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Research paper

# Analysis of risk factors and prevalence of haemoplasma infection in dogs

L.C. Aquino[a,*], J. Kamani[d], A.M. Haruna[e], G.R. Paludo[a], C.A. Hicks[b], C.R. Helps[b,c], S. Tasker[b,c]

[a] Laboratory of Veterinary Clinical Pathology, College of Agronomy and Veterinary Medicine, University of Brasília, Campus Universitário Darcy Ribeiro, Brasília 709010-900, Brazil
[b] School of Veterinary Sciences, University of Bristol, Langford House, Langford, Bristol BS40 5DU, UK
[c] Molecular Diagnostic Unit, Langford Veterinary Services, Langford, Bristol BS40 5DU, UK
[d] Parasitology Division, National Veterinary Research Institute (NVRI) Vom, Plateau State, Nigeria
[e] State Veterinary Hospital Jos, Ministry of Agriculture and Natural Resources, Plateau State, Nigeria

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ABSTRACT

*Mycoplasma haemocanis* (Mhc) and '*Candidatus* Mycoplasma haematoparvum' (CMhp) are canine haemoplasma species that can induce anaemia in immunocompromised and/or splenectomised dogs. This study aimed to determine the prevalence and phylogeny of canine haemoplasma species in dogs from Nigeria and describe any risk factors for infection. Canine haemoplasma species-specific and generic haemoplasma qPCR assays were used. The species-specific qPCR assays found Mhc infection in 18 of 245 dogs (7.3%), and CMhp infection in only one dog (0.4%). The generic haemoplasma qPCR assays were positive in 44 of 245 (17.9%) dogs. Twenty-five dogs had discordant qPCR results in that they were generic haemoplasma qPCR positive but species-specific qPCR negative. Further evaluation of these dogs by 16S rDNA sequencing gave limited results but 5 were confirmed to be infected with non-haemoplasma species: 2 *Anaplasma phagocytophilum*, 1 *Anaplasma ovis*, 1 *Serratia marcescens* and 1 *Aerococcus* spp. The 16S rRNA gene sequences from Mhc species showed > 99.8% identity with each other and > 99.6% identity with GenBank sequences, and resided in a single clade with other global Mhc and *Mycoplasma haemofelis* sequences, indicating low 16S rRNA genetic variability amongst this canine haemoplasma species.


## 1. Introduction

Haemoplasmas are small bacterial haemotropic mycoplasmas that parasitize the surface of erythrocytes in a wide range of mammal species (Messick, 2004). Dogs are mainly infected with two haemoplasma species: *Mycoplasma haemocanis* (Mhc) and '*Candidatus* Mycoplasma haematoparvum' (CMhp). Infections are usually chronic and subclinical in immunocompetent dogs but may lead to clinical signs related to haemolytic anaemia following splenectomy, immunosuppression or concurrent infections (Messick et al., 2002; Sykes et al., 2005).

Both Mhc and CMhp have been reported in dogs from Spain (Novacco et al., 2010; Roura et al., 2010), Trinidad (Barker et al., 2010), France (Kenny et al., 2004), Switzerland (Wengi et al., 2008), Portugal (Novacco et al., 2010), Italy (Novacco et al., 2010), Japan (Sasaki et al., 2008), United States (Compton et al., 2012), Greece (Tennant et al., 2011) and Australia (Barker et al., 2012; Hetzel et al., 2012). Previous studies (Kenny et al., 2004; Novacco et al., 2010) reported that dogs from warm subtropical climates may be at a higher risk of haemoplasma infection due to the concurrent presence of ectoparasites, such as *Rhipicephalus sanguineus*, which have been proposed as vectors (Kenny et al., 2004). However, data confirming vector transmission of canine haemoplasmas are lacking and little information exists regarding risk factors for canine haemoplasma infection in dogs from warmer tropical climates such as Africa and South America.

Recently, a study reported a high level of tick-borne pathogens such as *Hepatozoon canis*, *Ehrlichia canis*, *Rickettsia* spp., *Babesia rossi* and *Anaplasma platys* in two genera of ticks, *Rhipicephalus* and *Heamaphysalis*, in dogs from Nigeria, Africa (Kamani et al., 2013). Although haemoplasmas have been found in African dogs from Tanzania (Barker et al., 2010) and Sudan (Inokuma et al., 2006), no study has evaluated haemoplasma prevalence in dogs from Nigeria (Kamani et al., 2013).

* Corresponding author.
*E-mail addresses:* larissaquino@gmail.com, larissaquino@unb.br (L.C. Aquino).

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The aim of this study was to investigate any correlation between haemoplasma infection and potential risk factors in dogs. Additionally, we also assessed the prevalence of haemoplasmas in dogs from Nigeria using qPCR assays, and molecularly characterized the 16S rRNA gene of the identified haemoplasma species.

## 2. Materials and methods

### 2.1. Recruitment and data collection

Blood (EDTA) samples were obtained from dogs presenting to veterinary clinics in the city of Jos, Plateau State, Nigeria and from samples submitted to the Parasitology Division Laboratory, National Veterinary Research Institute, Vom, Nigeria. Packed cell volume (PCV) was determined on samples by the microhaematocrit centrifugation method, with anaemia defined as a PCV < 35%. Samples were stored at 4 °C and subsequently shipped to School of Veterinary Sciences, University of Bristol, UK for molecular analysis. Data regarding age, gender (including neutering status), breed, whether the dog was privately owned or from a breeding kennel, clinical health status (healthy or sick, based on the dog's history and clinical examination), presence of ticks, ectoparasite prophylaxis, splenectomy history and travel history was collected when available for each dog.

