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DETECTION AND MOLECULAR CHARACTERISATION OF *EHRLICHIA CANIS* IN NATURALLY INFECTED DOGS IN SOUTH WEST NIGERIA

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Canine ehrlichiosis is an important tick-borne rickettsial disease mainly caused by *Ehrlichia canis*. This study aimed to detect and characterise *E. canis* in dogs in Abeokuta, Nigeria by microscopy and nested PCR. Blood samples were collected from 205 dogs, thin smears were made, field-stained, and DNA was extracted from the blood samples. A partial region of the 16S rRNA gene was amplified by polymerase chain reaction (PCR) and sequenced unidirectionally. Ehrlichial morulae were detected in three dogs (1.5%). The PCR test revealed that 47 dogs (22.9%) were positive for *E. canis*. The lengths of the sequences obtained range from 374 bp to 376 bp with an average G-C content of 37% and 98–99% homology with the reference sequences in GenBank. The aligned autochthonous sequences were less polymorphic. The phylogenetic analysis separated sequences reported previously in Nigeria from the autochthonous sequences. The present work shows that the strain of *E. canis* detected in the study area is genetically different from those reported in the northern part of Nigeria and more closely related to sequences from Brazil and India.

Key words: *Ehrlichia canis*, dog, nested PCR, Abeokuta, 16s rRNA, prevalence

Canine ehrlichiosis is a rickettsial bacteria disease of dogs that has worldwide distribution (Engvall et al., 1996; McBride et al., 1996; Souza et al., 2010; Fourie et al., 2013; Milanjeet et al., 2014; Cardoso et al., 2016; Cicuttin et al., 2016; Kaewmongkol et al., 2016). Two forms of the disease are known in dogs and humans. In dogs, these include the monocytic form which is caused by *Ehrlichia canis* (*E. canis*) and the granulocytic form that is caused by *E. ewingii.* They are transmitted by the brown dog tick (*Rhipicephalus sanguineus*) and the lone star tick called *Amblyomma americanum*, respectively. In humans, the monocytic and granulocytic forms are caused by *E. chaffeensis* and *E. ewingii*, re-

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spectively, though *Anaplasma phagocytophilum* (formerly *E. equi*) and *E. canis* have also been reported in human ehrlichiosis (Dumler et al., 2005; Perez et al., 2006).

The clinical manifestation of canine ehrlichiosis varies depending on the immune status, the breeds of dogs infected and the strain of infecting *Ehrlichia* species. While *E. canis* is generally believed to cause more severe febrile condition in dogs, *E. ewingii* infection is less severe (Liddell et al., 2003). Signs of the disease in dogs may include but are not limited to fever, anaemia, haemorrhages, oedema, lymphadenopathy, thrombocytopenia, anorexia and weight loss (Cicuttin et al., 2016; Lauzi et al., 2016). The organism is characterised by the presence of intracytoplasmic inclusion bodies (morulae) in the monocytes/granulocytes of infected dogs, which can be detected by microscopic examination of peripheral blood (Dagnone et al., 2009). Other techniques such as serology can be used, though with its own limitation to differentiate between previous and ongoing infection of dogs (Cicuttin et al., 2016). In recent years, polymerase chain reaction (PCR) has been found to be more sensitive in the detection of *Ehrlichia* DNA in the blood of infected animals.

The prevalence and molecular characteristics of *Ehrlichia canis* have been studied extensively around the world (Suksawat et al., 2001; Unver et al., 2001; Vinasco et al., 2007; Aguiar et al., 2008; Harrus et al., 2011; Nazari et al., 2013; Milanjeet et al., 2014; Cardoso et al., 2016). One of these studies has revealed that more than one strain may exist in a region (Vinasco et al., 2007). In Nigeria, Kamani et al. (2013) shed light on the molecular characteristics of *E. canis* detected in three northern states of the country. Hence, this study characterised *E. canis* detected in naturally infected dogs in the western part of Nigeria and compared their sequences with those detected elsewhere in the world and those reported by Kamani et al. (2013) in Nigeria.

Materials and methods

Study area and sampled population

The study was carried out in Abeokuta metropolis, Ogun State, Nigeria. Ogun state is bounded by Lagos State to the south, Oyo and Osun States to the north, Ondo State to the east and the Republic of Benin to the west. A total of 205 dogs consisting local and exotic breeds were randomly sampled mainly from the dogs presented to the Veterinary Teaching Hospital, Federal University of Agriculture, Abeokuta and various veterinary hospitals in the city. The dogs were grouped to young (≤ 1 year) and adult (≥ 1 year) and their sexes were also recorded. The dogs sampled were predominantly exotic breeds, which included Alsatian (102), Rottweiler (18), Boeboel (44), unidentified breed (10) and mongrel (31). Pregnant dogs and those with a history of recent medication (such as

antibiotic therapy and anti-protozoan therapy) within the last four weeks were excluded from the study.

