the presence of AMR genes for seven (7) major classes of antimicrobial agents.

Key: The x-axis indicates percentage identity and coverage of AMR genes sequences identified from 12 contigs belonging to two hard tick sample datasets, including target length of the reference AMR genes sequences used.

6.2.6 Phylogenomic analysis

Phylogenomic analysis of the genomes generated from the tick sample datasets were built using information on taxonomic assignment from Anvi'o binning results and genomes identified as reference genomes via GTDB-Tk classifier. The maximum likelihood phylogenomic tree below grouped genomes generated in this project into five distinct clades, with *Vibrio cholerae* as an outgroup genome. *Vibrio cholerae* belongs to a major phylum of gram-negative bacteria called *Proteobacteria* and can be a suitable outgroup for a tree constructed using other members of this bacteria phylum. The maximum likelihood phylogenetic tree is drawn to display bootstrap values above >100%, and each clade assigned to individual genomes. The clade assigned to *Candidatus Coxiella mudrowiae* consists of two genomes, which were all recruited from two samples. In summary, five bacteria genomes belonging to five bacteria genera including *Coxiella, Ralstonia, Methylobacterium, Chryseobacterium* and *Microbacterium* species were generated from the hard tick sample datasets and this classification has been confirmed using a maximum likelihood phylogenomic tree (Figure 6.18).

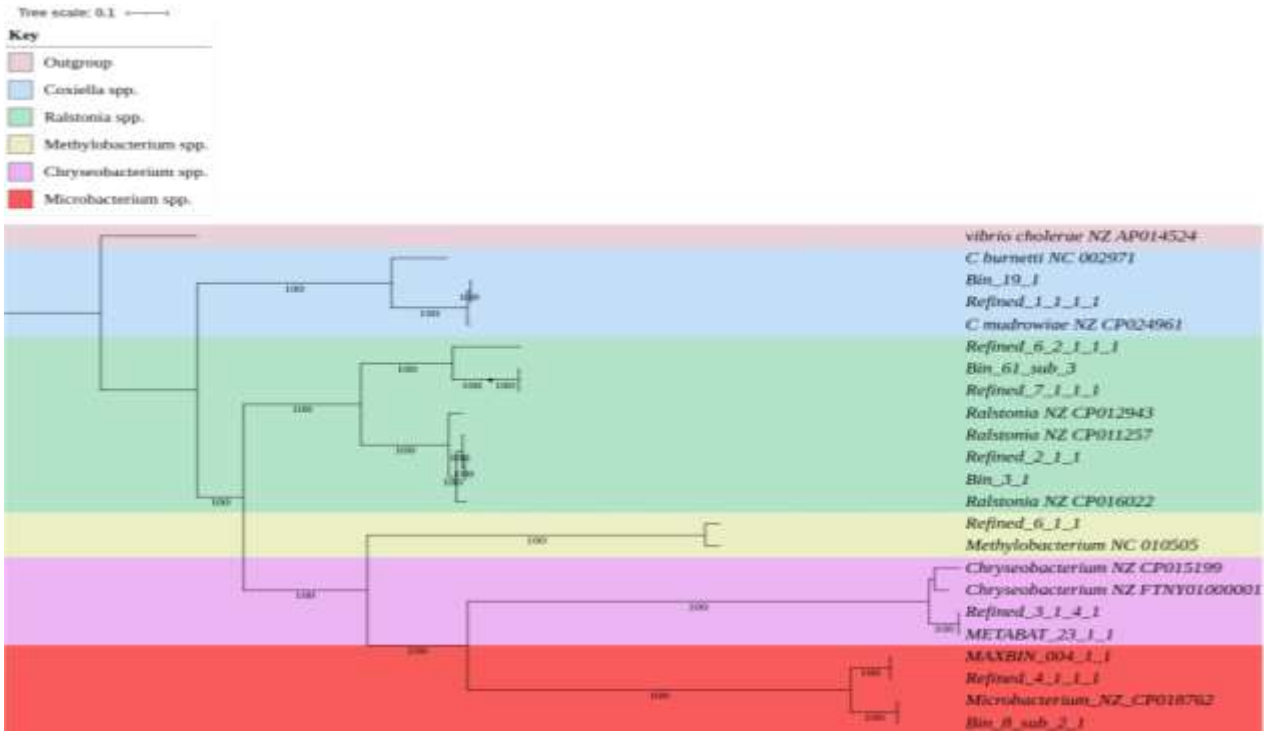


Figure 6.18: *Proteobacteria* genomes maximum likelihood phylogenomic tree drawn using genomes obtained from hard tick sample datasets and NCBI reference genomes, with *Vibrio cholerae* as an outgroup.

Key: GenBank reference sequences are labeled using bacteria genera, and GenBank accession numbers, while genomes obtained from this project are labeled with bin identification numbers to ensure uniformity throughout this study. The maximum likelihood phylogenetic tree is drawn to display bootstrap values above ≥100%.

6.3 Discussion and conclusion

Microbial communities in canine blood and hard ticks collected from Nigerian dogs, tick, and domestic chicken (*G. domesticus*) were assessed in this study using various bioinformatics tools following Illumina NovaSeq 6000 PE150 sequencing.

Identification of canine and hard tick host DNA was applied to sample datasets using a custom NCBI nucleotide blast search in Linux terminal. Statistical results from BAM files generated during host DNA removal showed a high percentage alignment between sequence reads and the corresponding host (canine and tick) reference genome for all sample datasets, while a smaller number of unmapped reads were used for downstream analysis. Over 95% of canine blood datasets aligned with the corresponding canine host reference genome. A total of 14,211,264 and 177,604,557 reads were obtained from canine blood and hard tick sample datasets, respectively, following quality trimming and removal of host DNA.

Sequence reads from both canine blood and hard tick datasets were assigned to multiple microbial taxa following centrifuge classification. The threshold for taxonomic assignment of reads for identification of microbes was set at a minimum of ten reads for genus and 50 reads for species level. These samples were collected onto Whatman's FTA cards (for ease of sample preservation and shipment) creating room for potential environmental contamination. This may have led to an elevated level of environmental bacterial reads in the metagenomics data generated. Previous studies have demonstrated that the presence of a high abundance of bacteria genera in metagenomics samples can be due to contamination of samples from soil or host skin (Wang et al., 2021). This is a factor for the presence of many of the microbial genera identified from the canine blood samples, as it does not seem feasible that these otherwise healthy dogs were septicaemic at the time of blood sample collection. A higher number of microbial sequence reads were identified from the tick sample

datasets where fewer reads mapped to the tick host. Recent studies suggest that *Rhipicephalus* and *Haemaphysalis* tick species harbour many microbial communities. The type of metagenomics sequencing methods and analysis pipeline used, can also determine the abundance of various microbial genera detected (Khoo et al., 2016). The presence of microbial communities belonging to bacterial and archaeal domains in canine blood sample datasets reported in this study can be regarded as environmental or tick gut contaminants as reported in previous studies (Tufts et al., 2020). However, two distinct microbial families including *Leishmaniinae* and *Plasmodiidae* are important microbes that were identified from canine blood sample datasets requiring further discussion irrespective of limitations encountered. The report of *Leishmania* species from canine blood in this study confirms previous studies reporting prevalence of 8.1% (n=545) in canine blood via PCR (Reithinger et al., 2000). Dogs are known reservoir hosts for *Leishmania*, previous studies have reported prevalence of 3.8% (n = 316) (Usman et al., 2020) from dogs in Northern Nigerian, and 13.0% (n = 200) from humans (Usman-Sani et al., 2019) in Benue state where over 50% of samples assessed in this study were collected. Rodents are also known reservoir hosts for *L. major* and there is also a likelihood of mechanical transmission and/or potential contamination of FTA cards via sandflies, and these findings require further confirmation.

