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Prevalence of *Babesia species* in hunting dogs in Ogun State South West Nigeria

Foluke A. Akande[1], Alex F. Adebowale[1], Micheal I. Takeet[1], Olugbenga K. Omisile [2]

[1]Department of Veterinary Microbiology and Parasitology, College of Veterinary Medicine, Federal University of Agriculture, Abeokuta, Nigeria, [2]Department of Epidemiology and Medical Statistics, College of Medicine University of Ibadan, Nigeria.

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Correspondence to:
Foluke A. Akande, akandefa@funaab.edu.ng

ABSTRACT

Dogs are commonly used for hunting in Nigeria and they may harbour parasites without showing any clinical features. Babesiosis is a worldwide, tick-borne, protozoal haemoparasitic disease caused by parasites of genus *Babesia*. This study assessed by means of microscopy and Polymerase Chain Reaction (PCR), the presence of *Babesia species* in 109 indigenous Nigerian hunting dogs. One hundred and nine hunting dogs were sampled from 21 different locations, from May 2014 to March 2015 with ages ranging from less than 1 to greater than 6 years. The average weight of the dogs studied was 12.20±0.33kg with the mean Packed Cell Volume (PCV) of 35.2±0.86% and mean age of 3.01±0.23 years. Using microscopy 24 (22%) of the dogs were positive for *Babesia* species. There were no statistically significant differences in the gender (p=0.84), age (p= 0.71), weight (p= 0.45) and PCV (p=0.87) of infected and non-infected dogs. With PCR assay, 52 (47.7%) of the sampled dogs were positive to *Babesia* spp. Of which 18 (34.6%) were male and 34(65.4%) were females. There were no statistical significance between the presence of *Babesia* spp. and sex (p=0.11) also with PCV (p=0.06) of hunting dogs but there were significant differences in their ages (p=0.04) and weights (p= 0.01)**.** Using PCR as a gold standard the specificity and sensitivity of microscopy for detecting Babesia were 77.2% and 21.2 % respectively. The positive predictive value for microscopy was 45.8% while the negative predictive value was 51.8% with a diagnostic accuracy of 50%. The implication of the presence of the parasite in the study areas is discussed.

1. INTRODUCTION

Hunting is common as a means of livelihood in Nigeria especially among the traditional religion worshippers. Hunting dogs in Nigeria are found mostly in the rural areas in which they are used for hunting, guarding, religious purposes and as source of income (Macpherson *et al.*, 2000).These hunting dogs are usually indigenous breeds of dogs that are found mostly in the rural areas. They are kept on free range and therefore come in contact with formites, ectoparasites, endoparasites and even directly with infected blood of hunted game animals. Sometimes hunting dogs may harbour these parasites without showing any obvious clinical sign(s) (Adejoke, 2005).

Canine babesiosis is a worldwide tick-borne disease that is predominantly causes by *Babesia gibsoni* and *Babesia canis* (Boozer and Macintire , 2003). It may present with a wide variety of clinical signs, ranging from hyper acute, shock-associated haemolytic crisis to inapparent and subclinical infection. (Homer *et al.*, 2000; Boozer and Macintire, 2003).

Following clinical suspicion, the diagnosis of *Babesia* spp. infection can be confirmed microscopically, serologically and by using molecular

detection methods (Oyamada *et al.*, 2005). Microscopy is simple, easily accessible and is reasonably sensitive in detecting intra erythrocytic parasite during acute infection. However, in cases of low parasitaemia and due to the morphological similarity between species, the microscopic analysis of canine babesiosis is complicated and could lead to incorrect diagnosis (Irwin, 2009). Molecular methods are the most sensitive and specific approach for detection and differentiation of *Babesia* spp. infections (Cardoso *et al.*, 2008; Solano-Gallego *et al.*, 2008).

Various studies have been carried out on prevalence of Babesisois in dogs in Nigeria: Oduye and Dipeolu (1976) in blood smears made from 500 dogs that were presented to veterinary clinics in Ibadan, Oyo State, and were examined microscopically; found 26.0% of the dogs to be infected with *Babesia canis* (sensu lato), while 20.2% were infected with *Babesia gibsoni*. *Babesia canis* (sensu lato) has been reported from Zaria, Kaduna State (Useh *et al.*, 2003). A low prevalence (2.8%) of *Babesia canis* (sensu lato) infection was found in a blood-smear-based survey among slaughtered dogs in Maiduguri, Borno State (Adamu *et al.*, 2012). Using molecular detection and characterization on blood specimens of 181 dogs that were presented to veterinary hospitals in four different sites, *Babesia rossi* was detected in 11.8% of the dogs in Rivers State while in Plateau State 14.6% dogs in Jos North and 4.8% dogs in Jos South had Babesia rossi. A single dog in Kaduna state was found to be positive for *Babesia vogeli* (Kamani et al., 2013).

