Genetic Detection and Phylogenetic Relationship of Babesia Species Infecting Domestic Dogs from Select Regions in Kenya

Ismail Thoya Ngoka (Dr.Ismailthoya@gmail.com)
Jomo Kenyatta University of Agriculture & Technology
Kevin Mbogo
Jomo Kenyatta University of Agriculture & Technology
Martina Kyallo
Biosciences Eastern & Central Africa Hub
David Obiero
Maasai Mara University
Roger Pelle
Biosciences Eastern & Central Africa Hub

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Abstract

The genus *Babesia* has more than 100 species that are transmitted by ticks with some being zoonotic. They can infect humans, livestock, and wildlife. Although canine babesiosis occurs locally, published studies on the species involved are limited. *Babesia* parasites cause severe disease in dogs which can be fatal. Drawbacks of the current control methods necessitate vaccine development.

The study objective was to identify the *Babesia* species infecting dogs from three Kenyan counties; Nairobi, Mombasa, Nakuru and determine their phylogenetic relationship. This will enable improved control and rule out zoonotic potential. The study period was October 2018 to November 2019.The study design was descriptive and sampling opportunistic. One hundred and forty-three dogs were sampled. From whole blood, total DNA was extracted using the TanBead extractor followed by PCR amplification targeting *Babesia* 18S rRNA. Positive samples were purified and sequenced using the Sanger Dideoxy method.CLC Genomics Workbench, GenBank™ and BLASTn™ on NCBI were used for sequence processing and analysis. Geneious prime™ was used for multiple sequence alignment and phylogenetic analysis.

The overall prevalence of *Babesia canis* was 9.0% (95% CI: 4.37–13.81). Two out of 13 positive samples (2/13) were identified as *Babesia canis vogeli*, with a prevalence of 1.4% (95% CI: 1.38–14.2, $n = 143$) while 11/13 were identified as *Babesia canis rossi*, with a prevalence of 7.69% (95% CI: 3.3–12, $n = 143$). The *Babesia rossi* sequences identified were closely related to sequences from black-backed jackals, while the *Babesia vogeli* ones were related to sequences from a pet cat in China. *Babesia rossi* which causes severe canine babesiosis was identified in 84.6% of the positive samples, immediate and aggressive clinical intervention is necessary. The possible sylvatic cycle of *Babesia rossi* and low levels of infections by *Babesia vogeli* should inform pertinent control measures.

Introduction

The genus *Babesia* comprises parasites belonging to the order Apicomplexa which infect vertebrate red blood cells and are transmitted by ticks (1). There are more than 100 species in this genus, based on their microscopic appearance; they are classified as either small or large piroplasms. *Babesia* species can infect livestock, wildlife, human beings, and occur worldwide (1).

Babesiosis is considered a disease of increasing public health importance – an emerging zoonosis (24). The following *Babesia* species have been shown to infect and cause Babesiosis in the domestic dog; *B. canis*, *B. rossi, B. vogeli, B. gibsoni, B. conradae*, and *B. vitallii* (21, 24). However, there are other yet to be characterized *Babesia* species that can infect dogs (24).

Babesia species of veterinary and medical significance include *B. divergens* a zoonotic parasite, which infects cattle, humans, non-human primates, and other wildlife; *Babesia microti* whose lifecycle revolves around tick vectors and rodent hosts is also zoonotic (1). *B.bovis* and *B.bigemina* are of major importance in the cattle production industry (4) and also infect wildlife such as the white-tailed deer (*Odocoileus virginianus*) and the African Buffalo-*Syncerus caffer* (1). Other *Babesia* species include

B.venatorum, which infect sheep but has also been implicated in a zoonotic event involving a dog in a report from China (7); *B.caballi* infect horses, donkeys, and mules and *B.motasi* which infect sheep and goats (1).

Infections by *Babesia* parasites are known to occur in Kenya and have been documented in dogs, cattle, and wild felids (2, 3, 4, 34). However, published studies on the *Babesia* species infecting dogs in Kenya are limited. Similar studies have been reported from other African countries (15,16,17,18,19,20).

Canine babesiosis causes a debilitating illness in dogs characterized by fever, hemolytic anemia, jaundice, anorexia, vomiting, and lethargy (24).Factors determining the clinical severity of the disease include the *Babesia* species causing infection with *B.rossi* known to cause the most severe disease (24). The disease compromises the welfare of dogs and renders them unable to carry out their prescribed role in society (3).

Drugs used for the medical management of canine babesiosis include imidocarb dipropionate and diminazene aceturate. However, the veterinary treatment costs borne by the dog owners are high and the recovery period from the disease lengthy (24).

Dogs in Kenya serve a variety of purposes, including livestock guarding and herding, providing security for business premises and homesteads as well as serving as companion animals (5). The role the dog plays in society, coupled with the local livestock keeping practices allows close interaction between dogs, humans, livestock and wildlife (6). This provides an opportunity for the spread of parasites and diseases between them.

The objective of this study is to identify the *Babesia* species circulating among dogs from select regions of Kenya and determine their phylogenetic relationship. This would enable a more tailored clinical intervention in dogs suffering from the disease as well as lay the foundation for the development of a much-needed vaccine against the disease (8). Current control measures against the disease rely on the use of acaricides against the tick vectors (17). The shortcomings of these methods include, poor owner compliance with the required periodic acaricide applications, toxic nature of the chemicals used and development of resistance in the target tick populations (25).Species identification and phylogenetic characterization of the *Babesia* parasites circulating among dogs will also inform on any existing zoonotic potential.