Although, zoonotic *Plasmodium knowlesi* has been reported in other animals species including primates (Su and Wu, 2021), to date, no reports on the prevalence of *Plasmodium* species in domestic dogs are available. A preliminary report pending peer review demonstrating 16.7% prevalence of *Plasmodium* species known to infect humans in domestic dogs using PCR has been submitted by researchers from Indonesia (Munirah et al., 2020). The Munirah et al. (2020) preliminary report cannot be considered conclusive or used to inform research or clinical practice owing to its status, and this report has not been replicated in other studies and is hard to confirm without more details on the methods and sequence generated. However, adhesion G protein-coupled receptor E1

(ADGRE1), a gene established to be responsible for severe malaria resistance in humans has been reported in African dogs. Recent studies to investigate genetic adaptation to tropical environment using the application of whole-genome sequencing analysis of nineteen Nigerian indigenous dogs reported the presence of a positively selected gene for malaria resistance in dogs including ADGRE1. Nigerian dogs are also infected with similar protozoan pathogens (including *Babesia* species) sharing similar pathogenesis with *Plasmodium* species (Liu et al., 2018c). This report does not necessarily translate into the presence of *Plasmodium* infection in Nigerian dogs due to the possibility of contamination of the FTA cards with environmental or insect DNA and should be interpreted with caution. Further discussion in this study will focus more on hard tick datasets as fewer microbes were identified reliably from canine blood samples.

The most abundant bacteria phyla from hard tick samples were *Proteobacteria*. Related results were reported in a recent study on hard tick metagenomics profiling in Benue state Nigeria where *Proteobacteria* were reported as the most abundant phyla from *R. sanguineus* (Agwunobi et al., 2021), and other parts of the world (Wang et al., 2021, Huggins et al., 2021b). Apart from *Proteobacteria* (including *Candidatus Coxiella mudrowiae, Ehrlichia, Wolbachia, Rickettsia, Orientia, Francisella, Piscirickettsia salmonis, Borrelia*), *Euglenozoa* (*Leishmania)*, *Apicomplexa (Plasmodium and Theileria)*, and *Tenericutes* (*Mycoplasma*) genera, all other microbes identified from hard tick samples are likely to be considered as environmental and tick gut contaminants (Tufts et al., 2020, Wang et al., 2021). Taxonomic identification of *Coxiella*, *Rickettsia*, *Francisella,* and *Wolbachia* in this study confirms a previous report in Australia where these endosymbionts were reported to be abundant in hard ticks (Gofton et al., 2015, Huggins et al., 2021b).

Although, *Candidatus Coxiella mudrowiae* was not the most abundant endosymbiont identified from ticks, its relative abundance across tick datasets (except for the domestic bird ticks) confirmed previous reports that *Coxiella* and *Rickettsia* endosymbionts are prevalent in West African ticks (Rene-Martellet et

al., 2017, Agwunobi et al., 2021). Studies reporting the genus *Coxiella* as the most abundant *Proteobacteria* in *Haemaphysalis* species have been described (Khoo et al., 2016, Ruiling et al., 2019). *Coxiella burnetii* is known to cause a highly infectious disease called Q-fever in humans and animals globally. Whole genome sequencing and phylogenetic analysis study on *Coxiella-like* endosymbiont has also demonstrated the emergence of *C. burnetii* from tick *Coxiella-like* endosymbionts, following vertical transmission of endosymbionts from adult ticks to larva via eggs (Duron et al., 2015b).

Like with *Coxiella* endosymbionts, previous studies demonstrated that *Francisella* endosymbionts are transmitted vertically by *Dermacentor reticulatus* tick species. Further evidence shows that where *Coxiella-like* endosymbiont is not transmitted vertically to tick offspring, *Francisella* becomes an alternative endosymbiont (Duron et al., 2015b) Multiple factors such as direct competition among symbionts, virulence, and transmission of bacterial endosymbiont from females to egg, larva, or embryo are established to be responsible for the differences in abundance of *Coxiella-like* endosymbiont in ticks. Any of these factors can lead to a decrease in the number of endosymbionts passed to offspring, resulting in variation in relative abundance of the symbiont (Mira and Moran, 2002). Although, ticks are known to survive in the absence of *Coxiella* endosymbionts (Khoo et al., 2016). Production of vitamins (folic acid) and sex determination in hard tick species is entirely dependent on the presence of *Coxiella* and *Rickettsia* endosymbionts (Vila et al., 2019). However, a low abundance of *Rickettsia* was reported in this study. Although, endosymbionts such as *Coxiella*, *Wolbachia, Francisella* and *Rickettsia* are relevant for adaptation, development, reproduction, immunity and fitness in tick hosts, little information is available on their overall effects on hard ticks, animal hosts and other microbes identified in ticks (Vila et al., 2019) requiring further investigation.

Nearly complete *Candidatus Coxiella mudrowiae* genomes were generated from hard tick sample datasets assessed in this study. Recent studies on a *Coxiella-*

like endosymbiont of *R. turanicus* (CRt) compared the genome size of CRt to genomes and established the absence of certain genes, and reduction in genome size, this led to assignment of a new taxonomy *Candidatus Coxiella mudrowiae* (Gottlieb et al., 2015). The reduction in CRt genome size has also been reported from *Coxiella-like* endosymbiont of *R. sanguineus* (CRs) in Israel (Tsementzi et al., 2018), and in two tick species from Palestine (Ravi et al., 2019). In addition, recent studies from Kenya reported a *Coxiella* endosymbiont of African tick *Amblyomma nuttali* (CAn) genome size of ~1Mb, confirming this study. Two genomes classified as *Candidatus Coxiella mudrowiae* recruited from two brown dog ticks (*R. sanguineus*) were successfully presented in this study. *Candidatus Coxiella mudrowiae* genomes obtained from hard tick sample datasets assemblies were highly similar with the *Candidatus Coxiella mudrowiae* genome from Israel (Tsementzi et al., 2018). However, draft genomes from this study varied in sizes, the genome identified as Bin_19_1 measure 1.4 Mb, while Refined_1_1_1_1 measured 2.1Mb in size.

Ehrlichia species were the most prevalent bacteria identified from both canine blood (POC field test) and hard tick (PCR) samples. While 1/4 tick samples were reported to originate from dog positive to *E. canis* via the POC test kit, 3/4 of the hard tick samples contained *Ehrlichia* DNA following NGS analysis. Due to the low quality of samples used in this study, a lot of pathogens that were positive via qPCR which is a more specific and sensitive test were not positive following NGS analysis. However, the present study confirms previous studies where *E. canis* species were reported from hard ticks collected from Nigeria (PCR only) (Kamani et al., 2013a), and other parts of the world using both PCR and Illumina sequencing (Huggins et al., 2021b, Harrus et al., 2011, Gofton et al., 2015).