Interestingly, *Babesia canis* (sensu stricto) and *Babesia rossi* co-infection was found in a dog that had never left Vom, Plateau State (Kamani *et al.*, 2010). This stimulated renewed interest in the epidemiology of canine babesiosis in Africa, as it was the first confirmation of the occurrence of *Babesia canis* in a geographical region were *Dermacentor reticulatus*, the only confirmed vector of *Babesia canis*, does not occur (Kamani *et al.*, 2010). According to Adamu *et al.*, (2014) the prevalence for *Babesia rossi* was 13% and *Babesia vogeli* 1% out of one hundred samples analyzed using the Reverse Line Blot (RLB) assay.

To the best of our knowledge and as at the time of this write up, there are no available publications on the prevalence of *Babesia species* in hunting dogs in Nigeria who are at the interface of transmission cycle having gone to the wild for hunting and are brought back to the sentinel of their owners in the owners' abode with the attached arthropods that are vectors of various diseases. Thus the aims of this study were to determine the prevalence of *Babesia species*, using both microscopy and molecular methods, in indigenous hunting dogs in Ogun State, South West, Nigeria and to compare the sensitivity and specificity of microscopy and molecular methods by taking molecular method as gold standard.

2. MATERIALS AND METHODS.

This study was carried out in Ogun state from May 2014 to April 2015 these falls within the dry season and early rains period when hunting sprees are known to be commoner. Ogun state is a state in Southwest Nigeria that is bordered by Lagos state to the South, Oyo and Osun states to the North, Ondo state to the east and Republic of Benin to the West. One hundred and nine actively hunting dogs were randomly selected for the study. The dogs were recruited from twenty one different hunters' associations which spread across six different Local Government areas of the state.

The hunters' associations were recruited by snowballing sampling technique and the dogs were sample by random sampling. Consent was obtained from both the leadership of the hunters and individual hunters for the recruitment of their dogs for the study. Puppies were excluded in the sampling, as puppies are not used for hunting.

Three milliliters of blood each was collected into an EDTA bottle from each of the dog using cephalic vein after proper restrain on sternal recumbence. These were transported on ice packs to the Veterinary Parasitology laboratory of the College of Veterinary Medicine, Federal University of Agriculture, Abeokuta, Nigeria for laboratory analysis. The age, sex and weight of all the hunting dogs sampled were noted and recorded on a data sheet.

The Packed Cell Volume (PCV) of each dog was determined and a thin blood film was prepared for microscopy as described by Jain, (1986). The demonstration of a pyriform and/or round shape piroplasms in the Red Blood Cells stained with Giemsa stain were taken to be positive for *Babesia species* as viewed at x100 magnification using immersion oil under the microscope (Irwin 2009). (Figure I)

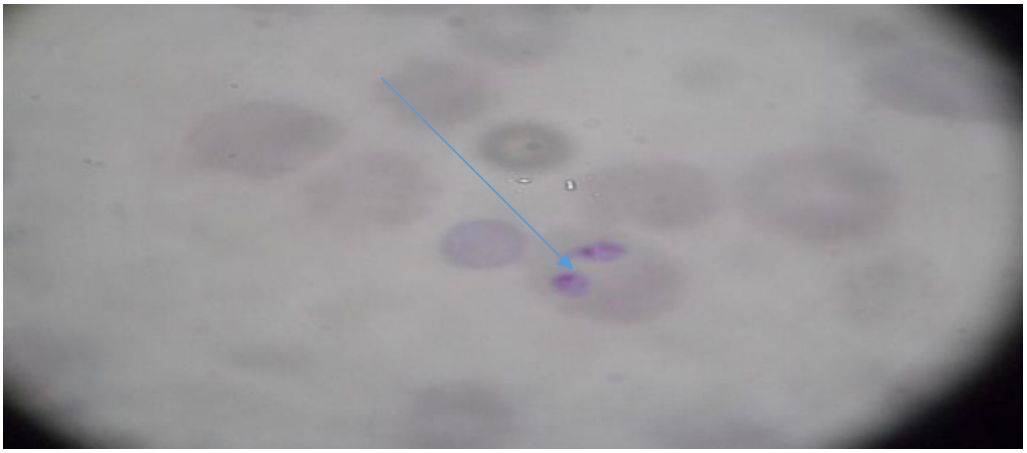

Figure I: Intraerythrocytic Pear-Shaped paired *Babesia Species (blue arrow) from one of the dogs in this study*