Materials And Methods

2.1) Study Areas and Description

The study was carried out in 3 counties in Kenya: Nairobi, Mombasa, and Nakuru.

Nairobi

It is a highly urbanized county and the commercial and administrative capital of Kenya (Fig.1). It has an area of 696.3km^2 with a high human population density of 4800/km^2 (16). The County lies at an altitude of 1669m with a warm and temperate climate. The average temperature is 19°C; annual rainfall is 869mm(37). There are significant levels of farming in the peri-urban zones. These include semi-nomadic pastoralism of cattle, sheep, and goats, and intensive rearing of dairy cattle, and poultry (9). Some districts within the county have free-roaming populations of goats, sheep, chicken, and other poultry e.g. ducks. Nairobi National park, located within the County is home to a large and diverse wildlife population such as the Cape buffalo, Maasai giraffe, Grants zebra, African leopard, lion, eland, impala, cheetah, etc (10, 34). Sections of the park borders are porous allowing constant interaction between wildlife, livestock (including dogs), and human beings (34). Anecdotal reports and published studies indicate that there is a large population of dogs, both stray and owned within Nairobi County. Some of the owned dogs are not kept under confinement and are allowed to roam freely. The dog population comprises both exotic and indigenous breeds with the majority being the latter (13).

Mombasa

The county is situated along the Indian Ocean coast of Kenya and consists of Mombasa Island and the surrounding mainland areas (Fig.1). It has an area of 294.9km^2 of which 65km^2 is water. Mombasa lies at an altitude of 23m above sea level and has a tropical climate. The average temperature is 26.7°C and the annual rainfall 1196 mm (37). The County has a human population of about 1.2 million. It is a highly urbanized county with livestock farming activities carried out in the peri-urban zones including, intensive poultry (broilers and layers) and dairy cattle farming. Free-roaming cattle, sheep, goats, and chickens are also observed in the suburban areas (9). There is a large population of dogs, both owned and stray, a large proportion of the owned dogs are allowed to freely roam around (11).

Nakuru

The county is located in the Rift valley region of Kenya (Fig.1). It lies at an altitude of 1871m with a mild climate which is generally warm and temperate. The average temperature is 17.5°C and annual rainfall around 895mm (37). The study area comprised of Naivasha town and its surroundings which has a human population of 181,966. Naivasha area is dominated by major geographical features such as Lake Naivasha, a freshwater lake, and Mt. Longonot with an elevation of 2,776m which is part of and surrounded by the Mt. Longonot National Park. The Park hosts a variety of wildlife including buffaloes, giraffes, plains zebra, Thomson's gazelle, and hartebeest (12). Lake Naivasha is an important wildlife area with hippopotamus and numerous bird species (9). Naivasha is an important livestock farming area that includes intensive dairy cattle farming, commercial beef ranching and poultry rearing. There is a significant population of free-roaming sheep, goats, chickens, and pigs. Nomadic pastoralism of cattle, sheep, and goats is also common in the county. There is a large population of dogs, both stray and owned especially in the peri-urban areas where a significant proportion of the owned animals are allowed to roam freely (13).

2.2) Sample Size Determination

The study objective was to determine which *Babesia* species are found circulating among dogs within the study locations. Literature review from similar studies in the region as well as local estimates based on clinical diagnosis of canine babesiosis indicated that prevalence for *Babesia* species infection among dogs was low (3,15,16).

The following formula was therefore used to determine sample size (38).

$$n = \frac{\text{Log } \beta}{\text{Log } \mathcal{P}}$$

At 95% confidence interval for detection of *Babesia* species, sample size is;

$$n = \frac{\text{Log } 0.05}{\text{Log } 0.9} = 28.4 \approx 30.0$$

The minimum number of samples provided by this formula is thirty. A minimum of 30 blood samples were therefore collected from different dogs in each county, a larger number of samples were however obtained. The total number of samples collected was one hundred and forty three (*n*=143).

2.2.1) Sampling

The study employed a descriptive design, where sampling was opportunistic. A total of 143 dogs were sampled (Fig.2). In all counties, samples were collected from dogs presented at the shelter facilities of the Kenya Society for the Protection and Care of Animals® (KSPCA). Samples were collected from apparently healthy dogs as well as individuals clinically symptomatic from a variety of ailments.

The dogs were of diverse backgrounds including stray, loosely owned, surrendered, and confiscation cases. The dogs were also of varied ages, sex, and breed (14).Figure 2 below presents the numbers in each demographic category used to classify the sampled canine population.

Age assessment was carried out using a combination of criteria, including teeth characteristics, size of the dog, hair coat, and state of ocular lens (26, 27). Sex was determined by visual inspection of the external genitalia (28). The breed was established by visually comparing the dog's physical attributes against established breed standards.

Approximately 2ml of blood was collected from the cephalic vein of each dog and loaded into 4ml EDTA tubes and refrigerated at 4˚C as previously described (35).

2.3) DNA extraction and characterization

Total DNA was extracted using the TanBead™ Automated DNA extractor. For extraction process optimization, two protocols were used: the Viral DNA™ extraction protocol and the Optipure™ protocol for blood DNA. Six samples were used for the optimization process. For each protocol, the DNA was extracted from 300µl of whole blood sample. 10µl of Proteinase K was then added into each of the

samples and mixed before transfer to the TanBead™ extractor for total DNA extraction.DNA quality and quantity were assayed through agarose gel electrophoresis and Nanodrop Spectrophotometry, a higher quantity and quality were obtained via the Optipure™ protocol. Thereafter total DNA extraction for the rest of the blood samples (*n=142*) was carried out using the Optipure™ protocol.