Orientia tsutsugamushi is another pathogen identified in the tick metagenomics taxonomic profile. To the best of our knowledge this pathogen has not been reported in Nigeria. *Orientia tsutsugamushi* is a rickettsial pathogen known to cause scrub typhus in humans and is transmitted through the bite of the larval

stage of scrub-itch (trombiculid) known to be prevalent in Nigeria (Okeke et al., 2020, Stekolnikov, 2018). Outbreaks resulting from this pathogen has been reported in Asian-Pacific regions (Luce-Fedrow et al., 2018, Vincent, 2016). Although, mites are known vectors for *Orientia* species transmission, this zoonotic pathogen has been reported in both canine blood and ticks via PCR (Namikawa et al., 2014), and only canine blood using serological techniques (Weitzel et al., 2018). Similarly, recent South African studies reported the presence of *Orientia tsutsugamushi* like pathogen from canine blood via qPCR (Kolo et al., 2016). No report has been documented in Nigeria at the time of writing this report. Although, these findings add to a growing body of evidence that this pathogen may be spread by ticks as well as mites and may be of significance in canine disease, further investigation is required.

Two genera (*Theileria orientalis* (in ticks), and *Plasmodium* species (both ticks and blood)) belonging to the phyla *Apicomplexa* known as obligate parasites of invertebrate and vertebrate animals. The most common species of *Theileria* reported in dogs from Africa and other parts of the world include *T. equi, T annulata* (Chomel, 2011) and. *T. orientali*s, the cause of serious outbreak of bovine theileriosis in New Zealand (Lawrence et al., 2021) and Australia (Eamens et al., 2013). Report of clinical disease caused by *T. orientalis* in domestic animals including cattle are rare in Africa, except for molecular prevalence reports from asymptomatic cattle (e.g., Ethiopia) (Gebrekidan et al., 2016). Canine theileriosis is an emerging febrile tick-borne disease of dogs caused by various *Theileria* species mentioned above. Ticks of the genera *Amblyomma, Hyalomma*, and those identified in this study (*Haemaphysalis,* and *Rhipicephalus)* are known to transmit canine theileriosis (Stockham et al., 2000). However, certain *Theileria* species infecting dogs are also thought to be transmitted vertically from bitches to puppies (Falkenö et al., 2013). Dogs are regarded as latent host for *T. orientalis* (Lawrence et al., 2021), the report of *T. orientalis* prevalence has been documented in apparently healthy dogs using PCR (Xu et al., 2015). *T. orientalis* is also prevalent in ticks that had previously

fed on livestock before attachment to dogs and these findings provides additional information on the number of *Theileria* species carried by hard ticks in Nigeria but require further investigation.

The identification of *Plasmodium* from ticks in this study support a previous study in Pakistan where *Plasmodium* was detected in *Rhipicephalus microplus* established to have fed on humans before sample collection in malaria endemic areas (Adegoke et al., 2020). *Plasmodium mexicanum,* a parasite of lizards transmitted by sand flies has been detected in *Ixodes pacificus* and *Geckobiella occidentalis* species of mites (Schall and Smith, 2006). The report of *Plasmodium* in this study seems to be an unusual finding, *Culex*, *Aedes*, and *Anopheles* mosquitoes are considered reservoir and competent vectors for the transmission of *Plasmodium* species and not hard ticks (Gutierrez-Lopez et al., 2020), this report should be interpreted with caution. In addition, we ground tick samples analyzed in this study during sample collection before placing on Whatman's FTA card for shipment, increasing the possibility of environmental contamination following mechanical feeding activities of mosquitoes on FTA cards that were left to dry at room temperature. Despite this, it is unlikely that the report of *Plasmodium* in this study could be due to environmental contamination requiring further investigation.

Moreover, the phyla Actinobacteria (canine blood: 0.23%, hard ticks: 3.51%) identified in this study contains *Mycobacterium* and *Microbacterium* genera. Nevertheless, the presence of non-specific *Mycobacterium* species in both canine blood and tick sample datasets support previous metagenomics studies on canine blood (Huggins et al., 2021b), and ticks (Carpi et al., 2011). Reports on the prevalence of *Mycobacterium* species from ticks are rare, apart from the report on *Mycobacterium* and other members of Proteobacteria species associated with *Ixodes ricinus* using metagenomic profiling (Carpi et al., 2011). Rare cases of *Mycobacterium* infection are also reported in dogs, canine tuberculosis is caused by two major species of the *M. tuberculosis complex* (Mtbc) including *M. tuberculosis* and *M. bovis*. Mtbc are reported to live and

survive in the soil for a lengthy period (Ghodbane et al., 2014, De Groote et al., 2006), and their presence may be due to environmental contamination of samples analysed in this study requiring further confirmation.

Additionally, two bacteria genera *Borrelia* and *Leptospira* classified under the phyla Spirochaetes (0.09%) were identified in this study. All canine blood samples tested negative to *B. burgdorferi sensu lato* following POC testing on blood and *Borrelia 16S* gene qPCR assay on canine blood and hard tick samples. Both canine blood and tick samples screened were negative for *Borrelia* species. The results of the metagenomics classification demonstrated the absence of *Borrelia* species in all the canine blood samples assessed. On the other hand, non-specific *Borrelia* species were identified from all the hard tick sample datasets assessed in this study, supporting a previous Nigerian studies demonstrating the presence of non-specific *Borrelia* species in ticks via PCR (Reye et al., 2012). The absence of reports of specific species of such as *B. burgdorferi* (s.l.) in this study, may be due to the absence of primary vectors like *Ixodes* tick *spp*. (Krupka and Straubinger, 2010, Rudenko et al., 2011).

Leptospira species were not reported from the canine blood samples however, this genus of bacteria was identified from all the four tick samples assessed. Contact with infected animals, urine, or wet surfaces is established to be the principal route for *Leptospira* transmission. These bacteria are known to survive in wet environment for an extended period, and outbreaks have been recorded in areas with frequent cases of flooding and Nigeria is not an exception. Rare reports of *Leptospira* species from ticks are available, with the first report in Poland where *Leptospira* species were reported in *Ixodes* species collected from areas known to have recent cases of flooding (Wójcik-Fatla et al., 2012), confirming this report is likely due to the presence of *Leptospira* as a potential tick flora or environmental contaminant.

The genera *Leishmania* belonging to the phyla *Euglenozoa* (0.15%) is another genus with high relative abundance from the hard tick samples. These were identified to a taxonomic species level as *Leishmania major*. These

trypanosomatids are obligatory intracellular parasites and are very unlikely to be environmental contaminants. Although *Leishmania* was identified in both canine blood and hard tick samples, higher abundance was recorded from the hard tick samples assessed. *Leishmania* are protozoal parasites causing Leishmaniases in both wild and domestic animals. They are considered to be a major public health threat globally (Alvar et al., 2012). Female sandflies from the family *Phlebotominae* have been established as the biological vectors for the transmission of *Leishmania* between animals, with a secondary transmission to humans. However, there are suggestions that ticks, and fleas may be responsible for the transmission of certain species of *Leishmania* including *L. infantum* (Dantas-Torres, 2011). Previous studies have demonstrated natural transovarial and transstadial passage of *L. infantum* from infected female *R. sanguineus* to newly emerging larvae (Dabaghmanesh et al., 2016). Quantification of *L. infantum* DNA from *R. sanguineus* female adults, eggs and larvae is also reported (Dantas-Torres et al., 2011). While the presence of *L. infantum* in *R. sanguineus* (7/91), and *Ixodes ricinus* (1/91) ticks, alongside other tick-borne pathogens such as *B. canis*, and *Rickettsia* have been reported (Trotta et al., 2012). In addition, a serological study reporting canine leishmaniasis prevalence of 4.40% (12/273) in Nigeria is documented (Adediran et al., 2016). Although, zoonotic cases of human leishmaniasis caused by *L. major* and *L. infantum* are underreported and considered a mild infection in Africa (Alvar et al., 2012, Kone et al., 2019). This parasite causes serious disease that may be difficult to treat, prevent and control in both humans and animals (Bamorovat et al., 2021), justifying why findings reported in this study require additional investigation.

