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Original Article

Analysis of risk factors and prevalence of haemoplasma infection in dogs

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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.

Keywords: haemoplasmas, Nigeria, dogs, prevalence, risk factors

1.1 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 *Haemaphysalis*, 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).

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.

1.2 Materials and Methods

1.2.1 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.

1.2.2 DNA extraction

A QIAextractor was used to extract DNA from 100µl of EDTA blood using the DX reagent kit (Qiagen) as per the manufacturer's protocol. The DNA was eluted in 100µl 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.

1.2.3 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.

1.2.4 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: 12.5µL of 2X Promega GoTaq Hot Start Colorless Master Mix (UK) with 0.2µM of each primer, 0.1µM probe, 4.5mM MgCl₂ final concentration and 5µl of DNA template with water to 25µL. All qPCRs were performed in Agilent MX3005P (Agilent, UK) thermocycler with initial incubation of 95°C for 2 minutes followed by

45 cycles of 95°C for 10 seconds and 60°C for 30 seconds 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.

1.2.5 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 \cong 1400bp 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 μ l of HotstarTaq Master mix with 0.2 μ M of each primer, 3.0mM of MgCl₂ and 5 μ l of DNA template with water to a final volume of 25 μ L. The reaction was performed in a SureCycler 8800 thermal cycler (Agilent Technologies, USA) with cycling conditions as follows: 95°C for 15 minutes followed by 45 cycles of 95°C for 15 seconds, 48°C for 30 seconds, 72°C for 2 minutes, and a final extension of 72°C for 10 minutes. The second conventional PCR amplified \cong 1100bp 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 μ L of 2X Promega GoTaq® Hot Start Colorless Master Mix (UK) with 0.2 μ M of each primer and 1 μ L of DNA template with water to a final volume of 25 μ L. The reaction was performed in a SureCycler 8800 thermal cycler (Agilent Technologies, USA) with cycling conditions as follows: 95°C for 5 minutes, followed

by 45 cycles of amplification (95°C, 10 seconds; 62°C, 30 seconds; 72°C, 90 seconds) with final extension of 72°C for 5 minutes. 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. These sequences were used in phylogenetic analysis from the distances matrices using the neighbour-joining method. The dataset was resampled 1000 times to generate bootstrap percentage values.

1.2.6 Statistical analysis

Data were entered into Excel[®] and statistical evaluation was carried out using SPSS for Windows (SPSS Inc., Chicago IL, USA). Results of the generic haemoplasma, Mhc and CMhp qPCR assays were tested for association with categorical variables (gender, breed, being privately owned or from a breeding kennel, clinical status, presence of ticks and ectoparasites prophylaxis) using the χ^2 test. Statistical comparison between Mhc and CMhp prevalence were calculated using the χ^2 test. The Kolmogorov-Smirnov test was used to test for normal distribution of the continuous variables PCV and age, and subsequently the Mann-Whitney U test was used to determine whether any significant difference existed between the results of the generic, Mhc and CMhp qPCR assays and each of PCV and age. Significance was assigned as a P value <0.05.

1.3 Results

A total of 246 samples were recruited for the study. Categorical descriptive data are summarized in Table 1. Age data were available for 245 dogs and ranged

from 2 to 9 years (median age: 2 years). All dogs in the study were entire except for two neutered male dogs aged 1 and 1.5 years. Two dogs had a history of travelling outside of Nigeria (one 4 year old male and a 5 year old female). No dogs were splenectomised. Due to the low numbers of neutered, splenectomised and travelled dogs, these variables were excluded from statistical analysis. PCV data were available for 228 dogs and ranged from 10 to 62% (median PCV: 42%). Of the 245 dogs evaluated for the presence of ticks 135 (55,1%) were found to be infected with ticks and all of these were identified as *Rhipicephalus sanguineus*.

Adequate DNA amplification, as indicated by an internal control GAPDH qPCR Ct value of ≤ 26 , occurred in 239 samples (median Ct: 15, range 12-26), whilst six had no GAPDH qPCR Ct values and had to be re-extracted, after which PCR analysis generated Ct values of ≤ 26 (range 12-19) in five of these dogs. In the one remaining dog the repeat extract generated a GAPDH qPCR Ct of 34 and was therefore excluded from the study. Thus 245 dogs comprised the final study population. All positive and negative controls for DNA extraction and qPCRs yielded positive and negative results, respectively.