DNA was extracted from individual blood samples collected using Quick-DNA universal ™ kit (Zymo Research Corporation) according to the manufacturer instruction. Genomic DNA extracted from reference isolates of *Babesia species* gotten from a dog positive for *Babesia canis* were incorporated into the study as positive controls and nuclease free water served as negative control. The amplification of DNA extracted from the blood was done using a genus specific set of primers, which amplifies a specific region of the 18S rRNA gene of *Babesia species*. A forward primer PIRO A1 (5'AGGGAGCCTGAGACGGCTACC3') and reverse primer PIRO-B (5'TTAAATACGAATGCCCCCAAC3') (Jefferies *et al.*, 2003) were used to amplify an approximately 450-bp region of the *Babesia* gene. The master mix of the PCR assay was prepared in a 25µL water-solution containing 10X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1U of Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA), and 0.2 mM of each primer.

DNA was amplified with a C1000 series thermal cycler (BIO RAD® USA) under the following conditions: an initial denaturation at 95ºC for 5 minutes, 40 repetitive cycles of denaturation at 95ºC for 30 seconds, annealing at 56ºC for 30 seconds, and elongation at 72ºC for 90seconds followed by a final elongation at 72°C for 5 minutes after which it was allowed to cool down. The PCR amplicons were visualized after electrophoresis in 1% Agarose® gel stained with SYBR® stain under UV Transilluminator (Spectroline® TC 312E), 1kb+® was used as the DNA marker to determine the size of the bands formed. Bands were captured and recorded using a digital camera. Presence of bands at 450bp were taken to be positive. (Figure II)

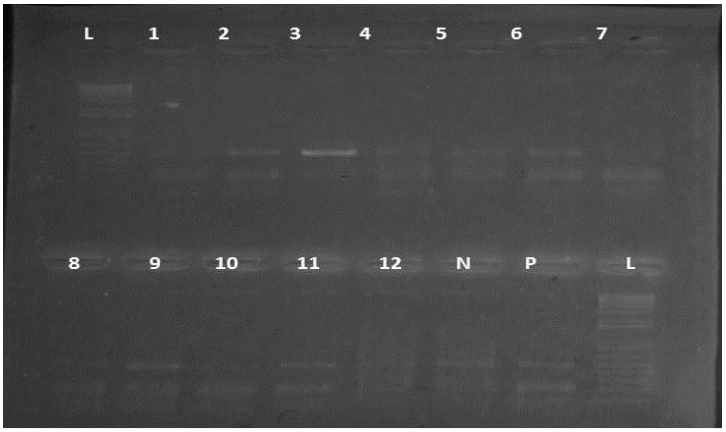

Figure II: *Babesia* spp PCR with primers PIROA/PIROB: lane L, 450bp DNA Marker; lane 1, 2 3 etc are from dogs from this study, lane 3 has a distinct 450bp band; lane 12, is negative; lane N, negative control; lane P, positi

Statistical Analysis

Data collected were analyzed using statistical package for Social Science (SPSS) Version 22. Relationship between *Babesia* infection and categorical variables were tested with chi-square test while the relationship between *Babesia* infection and continuous variables were tested with t-test (Miró, et al., 2015). A p-value of less than 0.05 was considered significant.

3. RESULT

A total of 109 dogs were recruited for the study comprising of 30 (27.5%) males and 79 (72.5%) females; 31 (28.4%) were within the age range of ≤ 1year, 67 (61.5%) were >1- 6 years old, and 11 (10.1%) dogs were within the age range of > 6 years old. The mean age was 3.01±0.23 years. The weight of the dog ranged from 4kg to 20kg with a mean of 12.2±0.33kg while the PCV ranged from 14 to 55% with a mean of 35.2± 0.86%.