The genus *Mycoplasma* belonging to the phyla *Tenericute* (0.13%) was identified from only hard tick sample datasets in this study, though specific species identification was not defined going by the minimum threshold set in this study however, respiratory/urogenital and haemotropic mycoplasmas have been reported in dogs. Haemotropic mycoplasma species including *M. haemocanis*

(erstwhile *Haemobartnonella canis* and *Eperythrozoon canis*), is the causative agent of vector-borne haemotropic mycoplasmosis in dogs. *M. haemocanis* is transmitted by fleas and *R. sanguineus*. Transstadial and transovarial transmission has been reported in ticks, meaning ticks could serve as reservoir host and vector (Greay, 2020). Recent report of *Mycoplasma* species in canine blood sample using a combination of PCR and next generation sequencing partially support this study (Huggins et al., 2021a, Huggins et al., 2021b). Similarly, our findings support recent studies that reported the prevalence of *Mycoplasma* from *R. sanguineus* in Australia (Greay, 2020), and from both ticks and blood samples collected from stray dogs via PCR in Thailand (Do et al., 2021). Haemotropic mycoplasmas have been reported from Nigerian dogs using PCR including South west (0.8%; n = 116) (Happi et al., 2018) and North central (17.1; n = 245) (Aquino et al., 2016), suggesting the risk of disease burden as reported in this study. However, the contribution of haemotropic *Mycoplasma* to canine disease burden require further investigation in Nigeria.

Finally, antimicrobial resistance (AMR) gene profiles were reported from assembled contigs generated using the hard tick datasets. No AMR genes were identified from contigs generated from the canine blood datasets. This result is in line with the results obtained from taxonomic classification of the assembled contigs as fewer microbes were identified from canine blood. Metagenomics is regarded as an efficient approach to monitoring global AMR gene prevalence in patients, food animals and environment (Williams et al., 2016, Danko et al., 2021). The report of AMR genes for macrolides, lincosamides, streptomycin and beta-lactam in this study, support previous studies on global microbiome and antimicrobial resistance profiles (Danko et al., 2021). Previous studies on the urban microbiome and AMR profiles have also shown beta-lactams to be the most prevalent AMR gene across the globe, and this finding is not entirely unexpected (Danko et al., 2021). Although, there is no specific information on how hard tick bacteria acquire AMR resistance gene in their environment, there are reports suggesting insects, rodents, and pets as reservoirs, vectors, and

sentinels of antimicrobial resistance and, this may be applicable to ticks (Gwenzi et al., 2021).

In conclusion, findings on metagenomics profiling provided insights into the prevalence of apicomplexan and bacterial microbiomes in both canine blood and hard ticks. Additional information was provided on vector-borne pathogens prevalent in dog ticks, alongside with pathogens known to be of public health threat to humans in Nigeria. Although, it is suggested reports from this study should be interpreted with caution due the presence of potential contaminants, these findings may also serve as additional baseline information to the existing data on canine pathogens and hard tick microbial communities or contaminants in Nigeria, and for future studies on vector-borne pathogens epidemiology in Nigeria.

Chapter 7: Summary, limitations, and recommendation for future work

7.1 Summary

The POC and molecular diagnosis showed that *B. burgdorferi* is unlikely to be present in Nigeria. It is also unlikely that *Rhipicephalus* species are responsible for *B. burgdorferi* transmission in Nigerian dogs, confirming previous reports that *B. burgdorfer*i and its principal vector *Ixodes* are more common in temperate regions.

However, in the face of mosquitoes as the principal vector for heartworm disease transmission, coupled with previous reports on the presence of *Dirofilaria* spp. in Nigeria, an exceptionally low prevalence of *Dirofilaria* species was recorded via POC antigen test, and no positive sample was recorded via the panfilarial qPCR protocol used in this study. Although, the widespread use of Ivermectin for parasitic treatment may be responsible, proper sample collection, diagnostic methods (combination of antigen test, Knott's technique, and PCR) are required to establish the true prevalence of canine filarial species in Nigeria. Negative results do not rule out the fact that *B. burgdorferi* (s.l.) and *D. immitis* are currently circulating in Nigerian dogs but demands for more extensive research to confirm the absence or presence of these zoonotic pathogens.

The presence of *B. vogeli*, *B. rossi*, and the absence of *B. gibsoni* in Nigerian dogs reported in this study supports previously studies on canine babesiosis in Nigeria. The cause of severe canine babesiosis (*Babesia* species) and ehrlichiosis (*Ehrlichia* species) are the most prevalent CVBPs infecting Nigerian dogs, following serological diagnosis (for *Ehrlichia* species) and PCR for both pathogens as shown in this study. This implies that there is a considerable risk of infection and disease burden in Nigerian dogs as the principal vector for these pathogens are established to be abundant in Nigeria. The inclusion of these pathogens in differential diagnosis during clinical

examination of dogs, and adherence to strategic tick control is recommended. A combination of both the POC and PCR-based test should offer the most accurate and reliable results for effective diagnosis of and treatment of CVBDs (specifically *E. canis*) and could be recommended to Nigerian veterinarians.

Assessment of risk factors associated with CVBPs using the POC test results (*Anaplasma* and *Ehrlichia*) showed that male dogs are more likely to be infested with ticks than females, and older dogs infested with ticks are likely to be seropositive to CVBPs. While the results of risk factor assessment using *Ehrlichia* and *Babesia* species PCR testing provided evidence that native dogs are less likely to test positive to CVBPs, and active infection with CVBPs is common during the rainy season of the year. Demonstrating evidence of breed variation and susceptibility to CVBPs, partly supporting a common belief shared by Nigerian veterinarians. Also, serological testing with POC kits provided information on immune experience to CVBPs in Nigerian older dogs including those infested with ticks, while PCR testing results provided evidence on active infection for dogs assessed. Although risk factors such as sex (male dogs), age (older dogs), breed (exotic dogs), and season of the year (rainy) cannot be easily modified, alternative methods of tick control including avoidance of roaming activities in male dogs, deliberate efforts to reduce exposure of older dogs or exotic breeds to tick infestation especially during the rainy season of the year is recommended.

The tropical lineage of *R. sanguineus* (s.l.) and *H. leachi* were demonstrated to be the most abundant canine hard tick population in Nigeria. Evidence for the presence of *Haemaphysalis* species tentatively identified as *H. longicornis* from Nigerian domestic birds is also provided in this study. In addition, the high prevalence of *Ehrlichia* species in *R. sanguineus*, confirms the serological and PCR testing on canine blood samples, and further provides information on the risk of disease burden for dogs and their owners.

The next generation sequencing approached used in this study provided insightful information on *Leishmania* species and microbial pathogens that

have not been reported in Nigerian hard ticks including *Plasmodium falciparum*, *Orientia*, and *Candidatus coxiella mudrowiae* etc. A further study is required to establish if these pathogens are environmental or tick contaminants and/or contribute to disease burden in Nigerian dogs.