The species-specific qPCRs found that 18 of 245 (7.3%) dogs were Mhc positive (median Ct 27, range 19-44) and one of 245 (0.4%) was CMhp positive (Ct 43). The Mhc prevalence was significantly higher than CMhp prevalence ($p=0.03$). No dog had concomitant infection with Mhc and CMhp.

The generic haemoplasma qPCR HF assay was positive alone (i.e. with a negative HM assay result) in 27 dogs (median Ct: 37, range 30-44) whilst the HM assay was positive alone (i.e. with a negative HF assay result) in two dogs (Cts of 24 and 39). The generic haemoplasma qPCR HM and HF assays were both positive for 13 dogs (HF median Ct: 23, range 15-31; HM median Ct: 26, range 13-40). The

generic haemoplasma qPCR was considered positive in any dog with any positive HF and/or HM result; thus 17.1% (42/245) of dogs were positive.

There was no significant association between positive generic haemoplasma qPCR or Mhc qPCR status and any assessed variables, including PCV (Table 1).

There was no significant association between generic haemoplasma PCR positive status nor Mhc PCR positive status and presence of anaemia (Table 1).

There were 25 dogs with discordant results between the generic haemoplasma qPCRs and species-specific qPCRs. Two different conventional PCRs were used to try to amplify and sequence the near complete 16S rRNA gene from these discordant dogs, but only nine generated visible PCR products with which to attempt sequencing. Five out of these nine dogs generated products using primers 8F and 1492R and four with primers MhfFw2 and MhfRev2. The other 16 discordant dogs didn't generate any visible PCR products, which was expected considering the high Ct values (median Ct: 39, range 33-44) obtained from the generic haemoplasma qPCRs.

The five 16S rRNA gene sequences generated using primers 8F and 1492R revealed two sequences 100% identical to *Anaplasma phagocytophilum* (KP745629), one sequence 99% identical to *Anaplasma ovis* (KJ410246), one sequence 100% identical to *Serratia marcescens* (KR133281) and one sequence 100% identical to *Aerococcus* spp. (KP943730). The four 16S rRNA gene sequences generated with primers MhfFw2 and MhfRev2 showed >99.5% identity with Mhc and Mhf worldwide-derived sequences. The phylogenetic tree revealed no obvious geographical or host specificity grouping of the Mhc sequences. All four Mhc sequences from our study fell within a single clade with other Mhc and Mhf sequences (Fig. 1).

1.4 Discussion

This study reports, for the first time, the prevalence of haemoplasma species in dogs from Nigeria. The overall prevalence was similar to that found in the south of France (Kenny et al., 2004), Spain (Roura et al., 2010) and Tanzania (Barker et al., 2010), but higher than in Switzerland (Wengi et al., 2008), Greece (Tennant et al., 2011), Trinidad (Barker et al., 2010), Italy (Novacco et al., 2010) and UK (Warman et al., 2010) and lower than in Portugal (Novacco et al., 2010), Sudan (Inokuma et al., 2006) and Australia (Barker et al., 2012). Considering Mhc infection, Nigerian dogs had a similar prevalence to that reported in Trinidad (Barker et al., 2010) and Greece (Tennant et al., 2011) but a higher prevalence than dogs from France (Kenny et al., 2004), Italy (Novacco et al., 2010), Switzerland (Wengi et al., 2008), UK (Warman et al., 2010) and Spain (Novacco et al., 2010). Considering CMhp infection, Nigerian dogs had a lower prevalence than all previous studies (Barker et al., 2010; Kenny et al., 2004; Novacco et al., 2010; Tennant et al., 2011; Warman et al., 2010), except from those in Switzerland (Wengi et al., 2008) and Spain (Roura et al., 2010). The findings in the previous canine haemoplasma studies from France (Kenny et al., 2004) and Sudan (Inokuma et al., 2006) found CMhp to be more prevalent than Mhc, which is in contrast to those of the current study and a previous study from Trinidad (Barker et al., 2010) where Mhc was significantly ($p=0.03$, current study) more prevalent than CMhp. In agreement with previous studies from Switzerland (Wengi et al., 2008), Australia (Hetzl et al., 2012), USA (Compton et al., 2012) and UK (Warman et al., 2010), our study found no dog infected with more than one haemoplasma species. This contrasts with the majority of haemoplasma prevalence studies (Barker et al., 2010; Kenny et al., 2004; Novacco et al., 2010; Roura et al., 2010; Tennant et al., 2011), which have found dogs to be co-infected with Mhc and CMhp.