Using microscopy 24 dogs were positive for *Babesia* species giving a prevalence of 22% out of which 7 (29.2%) were male and 17(70.8%) were females. There was no statistically significant difference in the gender of the dogs with or without Babesia parasites (p=0.84). Of the dogs positive for Babesia spp; 6 (25%) were ≤ 1 year of age and 13 (54.2%) were >1 -6years and 5 (0.9%) were between >6years There was no statistical significant difference in the ages of dogs with or without Babesia (p=0.71). The mean PCV value of infected dogs with *Babesia spp* (34.5 ± 10.8) was lower than the mean PCV of non-infected hunting dogs (35.17± 8.4) however this did not attain statistical significance at p=0.87. The mean weight of infected and non-infected dogs did not differ significantly (p= 0.45) (Table 1)

TABLE I: STATISTICAL ANALYSIS OF MICROSCOPY

Prevalence: 24 (22.0%; 95% CI: 14.1 – 29.9%)

	Positive	Negative	p-value
Gender (N, %)			
Male	7 (29.2)	23 (27.1)	
Female	17 (70.8)	62 (72.9)	0.838
Age (median, Range)	2.0 (0.5 - 13.0)	2.5 (0.75 - 10.0)	0.577
[1]Weight (Mean, SE)	12.7 (0.7)	12.1 (0.4)	0.450
PCV (Mean, SD)	35.5 (10.8)	35.1 (8.4)	0.870

[1]Covariates appearing in the model is evaluated at the Age: 3.0, PCV: 35.2

From the PCR results; overall, 52 (47.7%) of the sampled dogs were positive to *Babesia* spp. Of the dogs positive for *Babesia* species with PCR, 18 (34.6%) were male and 34(65.4%) were females. There were no statistical significance between the presence of *Babesia* spp. and the gender of the hunting dogs (p=0.11). Of the 52 samples positive for *Babesia* spp by PCR; 8 (15.4%) were within age group of ≤ 1, 39 (75%) were within age group of >1-6years and 5 (9.6%) were within age group of >6.There was a statistical significance difference between the ages of dogs that had *Babesia* and those that did not (p= 0.04). The mean weight of the infected dogs (11.3 ± 0.5) was lower than that of the non-infected dogs (13.0± 0.4 kg) and this was statistically significant (p= 0.01). The mean PCV of the infected dogs with *Babesia spp* based on PCR was 33.5± 8.4 % while the mean PCV of non-infected hunting dogs was 36.7± 9.1 but the difference was not statistically significant (p=0.05) {Table II}

TABLE II: Statistical analysis of pcr technique
Prevalence: 52 (47.7%; 95% CI: 38.2 – 57.2%)

	Positive	**Negative**	**p-value**
Gender (N, %)			
Male	18(34.6)	12(21.1)	
Female	34(65.4)	45(78.9)	0.113
Age (median, Range)	2.0 (0.7-13.0)	3.0 (0.5-10.0)	0.778
[1]Weight (Mean, SE)	11.3 (0.5)	13.0 (0.4)	0.008
PCV (Mean, SD)	33.5 (8.4)	36.7 (9.1)	0.058

[1]Covariates appearing in the model is evaluated at the Age: 3.0, PCV: 35.2

Using PCR as a gold standard the specificity and sensitivity of microscopy for detecting *Babesia* were 77.2% and 21.2% respectively. Microscopy had positive predictive value of 45.8% and negative predictive value of 51.8% while the diagnostic accuracy was 50%. (TABLE III)

TABLE III: COMPARISM

	PCR Positive	PCR Negative
Microscopic Positive	11 (21.2)	13 (22.8)
Microscopic Negative	41 (78.8)	44 (77.2)

Sensitivity: 21.2%. Specificity: 77.2%. PPV: 0.458. NPV: 0.518. Diagnostic accuracy of microscopy: 0.50

4. DISCUSSION

Microscopically 22% of the dog studied were positive for Babesia infection. This is lower than the prevalence of 38.5% reported by Okubanjo *et al.,* (2013) in Sokoto using microscopy also and this might be due to different geographical locations where the studies were carried out and the type of dogs that were sampled. The prevalence in this study is also much lower than 54% reported by Adamu *et al.,* (2014) in Jos, Nigeria. This is not unexpected as Adamu et al used serology which is known to detect both active and passive infections unlike microscopy which tend to detect only active parasites circulating in the system as at when sampled.