2.3.1) DNA extraction from a Canine Babesiosis +ve dog

To optimize PCR conditions for diagnostic PCR, total DNA was extracted according to the manufacturer's instructions (using QIAGEN DNeasy kit™ for blood and tissue using the nucleated tissue protocol) from blood drawn from a dog clinically diagnosed with babesiosis. The protocol was altered after adding lysis buffer, where the incubation temperature was set at 56°C at 1000rpm for 12hrs. The extracted DNA sample was then diluted to 25ng/µl using triple distilled Milli-Q water (35).

The extracted DNA was stored at -20°C (35).

2.4) Gradient Polymerase Chain Reaction (PCR)

Gradient PCR was used to determine optimum diagnostic PCR conditions for *Babesia* DNA. Total DNA from a dog positively diagnosed with babesiosis after clinical examination (as described under section *(2.3.1)* was used for optimization via gradient PCR. The conditions were as follows; 95°C for 3min,94°C for 45sec,52°C to 62°C range with 12 intervals and a median of 57°C each for 1 min,72°C for 1min,72°C for 10min, 15°C ∞ . An agarose gel (1.5%) was prepared to run the PCR products at 135V for 30min. The results were visualized using a gel documentation system and used to establish optimum PCR conditions (35).

2.4.1) Conventional PCR

The master mix was set as follows 2x AccuPower™ master mix 5µl, pF 0.33µl, pR 0.33µl, g DNA 2.5µl, double distilled water 1.84µl for a total volume of 10.0µl. Diagnostic PCR conditions used were, 95°C for 5min, 94°C for 30sec, 52°C for 1min, 72°C for 1min, 72°C for 10min,15°C ∞ . An agarose gel (1.5%) was prepared to run the PCR products at 135V for 30min. The results were visualized using a gel documentation system (35). The positive PCR products were purified using the QIAGEN quick gel purification kit™ according to the manufacturer's instructions and the products submitted for sequencing. The primers used were as outlined in Table 1.

Table 1.*Babesia* genus common primers used in PCR.

Primer	Oligo Sequence	Target Species
18S_rDNA_BTH_F	5’-CCT GMG ARA CGG CTA CCA CAT CT-3’ (23mer)	*Babesia* genus
18S_rDNA_BTH_R	5’-TTG CGA CCA TAC TCC CCC CA-3’(20mer)	*Babesia* genus
18S_rDNA_GF1	5’-GTC TTG TAA TTG GAA TGA TGG-3’(21mer)	*Babesia* genus
18S_rDNA_GR2	5’-CCA AAG ACT TTG ATT TCT CTC-3’(21mer)	*Babesia* genus

2.5) DNA Sequencing and Analysis

Sequencing was done using the Sanger Dideoxy method at the International Livestock Research Institutes’ Segoli Lab (35).The obtained sequences were analyzed using the CLC Genomics Workbench version 20.0.2 where the sequences were trimmed, conflicts resolved using the forward and reverse sequences, and a consensus sequence generated (35). The consensus sequence was analyzed using the BLASTn™ program of the National Center for Biotechnology Information to identify closely related sequences on GenBank™ (32). Multiple sequence alignment and a phylogenetic tree were constructed using Geneious™ Prime 2020.1.2, Build 2020-04-07 08:42, Java version 11.0.4+11 (64bit) using the MUSCLE program version 3.8.425 by Robert C. Edgar and Geneious tree builder. The genetic distance model used was Jukes-Cantor, while the tree build method was Neighbor-joining, the number of bootstrap replicates was 1000 (36).

The following sequences from GenBank™ with accession numbers, *Tryps simiae* Muhak AJ404608,*Theileria parva* South Africa HQ895984,*B.duncani* USA HQ289870,*B.vogeli* Spain AY150061,*B.vogeli* Brazil KT333456,*B.vogeli* Texas EU084681,*B.vogeli* Zambia LC331058,*B.vogeli* China KJ939326,*B.vogeli* Nigeria AB303076,*B.vogeli* Egypt MN625891,*B.vogeli* Japan AY077719,*B.rossi* Zambia LC331056,*B.rossi* South Africa KY463434,*B.rossi* Sudan DQ111760,*B.rossi* Nigeria JN982353, *B.rossi* China MH143395,*B.rossi* Turkey MF040149,*B.rossi* Texas HM585429, *B.coco* USA AY618928,*B.gibsoni* India KF928958,*B.venatorum* China KU204792 were used to analyze the Kenyan *Babesia canis* sequences. (32)

Results

3.1) Sampling

All the samples were collected from the shelter facilities of the Kenya Society for the Protection and Care of Animals of the respective counties. A total of 143 dogs were sampled as described under materials and methods. Figure 2 below depicts the demographic structure of the dogs sampled.

3.2) DNA Extraction Protocol Optimization

Two protocols, the Optipure™ protocol and the Viral blood™ DNA protocol were used in optimizing DNA extraction as described under materials and methods. Assessment of the two extraction protocols used

was done through agarose gel electrophoresis and Nanodrop Spectrophotometry. These indicated the superior DNA yields of the Optipure™ protocol. Thereafter 142 out of the 143 samples had their DNA extracted using the Optipure™ protocol. One sample was extracted using QIAgen DNeasy kit™ (covered under *2.3.1*)

3.3) PCR Conditions Optimization

Gradient PCR thermal cycler conditions were set based on the primer manufacturer's guidelines. *Babesia* species DNA extracted as described under materials and methods (*2.3.1*) from a dog clinically diagnosed with canine babesiosis was used.PCR results were visualized by agarose gel electrophoresis and gel documentation.