The differences in the prevalence of Mhc and CMhp found in previous studies compared to those found herein could reflect the dog population sampled. Our study primarily comprised privately owned pets with few kennelled dogs and this may have contributed to the low prevalence of Mhf and CMhp seen. A previous study (Kemming et al., 2004b) assessing haemoplasma infection in pet and kennelled dog populations found that kennelled dogs were at high risk of haemoplasma infection (35%), which is much higher than the haemoplasma prevalence found herein. One study in Australia (Barker et al., 2012), sampling free-roaming dogs, found a higher Mhc and CMhp prevalence than studies in pet dogs (Barker et al., 2010; Novacco et al., 2010; Roura et al., 2010; Wengi et al., 2008) like ours, although a direct comparison cannot be made as there were no data on outdoor access for the dogs in the current study. One study performed in dogs from Mediterranean countries found that being a crossbred was a risk factor for haemoplasma infection, however the majority of the crossbreds in that study lived in kennels, which was also identified as a risk factor for canine haemoplasma infection (Novacco et al., 2010; Tennant et al., 2011); it may well be that the living conditions are more important than breed as a risk factor for infection. Also, different dog populations have diverse behaviour activities that may be involved in other methods of haemoplasma transmission, such as direct inoculation and transplacental infection (Compton et al., 2012).

Different PCR assays with varying sensitivities may also contribute to the differences in canine haemoplasma prevalences reported in studies (Compton et al., 2012). Poor quality DNA and/or PCR inhibitors can result in false negative PCR results and consequently lower prevalences, if the PCR assays don't incorporate internal controls, as used in the current study.

Climate may also play a part, with an increased risk of haemoplasma infections in warmer countries such as Portugal (Novacco et al., 2010), Sudan (Inokuma et al., 2006), Tanzania (Barker et al., 2010) and the south of France (Kenny et al., 2004), which may be associated with potential vectors. In our study, the overall haemoplasma prevalence was comparable to that found in regions with a similar climate, such as the south of France (Kenny et al., 2004), Spain (Roura et al., 2010) and Tanzania (Barker et al., 2010). This, in combination with the near significant difference in the haemoplasma prevalence between dogs with and without ticks found herein (table 1), indicates that ticks may be potential vectors of haemoplasmas. In Nigeria, tick-borne pathogens have been previously (Ogo et al., 2012; Reye et al., 2012) identified in ticks and dogs. In the current study, significance was approached for an association between the ectoparasite prophylaxis and haemoplasma infection. Previous evidence of an association between the tick *Rhipicephalus sanguineus* and canine haemoplasma infection has been reported (Wengi et al., 2008); dogs infected with haemoplasmas in Switzerland had a history of travelling to countries where *R. sanguineus* is found. A Mediterranean study also documented an association between exposure to ticks and canine haemoplasma infection (Novacco et al., 2010). However, another study (Barker et al., 2010) failed to find a similar association, although it must be remembered that haemoplasmas can cause chronic infections and so recent tick exposure may not be apparent. Additionally, the mode of transmission of canine haemoplasmas in the field is uncertain (Barker et al., 2010).

There was no significant association between canine haemoplasma infection and age in our study, as has been reported previously (Barker et al., 2010; Kenny et al., 2004; Tennant et al., 2011; Wengi et al., 2008). One previous study (Barker et al.,

2010) found that males were more likely to be Mhc positive than females, and in the current study significance was approached for gender and Mhc infection.