7.2 Limitations

The piroplasm species PCR was an efficient protocol for the amplification of piroplasms 18S RNA gene from canine blood but was non-reproducible when applied to hard tick samples, impacting on piroplasm prevalence results obtained from hard ticks. The piroplasm 18S rRNA qPCR assay is designed to amplify over 400 bp of qPCR product creating room for a decrease in analytical specificity of this assay. Also, recent evidence demonstrates that the published 18S rRNA gene assay used in this study is unlikely to effectively detect other species of piroplasm including *Babesia divergens* (Fernandez et al., 2022). Application of an in-silico primer check tool (primer blast) as recommended by Bustin et al. (2009) confirmed the efficiency of the piroplasm primers to amplify 18S rRNA gene from algae etc. (e.g., symbiotic dinoflagellates: FJ823469) demonstrating why non-specific products were obtained following qPCR in ticks. The use of alternative qPCR assay for piroplasm qPCR with short qPCR amplicons to maximize high specific and efficiency (Svec et al., 2015) alongside conventional PCR assays to generate longer DNA sequences (where next generation sequencing is not achievable) for efficient identification of species is recommended for future studies.

It is possible that examination of fresh whole blood sample using a combination of microscopy (Knott's technique), antigen and PCR tests would have yielded > 0% prevalence or served as an additional diagnostic technique to confirm the absence or presence of *D. immitis* in this study. PCR is a sensitive diagnostic test known to depend on the number of circulating microfilariae. Negative PCR results reported may be associated with the absence of circulating microfilariae in dogs examined (Gioia et al., 2010, Borthakur et al., 2015). Previous studies have

reported a correlation between microscopic and PCR tests results (including Knott's technique) demonstrating a direct relationship between positive results obtained via microscopic parasitological technique and PCR positivity (Trancoso et al., 2020). Examination of fresh blood samples using a combination of these three diagnostic methods is suggested to eliminate this limitation and provide information on the true prevalence of *Dirofilaria* species in Nigeria dogs.

The generic *Ehrlichia* PCR protocol designed to amplify both *Ehrlichia* and *Anaplasma* species was also less efficient at detecting *Anaplasma* species, the use of efficient alternative generic PCR assay to amplify 23S rRNA from all members of the *Anaplasmataceae*, followed by generic and species-specific assays for *Anaplasma*, *Ehrlichia* and *Rickettsia* species detection as reported by Maksimovic et al. (2021) is recommended to avoid this limitation in for future studies.

The preliminary screening test using IDEXX test kits and PCR were not designed to include most of the endosymbionts or pathogens identified to be associated with hard ticks (including *Coxiella*, *Francisella, Wolbachia*, *Leishmania*, *Leptospira*, *Mycoplasma*, and *Plasmodium*) following NGS analysis. This created more room for doubts as to whether microbes identified were environmental or tick contaminants. Results on metagenomics profiling reported in this study should be interpreted with caution as environmental or host contaminants were not properly eliminated prior to DNA sequencing.

7.3 Recommendation for future work

A CVBP prevalence of 37.5% for indigenous and 55.9% for exotic dog breeds was reported in this study. An estimated sample size of 380 — 400 canine blood is recommended for future studies aimed at establishing the true prevalence of CVBPs among Nigerian dogs. Prevalence studies using additional lateral flow device test kits for the detection of CVBPs such as *Babesia* and *Leishmania* species prevalent in Nigeria rather than the use of IDEXX test kit which detects pathogens relevant to the North American region is recommended. Commercially available rapid POC antibody test kits for canine *Babesia* and *Leishmania* (e.g., rK39 RDT: Kalazar Detect Canine Rapid Test; Inbios) (Quinnell et al., 2013) species detection are recommended for future study.

This study was performed following collection of canine blood and ground tick samples (washed with 70% alcohol) on FTA cards across different veterinary clinics in Nigeria. While these findings are interesting, the use of whole blood and tick samples rather than smears on FTA cards is recommended to offer a more decent DNA quality and confirm results of the NGS methodology obtained in this study. Where the use of FTA card remains the only available option for sample collection and metagenomics, analysis of minimum negative control sequences from FTA cards with no samples is recommended. A combination of whole tick sample collection, washing with 10% bleach (sodium hypochlorite to eliminate contaminants), followed by 70% ethanol (preserve DNA) for 3 — 5 minutes, then additional wash (at least twice) with phosphate buffer saline (PBS) is recommended. This is reported to offer adequate DNA quality, eliminate environmental or tick contaminants, and provide more information on host-pathogen-vector transmission (Jiao et al., 2021, Martinez-Garcia et al., 2021, Karimian et al., 2022). Further studies to confirm if *Leishmania* and *Plasmodium* species are currently circulating between dogs and ticks in Nigeria is also suggested. DNA extraction and detection of *Leishmania* and *Plasmodium* from canine blood and specific hard

tick tissues (including midguts, salivary gland, and haemolymph if microscopy is required) using PCR and NGS is recommended to provide additional evidence on the prevalence of *Leishmania*, *Plasmodium* and other CVBPs in canine blood or ticks. Detection of TBPs from midgut and salivary glands of unfed ticks using PCR and NGS techniques is also relevant to assess the location, migration, and transmission of *Leishmania*, *Plasmodium* and other TBPs reported in this study.

In addition to the NGS sample requirement recommended above, future NGS study on CVBPs (using canine blood and tick) aimed at detecting protozoan haemoparasites is recommended to design or apply the use of published *18S rRNA* gene primers designed to exclusively amplify the conserved apicomplexan and kinetoplastid parasites (Huggins et al., 2019b). While the design or application of published bacterial primers to exclusively amplify bacterial 16S rRNA gene is recommended where only canine bacterial haemoparasites are investigated (Vasconcelos et al., 2021). Where more resources are available, the design or application of published multipronged CVBPs next generation sequencing approach to detect a wide range of bacterial, and protozoa haemoparasites is also recommended (Huggins et al., 2021b). Both methods are designed to exclusively amplify bacterial and protozoan DNA minimizing the presence of host reads in NGS data, with an efficient downstream bioinformatics analysis.

Another study focusing on individual dog breeds is recommended to confirm findings from this study, as breed-specific genetic variation in susceptibility, tolerance and resistance to disease may exist. A preliminary study to provide field or laboratory evidence for the presence of genetic variation for tolerance between native and exotic dogs could include an experimental design to determine the red blood cell (RBC) density and weight loss between native and exotic dogs diagnosed/infected with *Ehrlichia* or *Babesia* species. This evidenced-based study will aim at measuring the peak parasite density (microscopy or PCR), anaemia (packed cell volume and thrombocytopaenia),

in exotic and Nigerian indigenous dog breeds before, during and after infection. Studies to investigate and compare the variation in cellular or humoral response to CVBPs between indigenous and exotic breeds are also suggested to confirm the phenomenon that Nigerian indigenous dogs are less likely to test positive to CVBPs. Where funds are available, standard ELISA assay for the quantification of humoral immune response (IgG or IgM) to CVBP infection can be developed, while commercial cytokine detection assays (ELISA) can be used to measure circulating interleukins in exotic and indigenous dog breeds (Lee et al., 2021) to ascertain cellular immune response in these breeds. The chi-square test is a recommended useful tool for the analysis of variation in immune response between exotic and indigenous dogs.