A blood sample was collected from each dog using cephalic venipuncture into tubes containing ethylenediamine tetraacetic acid (EDTA) as anticoagulant. The collected blood samples were transported on ice packs to the laboratory. Thin blood smears were made from each sample collected as well as smears of the buffy coat from the spun capillary tubes, fixed with 98% methanol (Dudal diesel, Madison, USA) and then reverse stained with Field stain A and B (Biolab Diagnostics, USA). The slides were examined under an oil immersion objective of a light microscope (Olympus CX21, China). Sample in which intracytoplasmic inclusion bodies were demonstrated were adjudged positive for *Ehrlichia* spp. (Engvall et al., 1996). Blood samples not processed immediately for DNA extraction were stored at –20 °C until use.

DNA extraction and PCR assay for E. canis

Genomic DNA was extracted from whole blood using a commercially available DNA extraction kit (Quick-gDNA MicroPrep, Zymo Research Corporation, Irvine, USA) following the manufacturer's protocol, and the eluted DNA was stored at –20 °C until use as previously described (Takeet et al., 2013). Nested PCR that targeted partial regions of the 16S rRNA gene was carried out using the primers listed (Table 1). The primary and the nested reactions were carried out using primer sets ECC & ECB and ECAN5 & HE3 (Table 1), respectively. The primary reaction primers target 478 bp of *Ehrlichia* spp. while the nested reaction primers are specific for the amplification of 398 bp of *E. canis*.

Table 1

The sequences of oligonucleotides used in the nested PCR of *Ehrlichia canis* detection in naturally infected dogs in Abeokuta, Nigeria

Primer set	Primer sequence (5′ – 3′)	Reference	Band size
ECC	AGAACGAACGCTGGCGGCAAGC		
ECB	CGTATTACCGCGGCT GCTGGCA	Murphy et al. (1998)	478
ECAN5	CAATTATTTATAGCCTCTGGCTCTGGCTATAGG		
HE3	TATAGGT ACCGTCATTATCTTCCCTAT	Milanjeet et al. (2014)	398

Amplification was carried out in 20 µl final volume containing 10 µl of 2 × mastermix (Syd Labs, Inc., USA), 8 µl of nuclease-free water and 0.5 µl (about 10 pmol) each of forward and reverse primer, and 1 µl (about 50 ng) of the genomic DNA in a personal cycler (Biorad, USA). The cycling conditions for the primary PCR reactions were: 94 °C for 3 min initial denaturation, 30 cycles

of 94 °C for 60 s, 59.5 °C for 60 s and 72 °C for 120 s with final extension at 72 °C for 8 min. The nested PCR was carried out also in 20 µl final volume containing 10 µl of 2 × mastermix (Syd Labs, Inc., USA), 8 µl of nuclease-free water and 0.5 µl (about 10 pmol) each of forward and reverse primer, and 1 µl of the primary PCR product as the template. The PCR conditions were: initial denaturation at 94 °C for 3 min, followed by two amplification steps: the first step consisted of three cycles of 94 °C for 1 min, 55 °C for 2 min, 72 °C for 1.5 min, while the second step consisted of 37 cycles of 94 °C for 1 min, 55 °C for 2 min, 72 °C for 1.5 min with final extension at 72 °C for 8 min. In every amplification, a negative control was included using nuclease-free water instead of DNA.

The PCR products were visualised in 1.5% agarose gel that was stained with ethidium bromide (Amresco, USA) in 1 × TAE buffer (40 mM Tris acetate and 1 mM EDTA) and electrophoresed at 80 V for 60 min along with 10 µl of 100 bp DNA ladder (BioExpress, Kaysville, UT, USA), and then visualised with UV transilluminator (Spectroline, USA). Gel picture was captured using a hand-held camera (Samsung WB50F, China).

Sequence and phylogenetic analyses

To confirm the PCR assay results, four PCR products from the samples that showed expected band size were sequenced directly using Big Dye Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) in the Core Lab of Cornell University Central Laboratory, USA using forward primer ECAN5. Search for homologous sequences in the GenBank was performed using BLASTn (www.ncbi.nlm.nih.gov/BLAST). The obtained sequences were viewed, aligned and compared using BioEdit® (version 7.0.9.0) software. The sequences were also aligned with other published *E. canis*16S rRNA sequences of dogs from around the world. Phylogenetic analysis of the sequences, including those obtained from GenBank were constructed using Maximum Composite Likelihood and Neighbour-Joining methods (Tamura et al., 2004) with a bootstrap confidence interval of 1000 replicates in Molecular Evolutionary Genetic Analysis (MEGA 5.0). The sequences obtained from this study have been deposited in GenBank under the accession number KY434110, KY434111, KY434112 and KY434113.

Data analysis

The data were summarised using descriptive statistics. The prevalences of *E. canis* obtained by PCR and microscopy as well as the prevalences within ages, sexes, and breeds of dogs were compared using Student's *t*-test. $P < 0.05$ was considered statistically significant. The analysis was carried out in SPSS version 19 software.