No significant association was found between haemoplasma status and either PCV or the presence of anaemia, in accordance with previous studies (Barker et al., 2010; Hetzel et al., 2012; Novacco et al., 2010; Roura et al., 2010). This finding could be due to chronic haemoplasma infection in dogs (Kemming et al., 2004a; Messick et al., 2002; Wengi et al., 2008) or the mild pathogenicity of Mhc and CMhp in most infected dogs (Compton et al., 2012). One study tracked haemoplasma infected dogs and showed that, despite continuous PCR positive results, no anaemia occurred (Wengi et al., 2008). Additional factors, such as immunosuppression and splenectomy, are usually necessary to induce overt disease in haemoplasma-infected dogs (Novacco et al., 2010; Warman et al., 2010), and none of the dogs in our study were known to have such factors.

Cytological examination of blood smears were originally included in the study, however an 'Eperythrozoon species-like' parasite was the only organism thought to be seen on a blood smear from a single dog that was negative in all haemoplasmas PCRs. Yet, the observation of organisms on erythrocytes in blood smears is known to be unreliable in the diagnosis of haemoplasma infections, as PCR is the gold standard whilst cytology has been reported to have a sensitivity of 0 to 37,5% and specificity of 84 to 98% (Jensen et al. 2001; Westfall et al. 2001; Tasker et al. 2003a and Bauer et al. 2008), therefore cytological data did not warrant inclusion in the final study.

Eleven of the 13 dogs positive in both generic haemoplasma qPCR assays (HM and HF) had lower Cts for the HF assay compared to the HM assay, most likely indicating infection with a haemoplasma species in the haemofelis group as

previously reported (Tasker et al., 2010), whilst two dogs had lower Ct values for the HM assay compared to the HF assay, most likely indicating infection with a haemoplasma species in the haemominutum group (Tasker et al., 2010).

Twenty-five dogs were positive by the generic haemoplasma qPCR assays but negative by the haemoplasma species-specific qPCRs. The 16S rRNA gene sequences from four of these dogs revealed that they were identical to *M. haemocanis*. The samples that were Mhc on sequencing had low haemoplasma copy numbers (Cts > 30), which may be the reason for the discordant results. In five of the 25 discordant dogs, sequencing results revealed close identity of 16S rRNA gene sequences with non-haemoplasma species: two dogs were infected with *Anaplasma phagocytophilum*, which, with *Anaplasma platys*, is one of the canine anaplasmosis agents (Beall et al., 2008); one dog was infected with *Anaplasma ovis*, a species known to infect sheep (Giangaspero et al., 2015) but not yet reported in dogs; one dog was infected with *Serratia marcescens*, an opportunistic agent known to cause nosocomial infection in dogs (Lobetti et al., 2002; Perez et al., 2011) and one dog was infected with *Aerococcus* sp., recently reported to cause resistant urinary infection in one dog (Budreckis et al., 2015). These findings may indicate that the generic haemoplasma qPCRs can detect bacteria other than haemoplasmas and illustrates the importance of sequencing in animals yielding positive results with generic PCR assays. However it is possible that haemoplasma co-infections were present in these dogs but that the universal 16S rRNA gene PCR amplified other co-infections (e.g. *Anaplasma* spp.) present at higher levels in the blood. We felt that the use of generic haemoplasma PCRs was important to evaluate the possibility of infection with novel haemoplasma species with sequence differences affecting the primer and/or probe binding in the species-specific qPCRs. Attempts to amplify and sequence the 16S rRNA gene in the

remaining 16 discordant samples failed, so it is possible that other bacterial species or novel haemoplasma species were present in these samples, as previously reported (Hii et al., 2012; Varanat et al., 2011), but at a level too low to allow amplification with both conventional PCR assays.

The partial 16S rRNA gene Mhc sequences generated in our study showed no genetic variability compared to other Mhc sequences from dogs in different countries (Novacco et al., 2010; Tasker et al., 2003b) and they grouped with them on phylogenetic analysis (Fig. 1). Phylogenetic analysis showed the canine haemoplasma species to group into two distinct clades; one clade comprising CMhm and CMhp species, and the other consisting of Mhc and Mhf species, as reported previously (Tasker et al., 2003b). The newly derived Nigerian Mhc sequences from the present study showed >99.6% identity with sequences previously reported for Mhc and Mhf (Kenny et al., 2004; Messick et al., 2002). A similar result, in which Mhc and Mhf 16S rRNA gene sequences shared >99% identity, has been previously reported (Brinson and Messick, 2001). However, phylogenetic studies based on the RNase P RNA gene showed that Mhc is a distinct species infecting the dog (do Nascimento et al., 2012). Unfortunately further phylogenetic analysis was not possible in the current study as insufficient haemoplasma DNA was available from these dogs for further amplification studies.