Additionally, a large-scale genome-wide association study (GWAS) to compare the differences in copy number variation and single nucleotide polymorphism (SNPs) between indigenous and dog breeds exotic to Nigeria or reference genome from NCBI or ENA database is required. The aim would be to identify genetic markers responsible for susceptibility, tolerance, and resistance to CVBPs between dog breeds. This study will provide additional information on genetic factors responsible for increased or reduced susceptibility to CVBPs. GWAS similar to a previous study on diverse cattle breeds reporting the presence of noticeable genotypes linked to growth and regional adaptation to disease in trypanotolerant African cattle breed (N'Dama) (Kim et al., 2017) is recommended for future studies.

Another interesting study to explore is the analysis of host DNA or blood meal from tick samples to establish potential reservoir hosts for the maintenance of CVBPs. Blood meal analysis targeting amplification of conserved mammalian mitochondrial gene using PCR probes or high-resolution melting temperature (with 50 — 60% sensitivity), and an overly expensive but sensitive tick proteins mass spectroscopy (efficiency of 88% recorded) using established database of known host have been documented. However, the

former methods (PCR probes or high-resolution melting temperature) depend on remnants of blood meal in host-seeking ticks. Two or three host hard ticks need a single blood meal at each stage of their lifecycle, while moulting, developing to the next stage and seeking of a new host may occur several months after the initial blood meal. Blood meal analysis targeting mitochondrial genome may only attempt to detect remnants of the reservoir host blood meal DNA present after several months if not years post previous feeding, and it is likely to be less efficient and recommended for future studies. The latter (mass spectroscopy of tick proteins) is also less likely to be recommended as it expensive and has not been demonstrated in field studies (Goethert et al., 2021). The use of retrotransposons-based host blood meal analysis in hard ticks is recommended for future studies. Retrotransposons are genetic materials known to integrate into host genomes and replicate using RNA intermediate template, leaving multiple DNA copies in the host post replication. Real-time PCR assays targeting retrotransposons of suspected reservoir hosts in ticks including rodents and white-tailed deer have produced efficient and cost-effective results in field studies (Goethert et al., 2021).

Appendices

Appendix 1: Tick species population infesting Nigerian dogs and local birds

Table 8.1: showing hard tick species population infesting Nigerian dogs and local birds, prevalence of selected tick-borne pathogens tested using qPCR. The table also contains GenBank accession numbers identical with the nucleotide sequences following successful Sanger sequencing and NCBI nucleotide blast search.

	No of Adult ticks collected	Morphological identification	Hard ticks *16S rRNA* PCR identification	GenBank accession number	*E. canis*	*Piroplasm*	*B. burgdorferi*
Benue state Niger							
Canine ticks	5	*Haemaphysalis spp.*	*H. leachi*	MN661151			
	1	*R. sanguineus*	*R. sanguineus*	KT989305			
	7	*R. sanguineus*	*R. sanguineus*	MK158996	5	1	
	5	*R. sanguineus*	*R. sanguineus*	MT568621 Ilorin	4	1	
	1	*R. sanguineus*	*R. sanguineus*	MK941245			
	1	*R. sanguineus*	*R. sanguineus*	MT026921	1		
	3	*R. sanguineus*	*R. sanguineus*	MK159003	3	1	
	1	*R. sanguineus*	*R. sanguineus*	MN994317	1		
	1	*R. sanguineus*	*R. sanguineus*	MT320105	1	1	
	1	*R. sanguineus*	*R. sanguineus*	KC476294			
	1	*R. sanguineus*	*R. sanguineus*	JX195174	1	1	
	1	*R. sanguineus*	*R. sanguineus*	KT382453	1		
	1	*R. sanguineus*	*R. sanguineus*	MK159005		1	
	1	*R. sanguineus*	*R. sanguineus*	MT008196	1		
	1	*R. sanguineus*	*R. sanguineus*	MW560384	1	1	

	No of Adult ticks collected	Morphological identification	Hard ticks *16S rRNA* PCR identification	GenBank accession number	*E. canis*	*Piroplasm*	*B. burgdorferi*
	5	*R. sanguineus*	*R. sanguineus*	Poor quality	4	1	
	60	*R. sanguineus*	*R. sanguineus*	Not sequenced	34	8	
	96						
Local bird ticks	11	*Haemaphysalis spp.*	*Ha. bancrofti*	NC_041076	Neg.		
Total	107				57	16	
Federal Capital Territory (Abuja)							
Canine ticks	3	*R. sanguineus*	*R. sanguineus*	MK158996	1	2	
	2	*R. sanguineus*	*R. sanguineus*	MT568621 Ilorin	2		
	1	*R. sanguineus*	*R. sanguineus*	Poor quality	1		
	2	*R. sanguineus*	*R. sanguineus*	Not sequenced	2	2	
Total	8				6	4	
Oyo state (Ibadan)							
Canine ticks	1	*R. sanguineus*	*R. sanguineus*	MW560390			
	1	*R. sanguineus*	*R. sanguineus*	MH513312	1		
	1	*R. sanguineus*	*R. sanguineus*	KX793744	1		
	1	*R. sanguineus*	*R. sanguineus*	MK159003	1		
	2	*R. sanguineus*	*R. sanguineus*	Poor quality	1		
	2	*R. sanguineus*	*R. sanguineus*	Not sequenced	2		
Total	8				6		
Grand total	123				77	20	

Appendix 2: Additional information on Canine and tick metagenomics

Table 8.2: Showing the number and percentage of sequence reads survived following quality filtering and trimming.

S/No	Sample name	Raw reads	Trimmed reads	% Survived
1	Dog5 tick sample 5B lane 1 read 1	22504261	22502235	99.99099726
2	Dog5 tick sample 5B lane 1 read 2	22504261	22502235	99.99099726
3	Dog5 tick sample 5B lane 2 read 1	14160630	14159433	99.99154699
4	Dog5 tick sample 5B lane 2 read 2	14160630	14157979	99.98127908
5	Local bird 6 tick sample 6D lane 1 read 1	25676119	25675696	99.99835255
6	Local bird 6 tick sample 6d lane 2 read 1	25676119	25671514	99.98206505
7	Local bird 6 tick sample 6d lane 2 read 1	12271519	12271331	99.998468
8	Local bird 6 tick sample 6D lane 2 read 2	12271519	12268150	99.97254619
9	Dog223 tick sample 223D lane 1 read 1	22177911	22177048	99.99610874
10	Dog223 tick sample 223D lane 1 read 2	22177911	22176465	99.99348
11	Dog223 tick sample 223D lane 2 read 1	15071908	5670287	37.62156059
12	Dog223 tick sample 223D lane 2 read 2	15071908	15070330	99.98953019
13	Dog231 tick sample 231C lane 1 read 1	20302886	20301596	99.99364622
14	Dog231 tick sample 231C lane 1 read 2	20302886	20301695	99.99413384
15	Dog231, tick 231C lane 2 read 1	15697272	15696607	99.9957636
16	Dog231, tick 231C lane 2 read 2	15697272	15695957	99.99162275
17	Dog35 blood sample 35 lane 1 read 1	24538081	24536040	99.99168232
18	Dog35 blood sample 35 lane 1 read 2	24538081	24536737	99.9945228
19	Dog35 blood sample 35 lane 2 read 1	12884040	12883057	99.99237041
20	Dog35 blood sample 35 lane 2 read 2	12884040	12883232	99.99372868