The ideal thermal cycler conditions were thus established to be as follows, 95°C for 5min, 94°C for 0.30sec, 53°C for 1 min, 72°C for 1min, 72°C for 10min, 15°C for ∞.

3.4) Diagnostic PCR for all the Extracted DNA samples

Using the established PCR conditions, all the DNA samples were screened for *Babesia* species DNA. Out of the 143 samples, 13 samples were positive for possible *Babesia* species DNA after processing and visualization via agarose gel electrophoresis and gel documentation by forming a band at the expected 500-700bp range.

3.5) DNA Submission for Sequencing and Bioinformatic Analysis

The 13 positive samples were purified and submitted for sequencing via the Sanger Dideoxy method at the International Livestock Research Institutes' Segoli lab. The raw sequences were then analyzed using bioinformatics software and methods including CLC genomics workbench, NCBI-BLASTn™ and Geneious™ prime software as described under materials and methods.

Using NCBI-BLASTn™, two of the sequences were identified as *Babesia canis vogeli*, while the remaining 11 sequences were identified as *Babesia canis rossi* on GenBank™.

The two *Babesia canis vogeli* sequences were submitted to and assigned the following GenBank™ accession numbers MT740261 and MT740272.

Likewise the eleven sequences identified as *Babesia canis rossi* sequences were submitted to and assigned the following GenBank™ accession numbers MT740262, MT740263, MT740264, MT740265, MT740266, MT740267, MT740268, MT740269, MT740270, MT740271, and MT740273.

Additionally 21 sequences from GenBank™ were used to analyze the Kenyan *Babesia canis* sequences as described under materials and methods.

Geneious™ prime software was used in the construction of a phylogenetic tree.

The Kenyan *Babesia canis rossi* sequences clustered closely around each other. The two *Babesia rossi* sequences from Nakuru formed a sub-cluster that was in close association with the other Kenyan *B.canis rossi* (Fig.6).

On NCBI-BLASTn™ other *B.rossi* sequences closely related to the Kenyan sequences included KY463431.1 from black-backed jackals in South Africa with a P.I (percentage identity) of 99.52 % (40), DQ111760.1 from dogs in Sudan with a P.I of 99.52 % (33), and LC331056.1 from dogs in Zambia with a P.I of 99.36% (50).

The most divergent *B.rossi* sequence within the Kenyan cluster was F12 (MT740273.1) from Nairobi County with a P.I range between 97.88% for MT740267.1 (Nvsa_B) to 99.39% for MT740262.1 (Nvsa) from Nakuru County (Fig. 6).

The two *Babesia canis vogeli* samples detected from Nairobi County, clustered closely to each other and other *Babesia canis vogeli* from Africa on the phylogenetic tree. On NCBI-BLASTn™, *Babesia canis vogeli* identified in samples from Nairobi County were closely related to *B.vogeli* from Nigeria AB303076.1 with a P.I of 98.89% (36), *B.vogeli* from Egypt MN625891.1 with a P.I of 99.42% (52). Other closely related sequences include *B.vogeli* from Japan AY077719.1. The two *B.vogeli* sequences obtained from this study, “B.vogeliNrb and B.vogeliNrbB” had a 99.0% P.I to a *B.vogeli* sequence from a pet cat in China (42).

The overall prevalence of the 13 sequences identified as *Babesia canis* translates to 9.0% with a 95% confidence interval of 0.0437 to 0.1381.

More specifically, the detected *Babesia canis rossi* sequences had a prevalence of 7.69% and 95% Confidence Interval of 0.033 to 0.12 while *Babesia canis vogeli* had a prevalence of 1.4% and 95% Confidence interval of 0.0138 to 0.0142

The characteristics of the dogs, samples, and species of *Babesia* identified are presented in Table 2.

Table 2. Distribution *of Babesia* species based on the population characteristics of age, sex, breed and county

		Age			Totals	Sex		Totals	Breed		Totals
		J	A	O		M	F		L	E	
Nairobi	***B.canis rossi***	2	5	1	8	5	3	8	6	2	8
	B.canis vogeli	-	2	-	2	1	1	2	2		2
Nakuru	***B.canis rossi***	-	2	-	2	1	1	2	2	-	2
	B.canis vogeli	-	-	-	-	-	-	-	-	-	-
Mombasa	***B.canis rossi***	-	1	-	1	-	1	1	1	-	1
	B.canis vogeli	-	-	-	-	-	-	-	-	-	-

Notes

J	Juvenile	≤ 1yr old
A	Adult	1yr ≤ 5yrs old
O	Old	5yrs old
M	Male	-
F	Female	-
E	Exotic breed	-
L	Local breed	-

The proportion of *B.canis rossi* detected (11/13) was 84.6%, while *B.canis vogeli* (2/13) was 15.4%.

Out of the 13 positive cases, 23.08% were juveniles <1year old, while 69.23% were adult animals >1year ≤5 years old and 7.69% were older dogs 5years old.