The current study represents the first report of the prevalence of canine haemoplasmas in dogs from Nigeria together with near complete 16S rRNA gene sequencing for Mhc in a number of dogs. The latter found very little evidence of genetic variability with other Mhc species.

1.5 Conclusion

In conclusion, the overall prevalence of haemoplasma species from dogs in Nigeria was comparable to other countries with a similar warm climate. Close genetic identity exists among 16S rRNA gene sequences from worldwide Mhc species. No association with anaemia reinforces the likely low pathogenicity of the canine haemoplasma species.

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Conflict of interest

None of the authors of this article has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

References

- Aquino, L.C., Hicks, C.A., Scalon, M.C., Lima, M.G., Lemos, M.D., Paludo, G.R., Helps, C.R., Tasker, S., 2014. Prevalence and phylogenetic analysis of haemoplasmas from cats infected with multiple species. *J Microbiol Methods* 107C, 189-196.
- Barker, E.N., Langton, D.A., Helps, C.R., Brown, G., Malik, R., Shaw, S.E., Tasker, S., 2012. Haemoparasites of free-roaming dogs associated with several remote Aboriginal communities in Australia. *BMC Vet Res* 8, 55.
- Barker, E.N., Tasker, S., Day, M.J., Warman, S.M., Woolley, K., Birtles, R., Georges, K.C., Ezeokoli, C.D., Newaj-Fyzul, A., Campbell, M.D., Sparagano, O.A., Cleaveland, S., Helps, C.R., 2010. Development and use of real-time PCR to detect and quantify *Mycoplasma haemocanis* and "*Candidatus Mycoplasma haematoparvum*" in dogs. *Vet Microbiol* 140, 167-170.
- Bauer N., Balzer H.J., Thure S., Moritz A., 2008. Prevalence of feline haemotropic mycoplasmas in convenience samples of cats in Germany. *Journal of Feline Medicine and Surgery* 10, 252-8.
- Beall, M.J., Chandrashekar, R., Eberts, M.D., Cyr, K.E., Diniz, P.P., Mainville, C., Hegarty, B.C., Crawford, J.M., Breitschwerdt, E.B., 2008. Serological and molecular prevalence of *Borrelia burgdorferi*, *Anaplasma*