S/No	Sample name	Raw reads	Trimmed reads	% Survived
21	Dog35 blood sample 35 lane 3 read 1	14645088	14644138	99.99351318
22	Dog35 blood sample 35 lane 3 read 2	14645088	14644386	99.99520658
23	Dog144 blood sample 144 lane 1 read 1	25994025	25991545	99.99045935
24	Dog144 blood sample 144 lane 1 read 2	25994025	25992825	99.99538355
25	Dog144 blood sample 144 lane 2 read 1	12224401	12223154	99.98979909
26	Dog144 blood sample 144 lane 2 read 2	12224401	12223576	99.9932512
27	Dog153 blood sample 153 lane 1 read 1	38963995	38958615	99.98619238
28	Dog153 blood sample 153 lane 1 read 2	38963995	38961712	99.99414074
29	Dog168 blood sample 168 lane 1 read 1	23914516	23913426	99.9954421
30	Dog168 blood sample 168 lane 1 read 2	23914516	23912581	99.99190868
31	Dog168 blood sample 168 lane 2 read 1	12248117	12247678	99.99641578
32	Dog168 blood sample 168 lane 2 read 2	12248117	12247073	99.99147624
33	Dog194 blood sample 194 lane 1 read 1	24915067	24912251	99.9886976
34	Dog194 blood sample 194 lane 1 read 2	24915067	24913939	99.99547262
35	Dog194 blood sample 194 lane 2 read 1	13024029	13022792	99.99050217
36	Dog194 blood sample 194 lane 2 read 2	13024029	13023220	99.9937884
37	Dog210 blood sample 210 lane 1b read 1	9851539	9850994	99.99446787
38	Dog210 blood sample 210 lane 1b read 2	9851539	9851078	99.99532053
39	Dog210 blood sample 210 lane 1 read 1	27199646	27198050	99.99413228
40	Dog210 blood sample 210 lane 2 read 2	27199646	27198511	99.99582715
41	Dog210 blood sample 210 lane 3 read 1	11889800	11889210	99.99503776
42	Dog210 blood sample 210 lane 3 read 2	11889800	11889419	99.99679557
	Total	800309700	790847797	98.81771982

Table 8.3: Showing statistics on assembly contigs generated from canine blood datasets using Megahit. Statistical data on all contigs presented were generated using Bbmap tools script.

B35 assembled contigs stat using Bbmap tool script								
A	C	G	T	N	IUPAC	Other	GC	GC_stdev
0.2379	0.2674	0.2648	0.2299	0	0	0	0.5321	0.1357

Main genome scaffold total:	4655		
Main genome contig total:	4655		
Main genome scaffold sequence total:	9.922 MB		
Main genome contig sequence total:	9.922 MB	0.000% gap	
Main genome scaffold N/L50:	1052/2.204 KB		
Main genome contig N/L50:	1052/2.204 KB		
Main genome scaffold N/L90:	3724/1.123 KB		
Main genome contig N/L90:	3724/1.123 KB		
Max scaffold length:	30.171 KB		
Max contig length:	30.171 KB		
Number of scaffolds > 50 KB:	0		
% Main genome in scaffolds > 50 KB:	0.00%		
Minimum scaffold length	Number of contigs	Total contigs length	scaffold contig coverage
All	4,655	9,922,102	100.00%
500	4,655	9,922,102	100.00%
1 KB	4,655	9,922,102	100.00%
2.5 KB	810	4,394,854	100.00%
5 KB	258	2,561,541	100.00%
10 KB	88	1,374,659	100.00%
25 KB	7	193,846	100.00%

B144 assembled contigs stat using Bbmap tool script								
A	C	G	T	N	IUPAC	Other	GC	GC_stdev
0.2464	0.2601	0.2562	0.2373	0	0	0	0.5163	0.1128

Main genome scaffold total:	3527		
Main genome contig total:	3527		
Main genome scaffold sequence total:	6.803 MB		
Main genome contig sequence total:	6.803 MB	0.000% gap	
Main genome scaffold N/L50:	993/1.983 KB		
Main genome contig N/L50:	993/1.983 KB		
Main genome scaffold N/L90:	2888/1.111 KB		
Main genome contig N/L90:	2888/1.111 KB		
Max scaffold length:	16.422 KB		
Max contig length:	16.422 KB		
Number of scaffolds > 50 KB:	0		
% Main genome in scaffolds > 50 KB:	0.00%		
Minimum scaffold length	Number of contigs	Total contigs length	scaffold contig coverage
All	3,527	6,803,262	100.00%
500	3,527	6,803,262	100.00%
1 KB	3,527	6,803,262	100.00%
2.5 KB	598	2,532,258	100.00%
5 KB	136	1,003,577	100.00%
10 KB	17	217,602	100.00%

B153 assembled contigs stat using Bbmap tool script								
A	C	G	T	N	IUPAC	Other	GC	GC_stdev
0.2389	0.2643	0.2645	0.2323	0	0	0	0.5289	0.1168

Main genome scaffold total:	3136

Main genome contig total:	3136	
Main genome scaffold sequence total:	6.315 MB	
Main genome contig sequence total:	6.315 MB	0.000% gap
Main genome scaffold N/L50:	827/2.131 KB	
Main genome contig N/L50:	827/2.131 KB	
Main genome scaffold N/L90:	2541/1.13 KB	
Main genome contig N/L90:	2541/1.13 KB	
Max scaffold length:	22.087 KB	
Max contig length:	22.087 KB	
Number of scaffolds > 50 KB:	0	
% Main genome in scaffolds > 50 KB:	0.00%	

Minimum scaffold length	Number of contigs	Total contigs length	scaffold contig coverage
All	3,136	6,314,626	100.00%
500	3,136	6,314,626	100.00%
1 KB	3,136	6,314,626	100.00%
2.5 KB	608	2,654,628	100.00%
5 KB	153	1,125,685	100.00%
10 KB	16	210,096	100.00%

B168 assembled contigs stat using Bbmap tool script

A	C	G	T	N	IUPAC	Other	GC	GC_stdev
0.2408	0.2639	0.2622	0.2331	0	0	0	0.5261	0.1177

Main genome scaffold total:	3160
Main genome contig total:	3160

Main genome scaffold sequence total:	5.880 MB		
Main genome contig sequence total:	5.880 MB	0.000% gap	
Main genome scaffold N/L50:	934/1.909 KB		
Main genome contig N/L50:	934/1.909 KB		
Main genome scaffold N/L90:	2603/1.113 KB		
Main genome contig N/L90:	2603/1.113 KB		
Max scaffold length:	16.074 KB		
Max contig length:	16.074 KB		
Number of scaffolds > 50 KB:	0		
% Main genome in scaffolds > 50 KB:	0.00%		
Minimum scaffold length	Number of contigs	Total contigs length	contig coverage
All	3,160	5,880,496	100.00%
500	3,160	5,880,496	100.00%
1 KB	3,160	5,880,496	100.00%
2.5 KB	498	2,003,008	100.00%
5 KB	98	673,281	100.00%
10 KB	7	91,978	100.00%

B194 assembled contigs stat using Bbmap tool script								
A	C	G	T	N	IUPAC	Other	GC	GC_stdev
0.2433	0.263	0.26	0.2336	0	0	0	0.523	0.1114
Main genome scaffold total:	3561							
Main genome contig total:	3561							
Main genome scaffold sequence total:	6.633 MB							
Main genome contig sequence total:	6.633 MB	0.000% gap						
Main genome scaffold N/L50:	1025/1.889 KB							
Main genome contig N/L50:	1025/1.889 KB							
Main genome scaffold N/L90:	2930/1.103 KB							

