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Short communication

Molecular detection and characterization of zoonotic *Anaplasma* species in domestic dogs in Lusaka, Zambia

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ABSTRACT

Although tick-borne pathogens, *Anaplasma platys* and *Anaplasma phagocytophilum* are recognized as zoonotic agents associated with appreciable morbidity and mortality in dogs and humans worldwide, there is limited information on these infections in many African countries, including Zambia. The purpose of this study was to detect, identify and phylogenetically characterize *Anaplasma* species from dogs in Chilanga District in Lusaka Province, Zambia. A total of 301 blood samples were collected from apparently healthy and semi-confined dogs. Initial screening by polymerase chain reaction with specific primers targeting the 16S *rRNA* gene of *Anaplasma* species revealed that 9% (27/301) of our samples were positive. Subsequent sequence and phylogenetic analysis of a longer fragment of the 16S *rRNA* and citrate synthase (*gltA*) genes of four positive samples showed the presence of *A. platys* and an *Anaplasma* species, which was closely related to those detected in dogs in South Africa. This is the first report on molecular identification and characterization of canine-associated zoonotic *Anaplasma* species in Zambia.

1. Introduction

Anaplasmosis, caused by species of the genus *Anaplasma*, in the family Anaplasmatacae, is an emerging infectious disease affecting both animals and humans (Demma et al., 2005; Nicholson et al., 2010). Within the genus, only *Anaplasma platys* and *Anaplasma phagocytophilum* are known to be zoonotic. The dog is considered the natural host for *A. platys* whilst both dogs and humans are considered accidental hosts for *A. phagocytophilum*, which naturally infects ruminant and rodent species (Nicholson et al., 2010).

A. phagocytophilum, transmitted by *Ixodes* ticks, is considered to be an emerging zoonotic pathogen with many reports in USA, Europe, Russia, North Africa and sub-Saharan Africa (Dumler et al., 2001; Djiba et al., 2013). There is genetic diversity within the *A. phagocytophilum* species, with the different variants likely having different ecological cycles (Dugat et al., 2015). In South Africa, there have been reports of an *Anaplasma* species (detected in dogs), which is genetically closely related to *A. phagocytophilum* and is suspected to be transmitted by *Rhipicephalus sanguineus* sensu lato (Inokuma et al., 2005; Kolo et al., 2016).

A. platys is suspected to be transmitted by the *R. sanguineus* group of ticks (Inokuma et al., 2000), which are widely distributed in Africa (Walker et al., 2003). Recently there have been confirmed cases of human infections, making this pathogen a newly emergent zoonotic agent (Maggi et al., 2013; Arraga-Alvarado et al., 2014).

The long-standing close relationship between humans and dogs serves as a risk factor for interspecies transmission of pathogens between these mammals (Otranto et al., 2009). Due to their outdoor

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nature and close association with humans, dogs can act as good sentinels for human tick-borne infections (Hornok et al., 2013).

There has been one report of *A. phagocytophilum* infection of non-human primates in Zambia (Nakayima et al., 2014), with no information available on the prevalence in dogs, as highlighted by the absence of such reports in a recent review on the role of dogs in the transmission of zoonotic parasites in Zambia (Siwila, 2016). The purpose of this study was thus to screen and genetically characterize zoonotic *Anaplasma* species associated with domestic dogs in Lusaka Province based on the 16S ribosomal ribonucleic acid (*rRNA*) and the citrate synthase (*gltA*) genes.

2. Materials and methods

2.1. Sample collection

In the period between February and May 2016, a total of 301 blood samples were collected from dogs in Chilanga District in Lusaka Province. The sampling points used in the study were three (3) veterinary camps namely, Chilongolo (17), Mapepe (136) and Mwembeshi (148).

2.2. Molecular detection of Anaplasma species

DNA was extracted from the collected blood samples using the QIAmp DNA extraction mini kit (Qiagen) according to the manufacturer's recommendations. For molecular screening of *Anaplasma* species, the extracted DNA was used in polymerase chain reaction (PCR) to amplify a 250-bp region of the 16S *rRNA* gene. PCR was conducted using the Dream Taq™ DNA polymerase kit (Thermo Fisher Scientific Inc.) following the manufacturer's protocol. The primers EHR 521 (5′-TGTAGGCGGTTCGGTAAGTTAAAG-3′) and EHR 747 (5′-GCACTCATCGTTTACAGCGTG-3′) and thermal cycling conditions used were as previously described (Hodzic et al., 1998). PCR products were visualized under UV light following electrophoresis on 1% agarose gel stained with ethidium bromide.