- phagocytophilum, and Ehrlichia species in dogs from Minnesota. Vector borne and zoonotic diseases 8, 455-464.
- Brinson, J.J., Messick, J.B., 2001. Use of a polymerase chain reaction assay for detection of *Haemobartonella canis* in a dog. Journal of the American Veterinary Medical Association 218, 1943-1945.
- Budreckis, D.M., Byrne, B.A., Pollard, R.E., Rebhun, R.B., Rodriguez, C.O., Jr., Skorupski, K.A., 2015. Bacterial urinary tract infections associated with transitional cell carcinoma in dogs. J Vet Intern Med 29, 828-833.
- Compton, S.M., Maggi, R.G., Breitschwerdt, E.B., 2012. Candidatus Mycoplasma haematoparvum and Mycoplasma haemocanis infections in dogs from the United States. Comp Immunol Microbiol Infect Dis 35, 557-562.
- Criado-Fornelio, A., Martinez-Marcos, A., Buling-Saraña, A., Barba-Carretero, J.C., 2003. Presence of Mycoplasma haemofelis, Mycoplasma haemominutum and piroplasmids in cats from southern Europe: a molecular study. Vet Microbiol 93, 307-317.
- do Nascimento, N.C., Santos, A.P., Guimaraes, A.M., Sanmiguel, P.J., Messick, J.B., 2012. Mycoplasma haemocanis--the canine hemoplasma and its feline counterpart in the genomic era. Vet Res 43, 66.
- Giangaspero, A., Marangi, M., Papini, R., Paoletti, B., Wijnveld, M., Jongejan, F., 2015. Theileria sp. OT3 and other tick-borne pathogens in sheep and ticks in Italy: molecular characterization and phylogeny. Ticks Tick Borne Dis 6, 75-83.
- Hetzel, N.J., Barker, E.N., Helps, C.R., Tasker, S., Arteaga, A., Barrs, V.R., Beatty, J., 2012. Prevalence of canine haemotropic mycoplasma infections in Sydney, Australia. Vet Rec 171, 126.
- Hii, S.F., Kopp, S.R., Thompson, M.F., O'Leary, C.A., Rees, R.L., Traub, R.J., 2012. Canine vector-borne disease pathogens in dogs from south-east Queensland and north-east Northern Territory. Australian Veterinary Journal 90, 130-135.
- Inokuma, H., Oyamada, M., Davoust, B., Boni, M., Dereure, J., Bucheton, B., Hammad, A., Watanabe, M., Itamoto, K., Okuda, M., Brouqui, P., 2006. Epidemiological survey of Ehrlichia canis and related species infection in dogs in eastern Sudan. Annals of the New York Academy of Sciences 1078, 461-463.
- Jensen W.A., Lappin M.R., Kamkar S., Reagan W.J., 2001. Use of a polymerase chain reaction assay to detect and differentiate two strains of *Haemobartonella felis* in naturally infected cats. American Journal of Veterinary Research 62, 604-8.
- Kamani, J., Baneth, G., Mumcuoglu, K.Y., Waziri, N.E., Eyal, O., Guthmann, Y., Harrus, S., 2013. Molecular detection and characterization of tick-borne pathogens in dogs and ticks from Nigeria. PLoS neglected tropical diseases 7, e2108.
- Kemming, G., Messick, J.B., Mueller, W., Enders, G., Meisner, F., Muenzing, S., Kisch-Wedel, H., Schropp, A., Wojtczyk, C., Packert, K., Messmer, K., Thein, E., 2004a. Can we continue research in splenectomized dogs? Mycoplasma haemocanis: old problem--new insight. European surgical research. Europäische chirurgische Forschung. Recherches chirurgicales europeennes 36, 198-205.