Based on the sex of dogs sampled, 53.85% were males and 46.15% were females. Sequence analysis of the positive cases indicated a county distribution of *Babesia canis rossi* in Nairobi with 8 cases, while Nakuru and Mombasa counties having 2 cases and 1 case respectively

Discussion

The results from the current study reveal that Kenyan dogs from regions sampled are infected with at least two *Babesia canis spp. Babesia canis rossi* and *Babesia canis vogeli* were detected at a prevalence of 7.69% and 1.4% respectively. These results agree with findings from similar studies in other African countries, Oyamada et al (2005) determined the overall prevalence for *B.canis vogeli* and *B.canis rossi* in a study from South Sudan to be 9.0% which compares well to the results obtained from this study (Table 2, 33). Similarly, the prevalence of *B.vogeli* and *B.rossi* in the South Sudan study was 2.6% and 6.4% respectively (15). In Uganda, *B.rossi* was detected at a prevalence of 7.8 % (16), while in Zambia, *B.rossi*

was detected at a prevalence of 8.0% (17).In Nigeria, *B.rossi* and *B.vogeli* were detected at the much lower prevalence of 2.0% and 0.3% respectively (18). All these study results compare well with the Kenyan findings where the prevalence of *Babesia canis vogeli* was also lower than that of *Babesia canis rossi*. Studies from African countries reported *B.vogeli* prevalence ranging between a low of 1.4% to a high of 5.8% (19, 20).The prevalence result from this study for *B.vogeli* was 1.4% (19, 20).

The presence and degree of infestation by competent tick vectors is an important factor determining infection by and therefore prevalence of *Babesia* parasites (1). *B.rossi* is mainly transmitted by *Haemophysalis leachi* while *B.vogeli* is spread *Riphicephalus sanguineus*.

Lower levels of tick vector infestation also probably accounted for the reduced prevalence figures compared to other African countries observed for both *B.rossi* and *B.vogeli* in the Nigerian study (18).

Clinically, canine babesiosis in Kenya is associated with severe morbidity and high mortality especially for cases that go untreated (3). This finding concurs with the current study findings where *Babesia canis rossi,* which is known to be the most virulent species, (24) was observed in 84.6% of *Babesia canis* positive cases while the less virulent *Babesia canis vogeli* was observed in only 15.4% of cases. *Babesia canis rossi* has been routinely detected in studies from various African countries including South Sudan (15), South Africa (8, 21), Nigeria (18), Zambia (17), and Uganda (16). Countries it has not been detected in include Angola and Namibia, but the non-detection could be attributable to sample size and study design for the Namibia case where only 1 dog was sampled (19).While in Angola, the geographical limitation of the samples used in the study probably excluded the parasites' detection (20).

Canine *Babesia* species not detected in this study but observed in similar studies from other African countries include *B.gibsoni* observed in Zambia (17), Angola (20) and South Africa (21). Reports indicate that *B.gibsoni* is mainly transmitted by *Rhiphicephalus sanguineus* ticks which are also the main vectors for *B.vogeli*.

On the phylogenetic tree (Fig.6), the Kenyan *Babesia rossi* sequences although also associating with sequences from other African countries clustered closely together indicating the possible existence of a local sub-population. The two *Babesia rossi* sequences from Nakuru County also formed a sub-cluster indicating a sub-population from that region. The black-backed jackal (*Canis mesomelas*) is known to be the reservoir host for *Babesia rossi* (22). The *B.rossi* sequences from Kenya, South Sudan, and Zambia were identical to a *B.rossi* sequence obtained from a Black-backed Jackal (*Canis mesomelas*) in South Africa (22).*B.rossi* is widely considered to originate from and occur only in Africa. Countries outside the African continent where it has been detected include Turkey (30), the USA (31) and China. In the American study, *B.rossi* was detected in a dog recently imported from South Africa (31). Surprisingly, the Turkish study detected *B.rossi* circulating in the tick species, *Haemophysalis parva* found infesting wild animals such as boars and hares, the tick vector in Africa is *Haemophysalis leachi* (22*)*. Further research into the Turkish *B.rossi* is necessary to determine its relationship to the parasite originating from Africa as well as other aspects such as pathogenicity to domestic dogs.

B.canis vogeli is global in distribution (1), the 2 Kenyan sequences identified as *B.canis vogeli* clustered closely to each other and to the *B.vogeli* sequences from other African countries including South Sudan (15), Nigeria (18), and Egypt (29) on the phylogenetic tree. Of note, *B.canis vogeli* although primarily known as a dog parasite has been detected in felids such as domestic cats in China (23) and Lions in Zimbabwe (33).In Kenya, species classified as part of *the B.canis* complex have been identified in wild felids such as lions (*Panthera leo*) from the Nairobi National Park (34).

In this study, 69% of *Babesia canis* positive cases were in adult dogs between 1 and 5yrs old followed by Juvenile dogs less 1yr old at 23%. While the proportion of infected dogs older than 5yrs was 7%.The results are in agreement with findings from a Zambian study; dogs between 2 and 5yrs old comprised 29.47% of positive cases, while dogs between 0.6 and 1yr old comprised 22.11%, dogs older than 5yrs had the smallest proportion of cases (17). Similar findings were observed in a Nigerian study where 66.7% of *Babesia canis* positive cases were adult dogs between 1 and 3yrs old, and 33% of cases were less than 1yr old (18). Sampling bias probably undermined findings as most dogs sampled in these studies were adults between 1 and 5yrs old.

Co-infection between different *Babesia* species although not common was observed in the studies from Zambia, *B.rossi* and *B.gibsoni* (17): in South Africa, *B.rossi* and *B.vogeli* (21).Mixed infections between a *Babesia* species and parasites from different genera were more frequently observed, in South Sudan and Angola (15, 20).This study was specifically designed to detect parasites belonging to the *Babesia* genus. No cases of co-infection between different species of the *Babesia* genus were observed.