This study showed that 47.7% of the hunting dogs in Abeokuta were exposed to infection by *Babesia* spp when analysed with Polymerase Chain Reaction. The prevalence in this study is higher than 2.3% reported in a general survey by Sasaki *et al.,* (2007) in Ibadan and 7.2% reported by Kamani et al., (2013). A plausible reason for this difference in prevalence is the fact that those two studies were among dogs that were presented to teaching hospitals while our own study was on hunting dogs that are in constant exposure to the bush environment where ticks are in ample supply. Hunting may have infact predispose to them to *Babesia* infection because of their constant contact with ticks.

The prevalence value recorded in this study is higher than 4.3% recorded in hunting dogs in Italy (Ebani *et al.,* 2015) and 16.1% in rural dogs in Brazil (Costa *et al.,* 2015). The different climatic and environmental conditions of the hunting dogs might be the possible reasons for the differences in the prevalence. Hunting dogs in this study are known to roam freely with little or no care from the owners. Furthermore, hunting areas are characterized by conditions favourable for the arthropods diffusion, as abundant vegetation and presence of other animal species, in particular wild canids that serve as reservoir for *Babesia* spp.

As at the time of this study and to the best of our knowledge there were no data on hunting dogs infected with canine babesiosis in Nigeria thus we may not be able to have a base data for comparism.

In this study, sex did not appear to be a factor affecting the prevalence *Babesia* spp in hunting dogs. This finding is in accordance with Cardozo *et al.,* (2010) and Salem and Farag, (2014) who found no difference in sex susceptibility between males and females studied for *Babesia* spp.

The prevalence was significantly higher among older dogs compared to those that were less than one year; this could be due to maternal immunity that the under one year old has which has not wane off. These older dogs had probably been exposed to an environment infected by *Babesia* spp contaminated ticks for a longer time compared to the younger animals. This is observation is similar to those of Taylor

et al, (2007); Ivanov and Tsachev, (2008) and Salem and Farag, (2014) who explained the variation of the disease across age categories. However, Hornok *et al.*, (2006) reported that there is no clear distinction between the prevalence of the disease in age categories thus with no statistical significance in the age group studied this study is in accordance with the former authors.

Although there was statistically significant difference in the packed cell volume of the hunting dogs with *Babesia* infection and those without *Babesia*, the mean PCV value of the infected dogs fell below the normal range of 37-55% (Kahn *et al.*, 2006). This also indicates that anaemia is associated with the disease condition. Various authors have suggested that babesiosis causes heamolysis and attendant reduction in blood volume. (Bohm *et al.*, 2006; Irwin, 2010; Birkenheuer, 2014, Koster *et al.*, 2015)

This study showed that microscopy has moderate specificity but low sensitivity for the detection of Babesiosis. Microscopy may lack sensitivity in dogs clinically suspected of babesiosis, possibly due to low parasitaemia (Irwin, 2009; Otranto. 2009). Moreover, it is not possible to differentiate species or subspecies of pathogens by sequence analysis as it may be done following PCR (Matjila et al., 2004, Oyamada et al., 2005, Koster et al., 2015). However, the cost and time required may be major limitations for the use of PCR in clinic-based practice (Sasaki *et al.*, 2007, Cardozo *et al.*, 2010, Salem and Farag, 2014).

It is noted that molecular diagnosis was done in this study to detect the generic specific *Babesia* parasite at 450bp. However multiple bands were seen in some of the gel positive to the parasite. This may however be explained by PCR amplification of 18S rRNA partial sequences that allows the identification of different *Babesia* species that were previously indistinguishable due to similarity in the parasites' morphology (Birkenheuer *et al.*, 2004, Duh *et al.*, 2004, Matjila *et al.*, 2004, De Sa *et al.*, 2006). Hence, further characterization is needed to determine the strain of *Babesia* spp that are prevalent in the study area.

5. CONCLUSION

In conclusion, Babesiosis is common among hunting dogs in Abeokuta and this poses a risk of transmission to other animals especially the domesticated ones and this might affect their weight and packed cell volume apart from acting as reservoirs for continuous transmission. The demonstration of more than one bands on gel image which suggest the possibility of dealing with more than one strain of Babesia canis could mean that broad spectrum treatment will be more appropriate in treating babesiosis among the hunting dogs in Abeokuta and by extension, Nigeria. Further work should be done on sequencing of the Babesia parasites discovered to authenticate the circulating strains in the study area to assist in the treatment options available to veterinarians in the area.

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