- Kemming, G.I., Messick, J.B., Enders, G., Boros, M., Lorenz, B., Muenzing, S., Kisch-Wedel, H., Mueller, W., Hahmann-Mueller, A., Messmer, K., Thein, E., 2004b. *Mycoplasma haemocanis* infection - a kennel disease? *Comparative Medicine* 54, 404-409.
- Kenny, M.J., Shaw, S.E., Beugnet, F., Tasker, S., 2004. Demonstration of two distinct hemotropic mycoplasmas in French dogs. *J Clin Microbiol* 42, 5397-5399.
- Lobetti, R.G., Joubert, K.E., Picard, J., Carstens, J., Pretorius, E., 2002. Bacterial colonization of intravenous catheters in young dogs suspected to have parvoviral enteritis. *J Am Vet Med Assoc* 220, 1321-1324.
- Messick, J.B., 2004. Hemotropic mycoplasmas (hemoplasmas): a review and new insights into pathogenic potential. *Vet Clin Pathol* 33, 2-13.
- Messick, J.B., Walker, P.G., Raphael, W., Berent, L., Shi, X., 2002. 'Candidatus mycoplasma haemodidelphidis' sp. nov., 'Candidatus mycoplasma haemolamae' sp. nov. and *Mycoplasma haemocanis* comb. nov., haemotropic parasites from a naturally infected opossum (*Didelphis virginiana*), alpaca (*Lama pacos*) and dog (*Canis familiaris*): phylogenetic and secondary structural relatedness of their 16S rRNA genes to other mycoplasmas. *Int J Syst Evol Microbiol* 52, 693-698.
- Novacco, M., Meli, M.L., Gentilini, F., Marsilio, F., Ceci, C., Pennisi, M.G., Lombardo, G., Lloret, A., Santos, L., Carrapiço, T., Willi, B., Wolf, G., Lutz, H., Hofmann-Lehmann, R., 2010. Prevalence and geographical distribution of canine hemotropic mycoplasma infections in Mediterranean countries and analysis of risk factors for infection. *Vet Microbiol* 142, 276-284.
- Ogo, N.I., de Mera, I.G., Galindo, R.C., Okubanjo, O.O., Inuwa, H.M., Agbede, R.I., Torina, A., Alongi, A., Vicente, J., Gortazar, C., de la Fuente, J., 2012. Molecular identification of tick-borne pathogens in Nigerian ticks. *Veterinary parasitology* 187, 572-577.
- Perez, C., Fujii, Y., Fauls, M., Hummel, J., Breitschwerdt, E., 2011. Fatal aortic endocarditis associated with community-acquired *Serratia marcescens* infection in a dog. *J Am Anim Hosp Assoc* 47, 133-137.
- Pitulle, C., Pace, N.R., 1999. T-cloning vector for plasmid-based 16S rDNA analysis. *Biotechniques* 26, 222-224.
- Reye, A.L., Arinola, O.G., Hubschen, J.M., Muller, C.P., 2012. Pathogen prevalence in ticks collected from the vegetation and livestock in Nigeria. *Applied and environmental microbiology* 78, 2562-2568.
- Roura, X., Peters, I.R., Altet, L., Tabar, M.D., Barker, E.N., Planellas, M., Helps, C.R., Francino, O., Shaw, S.E., Tasker, S., 2010. Prevalence of hemotropic mycoplasmas in healthy and unhealthy cats and dogs in Spain. *J Vet Diagn Invest* 22, 270-274.
- Sasaki, M., Ohta, K., Matsuu, A., Hirata, H., Ikadai, H., Oyamada, T., 2008. A molecular survey of *Mycoplasma haemocanis* in dogs and foxes in Aomori Prefecture, Japan. *The Journal of Protozoology Research* 18, 57-60.
- Sykes, J.E., Ball, L.M., Bailiff, N.L., Fry, M.M., 2005. 'Candidatus *Mycoplasma haematoparvum*', a novel small haemotropic mycoplasma from a dog. *Int J Syst Evol Microbiol* 55, 27-30.
- Tasker, S., Binns, S.H., Day M.J., Gruffydd-Jones T.J., Harbour D.A., Helps C.R., Jensen W.A., Olver C.S., Lappin M.R., 2003a. Use of a PCR assay to assess the prevalence and risk factors for *Mycoplasma haemofelis* and

- '*Candidatus Mycoplasma haemominutum*' in cats in the United Kingdom. *Veterinary Record* 152, 193–8.
- Tasker, S., Helps, C.R., Day, M.J., Harbour, D.A., Shaw, S.E., Harrus, S., Baneth, G., Lobetti, R.G., Malik, R., Beauvais, J.P., Belford, C.R., Gruffydd-Jones, T.J., 2003b. Phylogenetic analysis of hemoplasma species: an international study. *J Clin Microbiol* 41, 3877-3880.
- Tasker, S., Peters, I.R., Mumford, A.D., Day, M.J., Gruffydd-Jones, T.J., Day, S., Pretorius, A.M., Birtles, R.J., Helps, C.R., Neimark, H., 2010. Investigation of human haemotropic *Mycoplasma* infections using a novel generic haemoplasma qPCR assay on blood samples and blood smears. *Journal of medical microbiology* 59, 1285-1292.
- Tennant, K.V., Barker, E.N., Polizopoulou, Z., Helps, C.R., Tasker, S., 2011. Real-time quantitative polymerase chain reaction detection of haemoplasmas in healthy and unhealthy dogs from Central Macedonia, Greece. *J Small Anim Pract* 52, 645-649.
- Varanat, M., Maggi, R.G., Linder, K.E., Breitschwerdt, E.B., 2011. Molecular prevalence of *Bartonella*, *Babesia*, and hemotropic *Mycoplasma* sp. in dogs with splenic disease. *Journal of Veterinary Internal Medicine* 25, 1284-1291.
- Warman, S.M., Helps, C.R., Barker, E.N., Day, S., Sturgess, K., Day, M.J., Tasker, S., 2010. Haemoplasma infection is not a common cause of canine immune-mediated haemolytic anaemia in the UK. *J Small Anim Pract* 51, 534-539.
- Wengi, N., Willi, B., Boretti, F.S., Cattori, V., Riond, B., Meli, M.L., Reusch, C.E., Lutz, H., Hofmann-Lehmann, R., 2008. Real-time PCR-based prevalence study, infection follow-up and molecular characterization of canine hemotropic mycoplasmas. *Vet Microbiol* 126, 132-141.
- Westfall D.S., Jensen W.A., Reagan W.J., Radecki S.V., Lappin M.R., 2001. Inoculation of two genotypes of *Hemobartonella felis* (California and Ohio variants) to induce infection in cats and the response to treatment with azithromycin. *American Journal of Veterinary Research* 62, 687–91.