The public health importance of *Babesia* parasites is increasing. They are considered an emerging zoonosis (24). New *Babesia* species continue being discovered and many of the known *Babesia* species are still poorly characterized in aspects such as hosts, vectors, geographic range and pathogenicity. Example of these *Babesia* species include *B.divergens* like (MO1), *Babesia spp*. CA.1 and WA1 (1). Poorly characterized *Babesia spp.* known to infect dogs include *B.microti*-like from Spain and *Babesia spp*. CA.1 (24).

The recent implication of a dog in the transmission of the zoonotic *B.venatorum* (previously known to only infect sheep) to a human being (7) has served to reinforce the need to fully characterize canine *Babesia* species.

Conclusions

Results from this study indicate that majority of *Babesia* positive samples (84.6%) were due to *Babesia canis rossi* which is known to cause the most severe form of canine babesiosis. Veterinary clinicians, therefore, need to intervene with appropriate therapeutics urgently and aggressively upon diagnosing the disease to save valuable working dogs and companion animals.

Control measures against *B.rossi* must take into consideration the possible sylvatic cycle of the parasite including limiting interactions between wildlife and domestic animals. Developing the urgently needed

vaccine against *B.rossi* would also require research into the pathogens circulating in wildlife, especially in black-backed jackals (*Canis mesomelas*).

The phylogenetic analysis indicated sub-clustering between *B.rossi* sequences from Nakuru and Nairobi Counties. Similar sub-clustering was observed between the Kenyan *B.rossi* sequences and *B.rossi* sequences detected in other African countries. Therefore, further investigations to determine the level of diversity of *B.rossi* would be important for the development of effective control measures.

While adult dogs in this study appear to be disproportionately affected by canine babesiosis, observational studies that explore risk factors such as age are needed to corroborate these results.

Although *B.vogeli* has been observed to have a wide geographical range and the potential to infect multiple hosts, the parasite was detected in only 15.4% of the positive samples. This low level of infection coupled with the fact that *B.vogeli* is known to cause a mild form of canine babesiosis indicates the limited significance of this parasite for local clinical cases.

The primers used for this study were designed to target 18S rRNA common to the *Babesia* genus, the detected *Babesia* species, however, have no known zoonotic significance.

In summary, the *Babesia* spp. identified in this study plus the possible epidemiological transmission pathways would enable veterinary clinicians and researchers develop and use improved controls against canine babesiosis locally.

Declarations

Competing Interests

The authors declare that they have no competing interests.

Author Contributions

Ismail Thoya Ngoka -conceived the study, collected the samples, extracted DNA, carried out laboratory experiments including PCR, submitted samples for DNA sequencing, analyzed the data and drafted the manuscript, Roger Pelle -supervised the lab work and analysis of the data, Martina Kyallo-participated in and supervised the lab work and analysis of the data, Kevin Mbogo -supervised the entire project as well as participated in the refining of the study idea and design, David O. Oduori -participated in refining the study idea and drafting of the manuscript, All the authors have read the manuscript and approved its publication.

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ILRI).The sponsors had no role in conceptualizing, carrying out, analyzing, and interpreting the study results.

