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Original article

Detection and identification of blood-borne infections in dogs in Nigeria using light microscopy and the polymerase chain reaction

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ABSTRACT

Many sick dogs brought to the University of Ibadan Veterinary Teaching Hospital (UIVTH) are infested with ticks and are anemic. Up until recently, light microscopy (LM) has been the only available means used for detection of blood-borne infections. In other parts of the world, PCR-based assays been used as a gold standard for accurate diagnosis of blood-borne infections.

In this study, we used LM and broad-spectrum rRNA gene PCR-based assays on 116 blood samples from dogs brought to the UIVTH for detection of the 18S rRNA gene of *Babesia* and the 16S rRNA genes of *Ehrlichia* and hemotropic mycoplasmas. The relationship between clinicopathological findings and PCR results was evaluated. Age, sex, presence of ticks, anemia, co-infection status, and fever were also assessed in relation to PCR positivity to determine the risk factors using stepwise logistic regression analyses.

Light microscopic examination revealed an overall prevalence of infection of 14.7% (17/116). Organisms detected were *Babesia canis* (3.5%), *Ehrlichia canis* (10.3%) and *Trypanosoma congolense* (0.9%) and a single co-infection with *Babesia canis* and *Ehrlichia canis* (0.9%). PCR analysis revealed 89/116 (76.7%) positive samples. Infections with 1, 2 and 3 infectious agents occurred in 49 (55.1%), 36 (40.4%) and 4 (4.5%) samples, respectively. Specifically, among the 89 PCR positive samples, *Babesia spp.* (85.4%) was the most abundant infection followed by *Ehrlichia spp.* (46.1%) and hemoplasmas (13.5%). Sequencing of PCR products identified two samples (1.7%) that contained *Hepatozoon canis* DNA. Sequencing of hemoplasma positive samples identified '*Candidatus* Mycoplasma haemobos' in 0.8% of dogs. Using PCR, a 5-fold higher prevalence of blood-borne infections was found in the dogs (76.7%, 89/116) than with LM (14.7%, 17/116) alone"

Dogs between 1 and 12 months were the most frequently infected with multiple agents (47.2% double and 50.0% triple infections). Male dogs had the highest prevalence of infection (80.4%) and more triple infections (75.0%). A total of 57.3% of infected dogs were anemic. Anemic dogs were 2.77 times more likely to test positive for *Ehrlichia spp.* (OR: 2.77 95% CI: 1.25–6.16) and dogs with ticks were 3.6 times more likely to test positive for hemoplasmas (OR = 3.60 95% CI: 1.05-12.38).

This study underscores the abundance of blood-borne infections in dogs in Ibadan, Nigeria, which is underestimated using light microscopy. This is also the first evidence of existence of '*Candidatus* Mycoplasma haemobos' in a dog in Nigeria and in Africa. Consequently there is a need for molecular diagnostic facilities for routine screening of sick animals, as multiple infections were not found by light microscopy.

1. Introduction

Infections with blood-borne pathogens are common in dogs and can be associated with high morbidity and mortality. In Nigeria the main blood-borne pathogens in dogs are *Babesia spp.*, *Ehrlichia spp.*, *Hepatozoon canis*, *Theileria spp.*, and *Trypanosoma spp.* (Adamu et al., 2014; Happi and Anita, 2012; Kamani et al., 2013; Useh et al., 2003). These organisms can cause anemia, weight loss, and other clinical signs in dogs, and some are of zoonotic importance (Otranto et al., 2009).

While some infections result in overt clinical signs, others are subclinical (Hii et al., 2012). In most cases, the identification of anemia results in consideration of a blood-borne infection as a possibly underlying cause. In Nigeria, diagnosis is usually based on physical examination and microscopic detection of the pathogen in peripheral

Abbreviations: PCR, polymerase chain reaction; LM, light microscopy; UIVTH, University of Ibadan Veterinary Teaching Hospital

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blood, and rarely by molecular methods. This approach is insensitive and lacks specificity. With globalization, climate change, and increased movement of humans and animals across the continent, there is an additional challenge of introduction and re-emergence of new bloodstream pathogens in West Africa (Kamani et al., 2013). To better understand the epidemiology of blood-borne infectious agents in dogs in Africa, there is need to employ more robust, sensitive and rapid diagnostic assays such as PCR.

Several epidemiological surveys for blood-borne pathogens have been carried out on dogs in Nigeria but very few of them utilized molecular tools (Kamani et al., 2013; Ogo et al., 2012; Suksawat et al., 2001). These studies were focused on the most common pathogens such as *Babesia spp., Trypanosoma spp.,* and less so on *Hepatozoon and Ehrlichia* and very few on hemotropic mycoplasmas.

The objective of this study was to determine the identity and prevalence of blood-borne organisms of dogs in southwestern Nigeria using molecular tools. In addition, risk factors for these pathogens were assessed to aid diagnosis of these infections in the future.

2. Materials and methods

2.1. Study area and animals

The study was carried out on dogs evaluated for illness at University of Ibadan Veterinary Teaching Hospital (UIVTH), University of Ibadan, Nigeria. A total of 116 dogs of various breeds and ages (2 days–16 - years) seen between April and August 2013 were enrolled in the study. Signalment, clinical history and physical examination were available. Thirty-nine dogs were clinically suspected to be infected with blood-borne pathogens based on the presence of fever, icterus, tick infestation, or anemia.