Table 1. Characteristics of Nigerian dogs and haemoplasma PCR status

Variable	No. of dogs (%) (for categorical variables) or Median (range) (for continuous variables)						P value
	Total N (%)	Generic haemoplasma qPCR positive	Generic haemoplasma qPCR negative	P value	Mhc qPCR positive	Mhc qPCR negative	
Gender	245 (100%)						
Male	103 (42.3%)	21 (50%)	82 (40.7%)	0.173	12 (66.7%)	91 (40.6%)	>0.05
Female	142 (57.7%)	21 (50%)	121 (59.3%)		6 (33.3%)	136 (59.4%)	
Breed	210 (100%)						
Purebred	37 (17.6%)	3 (7.7%)	34 (19.8%)	0.071	1 (5.6%)	36 (18.8%)	0.20
Crossbred	173 (82.3%)	36 (92.3%)	137 (80.2%)		17 (94.4%)	156 (81.2%)	
Privately owned or breeding kennel origin	245 (100%)						
Privately owned	218 (89%)	38 (90.5%)	180 (88.6%)	0.312	18 (100%)	200 (89%)	0.38
Breeding kennel	27 (11%)	4 (9.5%)	23 (11.3%)		0	27 (11%)	
Clinical status	245 (100%)						
Healthy	85 (34.6%)	13 (31%)	72 (35.3%)	0.722	4 (22.2%)	81 (35.5%)	0.31
Sick	160 (65.4%)	29 (69%)	131 (64.7%)		14 (77.8%)	146 (64.5%)	
Presence of ticks*	245 (100%)						
Yes	135 (55.1%)	29 (69%)	106 (52.5%)	0.061	13 (72.2%)	122 (53.9%)	0.14
No	110 (44.9%)	13 (31%)	97 (47.5%)		5 (27.8%)	105 (46.1%)	
Ectoparasite prophylaxis	245 (100%)						
Yes	193 (78.5%)	31 (73.8%)	162 (79.4%)	0.415	11 (61.1%)	182 (79.8%)	0.076
No	52 (21.5%)	11 (26.2%)	41 (20.6%)		7 (38.9%)	45 (20.2%)	
Age (years)		1 (2-6)	2 (2-9)	0.320	1 (2-4)	2 (2-9)	0.188
Packed Cell Volume (PCV) (%)		38 (14-57)	43 (10-62)	0.131	36 (15-52)	43 (10-62)	0.095
Presence of anaemia							
Anaemic	70 (30.7%)	13 (29.5%)	57 (30.2%)	0.700	5 (31.3%)	64 (30.8%)	1.0
Non anaemic	158 (69.3%)	31 (70.5%)	132 (69.8%)		11 (68.8%)	144 (69.2%)	

§ P value indicates χ^2 test for categorical variables, Mann-Whitney U test for continuous variables.

* All ticks found were identified as *Rhipicephalus sanguineus*

Figure 1. Phylogenetic relations of previously published haemoplasma species 16S rRNA gene sequences and Mhc sequences from Nigerian dogs. Bootstrap percentage values are given at the nodes of the phylogenetic tree (only values >700 are shown). Evolutionary distances are to the scales shown. *Clostridium* spp. was used as an out-group. GenBank accession numbers are indicated in the figure. Mhc sequences (F2, G7, H7 and E8. GenBank nucleotide accession numbers: KP715857, KP715858, KP715859 and KP715860 respectively) were generated in the current study. Mhc = *Mycoplasma haemocanis*, CMhp = “*Candidatus Mycoplasma haematoparvum*”, Mhf = *Mycoplasma haemofelis*, CMhm = “*Candidatus Mycoplasma haemominutum*”.