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Figures

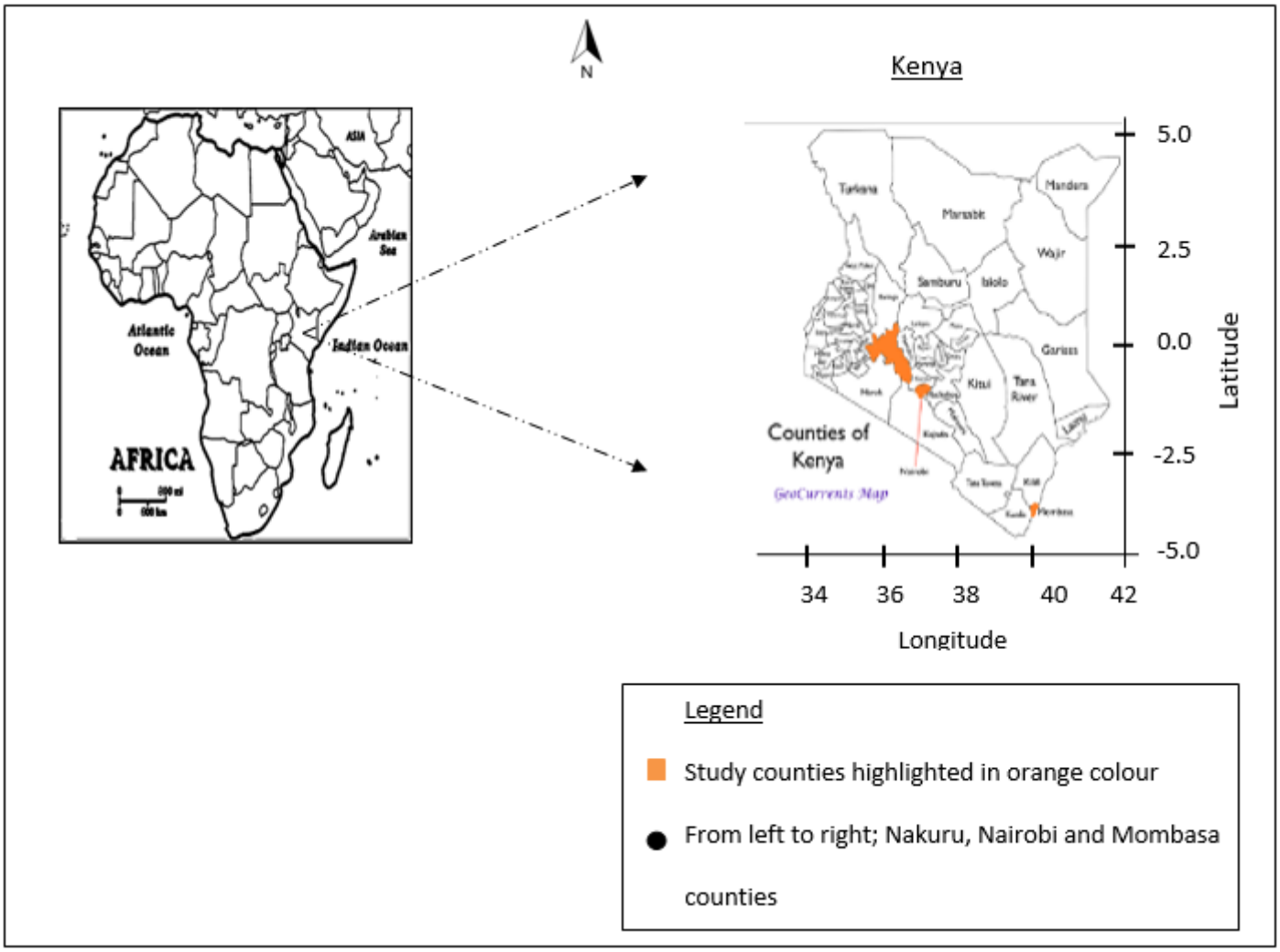

Figure 1

Map of Africa showing the position of Kenya in East Africa and a map of Kenya where the 3 study counties are highlighted in orange color.

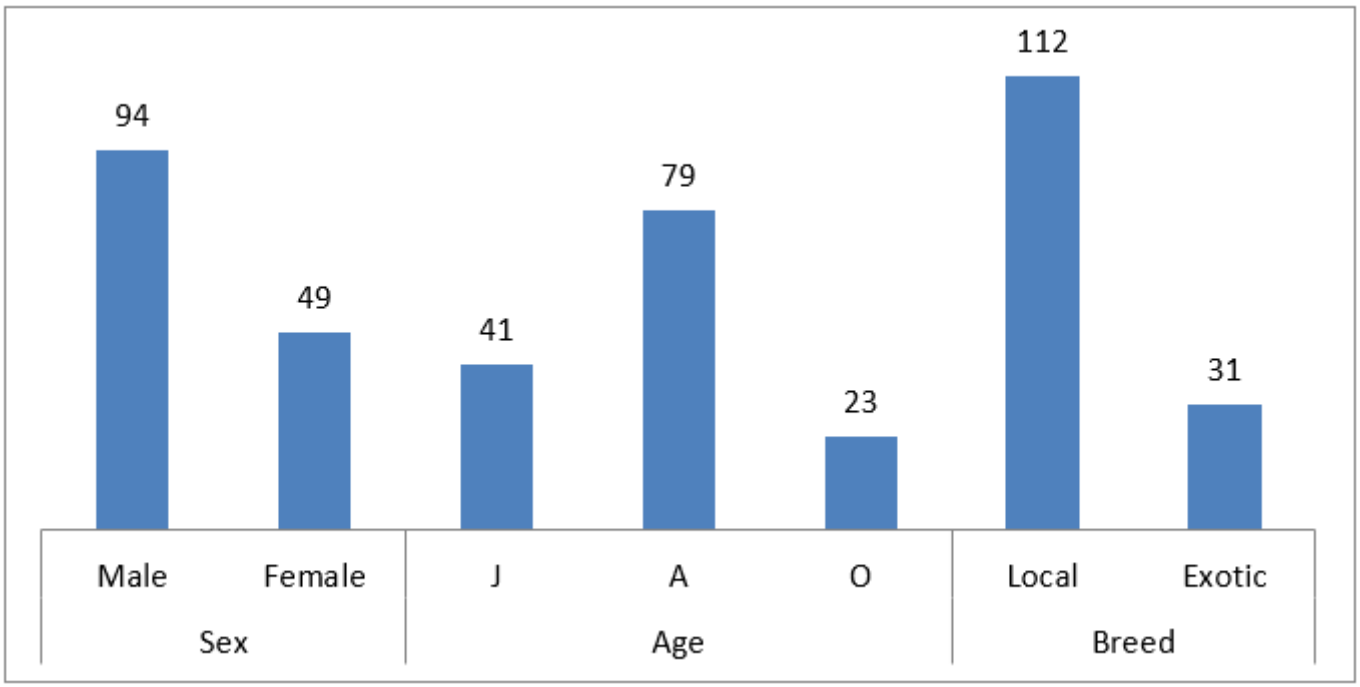

Figure 2

Sampling was random; whole blood was collected from dogs presented at the shelter facilities of the Kenya Society for the protection and Care of Animals in Nairobi, Mombasa, and Nakuru. The bias observed in the sampling pattern is not due to deliberate collection from the population sub-groups but may indicate the underlying canine population structure.

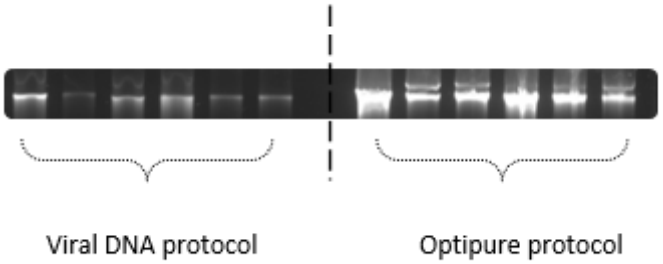

Figure 3

Agarose gel electrophoresis image of DNA extraction optimization, Optipure™ protocol with higher DNA yield.