### 2.2. DNA extraction

A QIAxtractor was used to extract DNA from 100 μl of EDTA blood using the DX reagent kit (Qiagen) as per the manufacturer's protocol. The DNA was eluted in 100 μl elution buffer and stored at −20 °C until use. Two negative controls using phosphate buffered saline were performed in parallel with the extraction of every set of 94 canine samples.

### 2.3. Species-specific haemoplasma qPCR assay

The samples were subjected to species-specific qPCRs for Mhc and CMhp, as previously described (Barker et al., 2010). Each assay was duplexed with a canine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) qPCR as an internal control to demonstrate the presence of amplifiable DNA and the absence of PCR inhibitors; a threshold cycle (Ct) of 26 was used as a cut off, above which the samples underwent repeat DNA extraction and repeat qPCR analysis. In each run of 94 samples, DNA from a known haemoplasma positive dog was used as a positive control and water as a negative control.

### 2.4. Generic haemoplasma qPCR assay

The samples also underwent previously described (Tasker et al., 2010) generic haemoplasma qPCR assays designed to detect novel haemoplasma species. These generic qPCR assays comprised two assays that amplify 16S rRNA gene sequences of the two main groups of haemoplasma species: the so-called haemominutum (HM) and haemofelis (HF) groups. These assays should detect any infecting haemoplasma species that would not be detected by the Mhc and CMhp specific qPCRs due to differences in their primer or probe binding sites. The protocol was adapted as follows: 12.5 μl of 2X Promega GoTaq Hot Start Colorless Master Mix (UK) with 0.2 μM of each primer, 0.1 μM probe, 4.5 mM $MgCl_2$ final concentration and 5 μl of DNA template with water to 25 μL. All qPCRs were performed in Agilent MX3005 P (Agilent, UK) thermocycler with initial incubation of 95 °C for 2 min followed by 45 cycles of 95 °C for 10 s and 60 °C for 30 s during which the fluorescence data were collected. In each batch of 94 qPCRs, DNA from known haemoplasma infected dog was used as a positive control, and water as a negative control.

### 2.5. Conventional PCRs and 16S rRNA gene sequencing

All dogs with discordant qPCR results (positive by the generic qPCRs but negative by the species-specific qPCRs) were submitted to two different conventional PCRs to amplify the near complete 16S rRNA gene and to perform DNA sequencing. The first conventional PCR amplified ≅1400 bp from the 16S rRNA gene and utilised 16S rRNA gene universal primers (8F and 1492R) as previously described (Pitulle and Pace, 1999). The reaction included 12.5 μl of HotstarTaq Master mix with 0.2 μM of each primer, 3.0 mM of $MgCl_2$ and 5 μl of DNA template with water to a final volume of 25 μl. The reaction was performed in a SureCycler 8800 thermal cycler (Agilent Technologies, USA) with cycling conditions as follows: 95 °C for 15 min followed by 45 cycles of 95 °C for 15 s, 48 °C for 30 s, 72 °C for 2 min, and a final extension of 72 °C for 10 min. The second conventional PCR amplified ≅1100 bp from the 16S rRNA gene of *Mycoplasma haemofelis* (Mhf)/Mhc and '*Candidatus* Mycoplasma haemominutum' (CMhm)/CMhp using previously described 16S rRNA gene species-specific primers (MhfFw2 and MhfRev2; CMhmFw2 and CMhmRev2 respectively) (Aquino et al., 2014). This reaction comprised 12.5 μl of 2X Promega GoTaq® Hot Start Colorless Master Mix (UK) with 0.2 μM of each primer and 1 μl of DNA template with water to a final volume of 25 μl. The reaction was performed in a SureCycler 8800 thermal cycler (Agilent Technologies, USA) with cycling conditions as follows: 95 °C for 5 min, followed by 45 cycles of amplification (95 °C, 10 s; 62 °C, 30 s; 72 °C, 90 s) with final extension of 72 °C for 5 min. Samples known to be positive for Mhc and Mhf were used as positive controls for the MhfFw2 and MhfRev2 primers and CMhp and CMhm positive samples were used as positive controls for the CMhmFw2 and CMhmRev2 primers. Water was used as a negative control in each PCR assay. PCR products were identified by electrophoresis in a 1.5% agarose gel stained with ethidium bromide. Samples presenting faint multiple bands were reamplified under the same conditions, with cycle numbers reduced to 20 and using <1 μl of amplicon from the previous PCR as template.

PCR products of the expected size were purified with the NucleoSpin® Gel and PCR Clean-up kit (MACHEREY NAGEL GmbH & Co.) according to the manufacturer's instructions, quantified with a Qubit™ fluorometer (Invitrogen™) and submitted to DNA Sequencing & Services (MRC PPU, College of Life Sciences, University of Dundee, Scotland, www.dnaseq.co.uk) for sequencing in the sense and antisense directions using Applied Biosystems Big-Dye Ver 3.1 chemistry on an Applied Biosystems model 3730 automated capillary DNA sequencer.

Sequence editing and analysis were performed in MacVector v13.0.3, Inc. Reconstruction of near-complete 16S rRNA gene sequences was performed from products of either the first or second conventional PCR as follows: the sequences derived from the universal 8F and 1492R primers were combined with sequences generated from internal primers (HBT-F and HBT-R) as previously published (Criado-Fornelio et al., 2003). The sequences derived from the MhfFw2 and MhfRev2 primers were combined with sequences generated by the use of additional internal primers previously described (Aquino et al., 2014). These newly derived 16S rRNA gene sequences were initially compared to sequences available in GenBank using BLAST and were then aligned with 16S rRNA gene sequences from canine haemoplasma species available from GenBank using Clustal-W to determine the approximate phylogenetic affiliation.

The 16S rRNA gene sequences from 4/17 Mhc species detected in this study were submitted to GenBank under the following accession numbers: KP715857, KP715858, KP715859 and KP715860.