2.3. Amplification, sequencing and phylogenetic analysis of the 16S rRNA and gltA genes

From the samples that showed the expected amplicon in the first PCR screening assay, four (sample 72–Mapepe, sample 99–Mapepe, sample 166–Mwembeshi, sample 181–Mwembeshi) were randomly selected for sequencing and phylogenetic analysis of a near full-length fragment of the 16S *rRNA* and part of the *gltA* genes. Amplification of the near full-length region of the16S *rRNA* gene was conducted using the primer pair FD1 (5′-CCGAATTCGTCGCAACAGAGTTTGATCC TGGCTCAG-3′) and Reverse primer B (5′-CCCGGGATCCAAGCTT GATCCTTCTGCAGGTTCACCTAC-3′) (Allsopp and Allsopp, 2001). Alternatively, two sets of primer pairs, FD1/EHR 747 and EHR 521/Reverse primer B were used to amplify either an approximately 800 or 900-bp region of the 16S *rRNA* gene, respectively. The *gltA* gene was amplified using the primer pair F4B (5′-CCGGGTTTTATGTCTACTGC-3′) and HG 1085R (5′-ACTATACCKGAGTAAAAGTC-3′), which gives a PCR product of about 950-bp (Inokuma et al., 2001). Also, for samples that showed low DNA yields, the *gltA* gene was amplified using the primer pair F1b: (5′-GATCATGARCARAATGCTTC-3′) and HG 1085R, which produced an amplicon of about 430-bp.

PCR products were purified from agarose gels using a Wizard® SV Gel and Clean-Up System (Promega) following the manufacturer's recommendations. Purified DNA was then sequenced directly using a BigDye Terminator Cycle Sequencing Ready Reaction Kit V3.1 (Applied Biosystems). Ethanol/EDTA/sodium acetate precipitation was then conducted on products obtained from the cycle sequencing reaction. These were separated on a 3130 Genetic Analyzer (Applied Biosystems). Nucleotide sequences were assembled and edited using GENETYX ATGC software, version 7.5.1 (GENETYX Co.,Tokyo, Japan). The sequences obtained were deposited in GenBank and were assigned accession numbers LC269820–LC269827.

For phylogenetic analysis, the sequences obtained in the present study were analyzed along with reference sequences retrieved from GenBank. The evolutionary relationships were inferred using the Neighbor-Joining method implemented in MEGA 6 software (Tamura et al., 2013). The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) and genetic tree topological support was assessed using the bootstrap method (1, 000 replicates).

2.4. Compliance with ethical standards

Ethical approval for this study was granted by the University of Zambia Biomedical Research and Ethics Committee (Reference number 009-09-15). Only dogs whose owners agreed to collect blood samples were included in the study.

3. Results

3.1. Prevalence of Anaplasma species in dogs

Of the 301 samples that were subjected to PCR for the detection of *Anaplasma* species 9% of our samples tested positive (27/301) [95% CI: 6.0–12.8%]. When analyzed according to the area of sampling, the obtained prevalences were 11.0% in Mapepe and 8.1% in Mwembeshi. No dogs were found positive for *Anaplasma* species in Chilongolo.

3.2. Sequence and phylogenetic analysis of the 16S rRNA and gltA genes

Of the four samples that were selected for sequencing, three (72, 99 & 166) shared 100% nucleotide sequence identity across the 1487-bp region of the 16S *rRNA* gene obtained. On sequence comparison with the basic local alignment search tool (BLAST) available from GenBank, the 16S *rRNA* nucleotide sequence of these samples was highly similar (100% sequence identity) to that of *A. platys* (accession no. EF139459), which was detected in a dog in Thailand (Pinyoowong et al., 2008). In contrast, the 16S *rRNA* nucleotide sequence (1416-bp) of the fourth sample (181) showed 99.6% similarity to *Anaplasma* sp. 'South Africa dog-1108′ (accession no. AY570538), found in South Africa (Inokuma et al., 2005). Sequence analysis of part of the *gltA* genes of the four samples revealed a similar trend, with three of them (72, 99 & 166) giving an identical 950-bp sequence that showed a 100% similarity to *A. platys* (accession no. KR011928) detected in a tick in China (Li et al., 2015). On the other hand, the *gltA* partial nucleotide sequence (427-bp) of the fourth sample (181) showed a 99.6% sequence identity to *Anaplasma* sp. 'South Africa dog-1108′ (accession no. AY570541).On phylogenetic analysis, whereas the 16S *rRNA* nucleotide sequence of three samples characterized in this study clustered with those of *A. platys* strains detected in various countries, that of one sample was closely related to three *Anaplasma* sp. detected in South African dogs (Fig. 1). Phylogenetic analysis based on the *gltA* gene followed a similar clustering pattern, with high bootstrap values (Fig. 2). To further ascertain the genetic relatedness between *Anaplasma* species identified in this study and that of *A. phagocytophilum* detected from non-human primates in Zambia previously (Nakayima et al., 2014), phylogenetic analysis based on a shorter fragment of the 16S *rRNA* gene was conducted. Indeed, the analysis confirmed the presence of at least three *Anaplasma* species [i.e. *A. phagocytophilum*, *A. platys* and *Anaplasma.* sp. (closely related to those detected in South African dogs) in Zambia (Supplementary Fig. S1).

4. Discussion

The main aim of our study was to screen for the presence of zoonotic