2.2. Light microscopy (LM) examination of blood from UIVTH dogs

Blood samples were collected into EDTA tubes for complete blood count and LM screening for infectious agents. Hematological analysis was done as described elsewhere (Schalm et al., 1975; Jain, 1986). The PCV was determined using the microhaematocrit method (Jain, 1986). Hemoglobin concentration was determined using the cyanmethemoglobin method (Jain, 1986). The platelet and WBC counts were determined using a improved Hawksley hemocytometer with premade diluents (Jain, 1986), while the differential leukocyte count was determined by counting from a total of 200 leukocyte cells from randomly selected fields. The leukocytes were classified into types, and the relative and absolute leukocyte values of each type were calculated (Schalm et al., 1975).

From each EDTA blood sampled, 500 µl were aliquoted into another tube and kept at − 20 °C for DNA extraction. Whole blood and buffy coat smears from each dog were made, air-dried and stained using Giemsa stain and examined by a veterinary clinical pathologist (AH) under LM at × 100 oil immersion for blood-borne microbes, including visible intraerythrocytic piroplasmas, intracytoplasmic morulae consistent with rickettsial organisms, *Hepatozoon* gamonts, or epierythrocytic bacteria (hemoplasmas). The criteria for LM identification of each of the organism were based on the morphological characteristics described by Harvey (2001).

2.3. DNA extraction

DNA was extracted from 200 µl of EDTA blood using the DNeasy blood and tissue kit (Qiagen LTD, USA) according to the manufacturer's protocol. The DNA was eluted with 100 ul elution buffer and kept at − 20 °C for PCR and sequencing. All DNA extraction was performed at the Africa Center of Excellence for Genomics of Infectious Diseases (ACEGID) laboratory, Redeemer' University, Ede, Nigeria.

2.4. Polymerase chain reaction and sequencing of amplified DNA products

Extracted DNA was used as template in a standard PCR assay using primers for detection of the 16S rRNA gene of hemotropic *Mycoplasma* spp. (Jensen et al., 2001), 18S rRNA gene of *Babesia* spp. (Sikorski et al., 2010), and the 16S rRNA gene of *Ehrlichia* spp. and *Anaplasma* spp. (Lappin et al., 2004). PCR amplification was performed as previously described (Sykes et al., 2005). Ultrapure water was used as a negative control.

The resulting PCR products were sequenced to confirm their identity as previously reported (Sykes et al., 2005). DNA sequencing was performed using automated methods (California University DNA Sequencing Facility, Davis, CA, USA). Sequences obtained were compared with those in the GenBank database using Basic Local Search Alignment Tools (www.ncbi.nlm.gov/BLAST).

2.5. Statistical analysis

Descriptive statistics were used to determine the infection rate in sampled dogs and to describe other factors associated with infection in dogs. Chi-squared analyses were performed to determine whether there were significant differences in the number of dogs infected based on age and sex. For this purpose, dogs were divided in 4 age groups of 0–12 months (43 dogs), > 1–2 years (19 dogs), > 2–5 years (44 dogs) and the group of 7 years (4 dogs) and a group of 6 dogs with no record of age. Stepwise logistic regression analyses were performed to determine the effects of several demographic and clinical variables on PCR positivity. All analyses were completed at the 0.05 significance level. Graphical and logistic regression analyses were completed using Excel (Microsoft, Redmond, WA), SAS (SAS Institute, Cary, NC), and GraphPad Prism 7 (La Jolla, CA).

3. Results

3.1. Distribution of sampled dogs

Of the 116 dogs, 43 were 1 year-old or younger, 23 were > 1–2 years, 33 were > 2–5 years, 6 were > 5–7 years, 4 were above 7 years of age, and 7 had unknown age. Sixty- seven dogs were females while 46 were males and 3 had no record of their sex. All of the dogs were ill dogs owned by individual households located in the vicinity of the teaching hospital.

3.2. Detection of blood-borne microorganisms by light microscopy

Of the 116 dog blood samples, 17 (14.7%) contained blood-borne microorganisms as identified using LM. Among these positive samples, *Ehrlichia canis* was the most abundant (12/17, 70.6%), followed by *Babesia canis* (4/17, 23.5%) and *Trypanosoma congolense* (1/17, 5.9%) (Fig. 1). The overall prevalence of infection was 10.3% for *E. canis,* 3.5% for *B. canis*, and 0.9% for *T. congolense* (Table 1).

3.3. Detection of blood-borne microorganisms by PCR and sequence analysis

Using PCR, a 5-fold higher prevalence of infection with blood-borne microorganisms was found in the dogs (76.7%, 89/116) than with LM alone. Blood-borne microorganisms identified using PCR were *Babesia spp.* (85.4%, 76/86 samples), *Ehrlichia spp.* (47.2%, 42/89 samples), and hemotropic mycoplasmas. (13.5%, 12/89 samples). The presence of *Ehrlichia* and *Babesia* in samples positive by LM was confirmed in all of these samples using PCR. Sequencing and analysis of PCR products revealed that all *Babesia* and *Ehrlichia* positive samples were *B. canis* and *E. canis* respectively. Most (11/12) hemotropic mycoplasmas were *Mycoplasma haemocanis*.

Using broad-spectrum rRNA gene PCR and sequencing, two