Main genome contig N/L90:	2930/1.103 KB		
Max scaffold length:	17.352 KB		
Max contig length:	17.352 KB		
Number of scaffolds > 50 KB:	0		
% Main genome in scaffolds > 50 KB:	0.00%		
Minimum scaffold length	Number of contigs	Total contigs length	scaffold contig coverage
All	3,561	6,632,913	100.00%
500	3,561	6,632,913	100.00%
1 KB	3,561	6,632,913	100.00%
2.5 KB	570	2,345,701	100.00%
5 KB	119	848,448	100.00%
10 KB	11	144,158	100.00%

B210 assembled contigs stat using Bbmap tool script stats.h

A	C	G	T	N	IUPAC	Other	GC	GC_stdev
0.2419	0.2614	0.2615	0.2352	0	0	0	0.5229	0.1231
Main genome scaffold total:	3546							
Main genome contig total:	3546							
Main genome scaffold sequence total:	7.688 MB							
Main genome contig sequence total:	7.688 MB	0.000% gap						
Main genome scaffold N/L50:	824/2.282 KB							
Main genome contig N/L50:	824/2.282 KB							
Main genome scaffold N/L90:	2824/1.139 KB							
Main genome contig N/L90:	2824/1.139 KB							
Max scaffold length:	31.695 KB							
Max contig length:	31.695 KB							
Number of scaffolds > 50 KB:	0							
% Main genome in scaffolds > 50 KB:	0.00%							

Minimum scaffold length	Number of contigs	Total contigs length	scaffold contig coverage
All	3,546	7,688,266	100.00%
500	3,546	7,688,266	100.00%
1 KB	3,546	7,688,266	100.00%
2.5 KB	686	3,518,293	100.00%
5 KB	212	1,927,696	100.00%
10 KB	59	873,078	100.00%
25 KB	3	88,660	100.00%

Table 8.4: Showing statistics on assembly contigs generated from tick sample datasets using Megahit. Statistical data on all contigs presented were generated using Bbmap tools script.

T5B assembled contigs stat using Bbmap tool script								
A	C	G	T	N	IUPAC	Other	GC	GC_stdev
0.2599	0.242	0.2413	0.2568	0	0	0	0.4833	0.0436
Main genome scaffold total:	373659							
Main genome contig total:	373659							
Main genome scaffold sequence total:	633.586 MB							
Main genome contig sequence total:	633.586 MB	0.000% gap						
Main genome scaffold N/L50:	124987/1.702 KB							
Main genome contig N/L50:	124987/1.702 KB							
Main genome scaffold N/L90:	313440/1.101 KB							
Main genome contig N/L90:	313440/1.101 KB							
Max scaffold length:	451.264 KB							
Max contig length:	451.264 KB							
Number of scaffolds > 50 KB:	3							
% Main genome in scaffolds > 50 KB:	0.13%							
Minimum scaffold length	Number of contigs	Total contigs length	scaffold contig coverage					
All	373,659	633,586,153	100.00%					
500	373,659	633,586,153	100.00%					
1 KB	373,659	633,586,153	100.00%					
2.5 KB	44,608	154,232,224	100.00%					
5 KB	3,384	21,918,830	100.00%					
10 KB	90	1,935,181	100.00%					
25 KB	5	891,168	100.00%					

50 KB	3	827,473	100.00%
100 KB	2	742,720	100.00%
250 KB	2	742,720	100.00%

NLB6D assembled contigs stat using Bbmap tool script

A	C	G	T	N	IUPAC	Other	GC	GC_stdev
0.2019	0.2996	0.2986	0.1998	0	0	0	0.5983	0.0923

Main genome scaffold total:	59655
Main genome contig total:	59655
Main genome scaffold sequence total:	154.470 MB
Main genome contig sequence total:	154.470 MB 0.000% gap
Main genome scaffold N/L50:	6515/2.881 KB
Main genome contig N/L50:	6515/2.881 KB
Main genome scaffold N/L90:	45170/1.13 KB
Main genome contig N/L90:	45170/1.13 KB
Max scaffold length:	733.274 KB
Max contig length:	733.274 KB
Number of scaffolds > 50 KB:	277
% Main genome in scaffolds > 50 KB:	19.10%

Minimum scaffold length	Number of contigs	Total contigs length	scaffold contig coverage
All	59,655	154,469,727	100.00%
500	59,655	154,469,727	100.00%
1 KB	59,655	154,469,727	100.00%
2.5 KB	8,668	83,009,019	100.00%
5 KB	2,750	63,667,766	100.00%
10 KB	1,455	54,995,645	100.00%

25 KB			647	42,231,241	100.00%				
50 KB			277	29,502,101	100.00%				
100 KB			95	17,259,060	100.00%				
250 KB			15	5,497,036	100.00%				
500 KB			1	733,274	100.00%				

T223D assembled contigs stat using Bbmap tool script

A	C	G	T	N	IUPAC	Other	GC	GC_stdev
0.2506	0.2512	0.2509	0.2474	0	0	0	0.5021	0.0412

Main genome scaffold total:			56964						
Main genome contig total:			56964						
Main genome scaffold sequence total:	78.587 MB								
Main genome contig sequence total:	78.587 MB	0.000% gap							
Main genome scaffold N/L50:	22184/1.312 KB								
Main genome contig N/L50:	22184/1.312 KB								
Main genome scaffold N/L90:	49395/1.046 KB								
Main genome contig N/L90:	49395/1.046 KB								
Max scaffold length:	87.601 KB								
Max contig length:	87.601 KB								
Number of scaffolds > 50 KB:			2						
% Main genome in scaffolds > 50 KB:			0.19%						

Minimum scaffold length	**Number of contigs**	**Total contigs length**	**contig coverage**
All	56,964	78,587,243	100.00%
500	56,964	78,587,243	100.00%
1 KB	56,964	78,587,243	100.00%
2.5 KB	1,519	5,743,148	100.00%
5 KB	206	1,642,838	100.00%

10 KB	27	467,480	100.00%	
25 KB	2	148,847	100.00%	
50 KB	2	148,847	100.00%	

T231C assembled contigs stat using Bbmap tool script

A	C	G	T	N	IUPAC	Other	GC	GC_stdev
0.2591	0.2425	0.2422	0.2562	0	0	0	0.4847	0.0393

Main genome scaffold total:	124386	
Main genome contig total:	124386	
Main genome scaffold sequence total:	179.342 MB	
Main genome contig sequence total:	179.342 MB	0.000% gap
Main genome scaffold N/L50:	47237/1.391 KB	
Main genome contig N/L50:	47237/1.391 KB	
Main genome scaffold N/L90:	107024/1.058 KB	
Main genome contig N/L90:	107024/1.058 KB	
Max scaffold length:	448.874 KB	
Max contig length:	448.874 KB	
Number of scaffolds > 50 KB:	7	
% Main genome in scaffolds > 50 KB:	0.79%	

Minimum scaffold length	**Number of contigs**	**Total contigs length**	**scaffold contig coverage**
All	124,386	179,342,386	100.00%
500	124,386	179,342,386	100.00%
1 KB	124,386	179,342,386	100.00%
2.5 KB	4,571	16,062,482	100.00%
5 KB	201	2,831,113	100.00%
10 KB	23	1,703,653	100.00%

25 KB	10	1,532,552	100.00%
50 KB	7	1,423,336	100.00%
100 KB	6	1,362,602	100.00%
250 KB	2	814,814	100.00%

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