Gradient PCR annealing temperature of 53°C at which band size and intensity was most distinct

Figure 4

Optimum annealing temperature observed at the 53° C mark

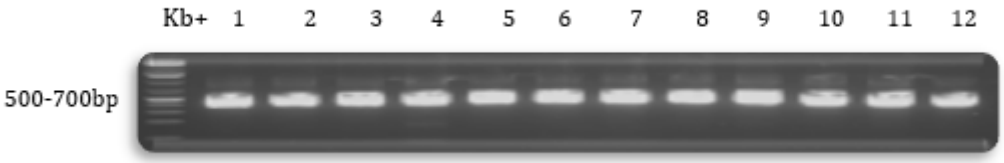

Figure 5

All the positive samples from the initial mass screening of the extracted total DNA underwent re–PCR to increase the DNA yield to facilitate further processing of the positive samples through sequencing. The positive samples generated a band at the expected 500-700bp position on agarose gel electrophoresis.

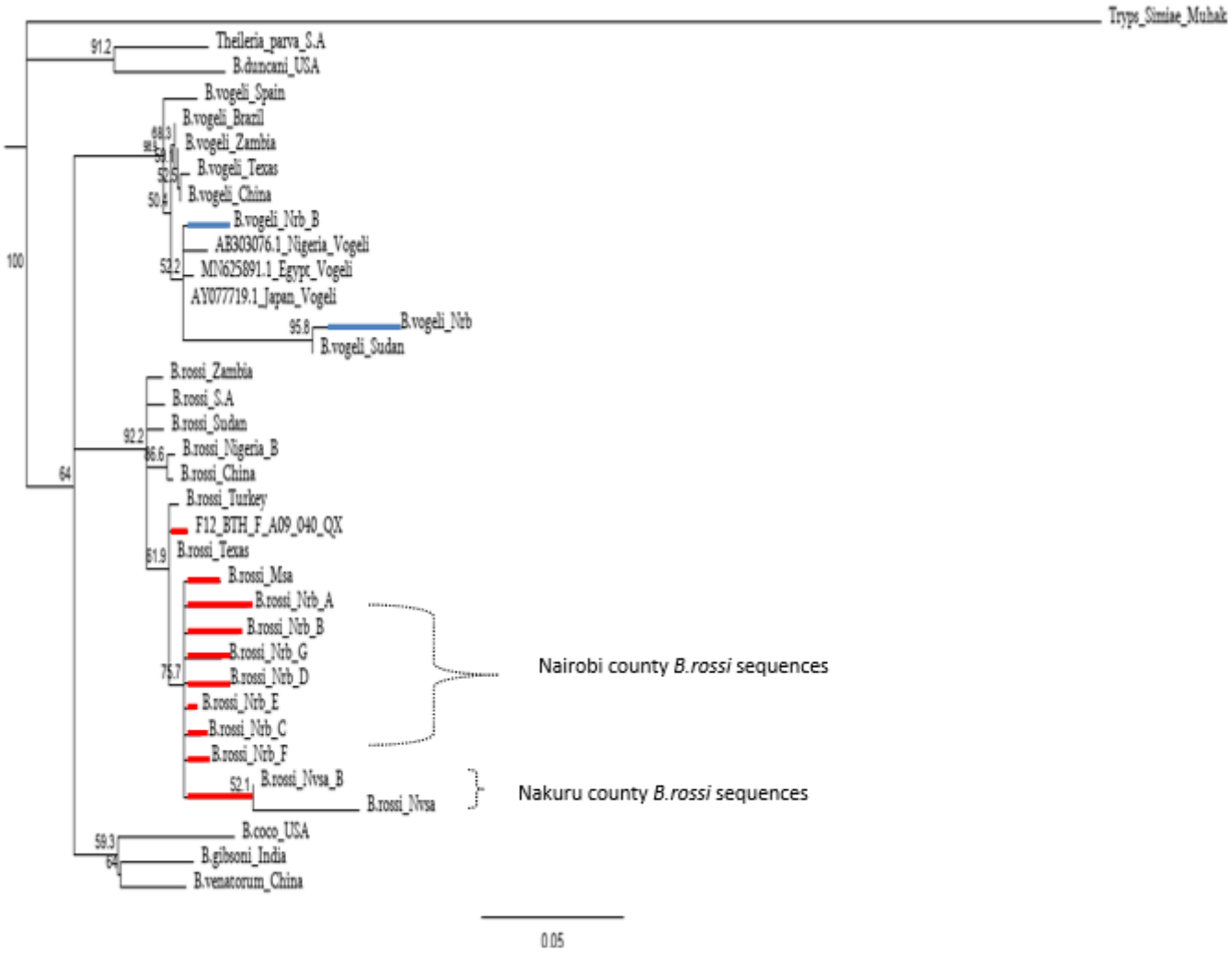

Figure 6

Phylogenetic Tree, the Kenyan B.rossi samples highlighted in red. B. rossi_ Msa; B.rossi_Nrb_A; B.rossi_Nrb_B; B.rossi_Nrb_G clustered around each other, the 2 positive samples from Naivasha formed a separate sub-cluster. The detected B. rossi were most closely related to samples from Turkey, China, Nigeria, Sudan, South Africa, and Zambia. The two B. vogeli samples highlighted in blue were closely related to samples from Sudan, Japan, Egypt, and Nigeria.