Ethical considerations

Ethical approval (No. FUNAAB/COLVET/CREC/009/17) was obtained from the Ethical Committee of the College of Veterinary Medicine, Federal University of Agriculture Abeokuta, Nigeria, before commencing the project.

Results

Detection of E. canis *by microscopy and PCR*

Out of the 205 dogs screened by microscopy, ehrlichial morulae were detected in the blood (two monocytes and one neutrophil) of three (1.5%) dogs (Fig. 1). Gel electrophoresis of the nested PCR products revealed 47 (22.9%) of the tested dogs to have a band size of about 398 bp which corresponded to the expected fragment size of the amplified partial region of the 16S rRNA gene of *E. canis*. The three samples that were positive by microscopy were also positive by PCR. Among the screened dogs, there were more females (103) than males (102). The occurrence of *E. canis* in mongrels (n = 2, 6.5%), was significantly ($P < 0.05$) lower compared to exotic breeds (n = 45, 25.9%), but the occurrence in female dogs (23.3%) was not significantly different from that in males (22.5%) ($X^2 = 0.02$, $P = 0.09$). As regards age, the occurrence of *E. canis* in dogs less than 12 months old (26%) was not significantly different from that in dogs older than one year (21%) ($X^2 = 0.068$, $P = 0.4$).

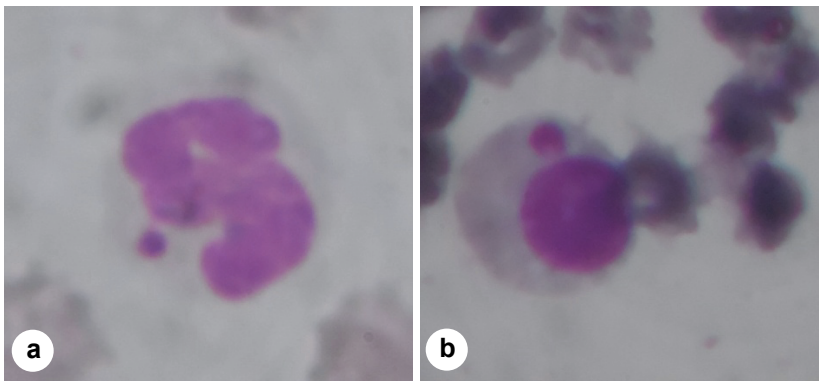

Fig. 1a–b. Microscopic screening of *Ehrlichia canis* in naturally infected dogs using a field-stained blood smear. a: Neutrophil infected by the morulae of *E. canis*; b: *E. canis* in an infected monocyte. Plates (a and b) were re-magnified at × 12 of a Samsung (WB50F) camera

Sequences and phylogenetic analysis

The lengths of the sequences obtained from this study range from 374 to 376 bp with an average G-C content of 37%. Homology search revealed that se-

quences obtained from this study had 98–9% homology with the sequences deposited in GenBank (DQ379966, DQ228505, KP844663, KF878949, KX180945, KX898137, LC057655, M73226, NR118741, JX893522, U54805, KP642754, KJ995842, KJ995837 and KF972451). The aligned autochthonous sequences were less polymorphic with sequences KY434110 showing insertion (C) at point 994 and sequences KY434110 and KY434111 showing alterations (A → C) at point 947. When these sequences were aligned with those previously reported in Nigeria by Kamani et al. (2013), there were considerable variations characterised by complete deletion (at points 926, 962 and 1045), insertion (at points 886, 894, 944, 994 and 1024) and alterations at 912 (T→A), 915 (A→C), 919–921 (AGT→GCT) and 941–942 (TA→AC).

The phylogenetic tree inferred by Maximum Likelihood and Neighbour Joining methods showed the same topologies (Figs 2 and 3). The trees clearly separated the sequences reported previously in Nigeria from the autochthonous sequences into two different clades. The sequences from this study clustered with those from India, Brazil and Angola, while those previously reported in Nigeria clustered among those from the USA, Turkey, Switzerland, Israel, Japan, South America, and Tahiti.

Discussion

This study attempted to shed more light on the occurrence of canine ehrlichiosis and the molecular characteristics of *E. canis* detected in naturally infected dogs in South West Nigeria by microscopy and by nested PCR that targeted a partial region of the 16S rRNA gene.

The occurrence of ehrlichial morulae in 1.5% of the dogs sampled and screened by microscopy could not be compared due to the paucity of data and reports on microscopic detection of the parasite in Nigerian dogs. This may be an indication of how difficult microscopic detection of *Ehrlichia* parasites could be in the blood. The detection of morulae in both monocytes and granulocytes in this study may suggest that both granulocytic and monocytic ehrlichiosis exist among the dog population in Nigeria; hence, there is a need for a wide-scale molecular screening to ascertain which of the *Ehrlichia* species is/are available in Nigeria as those found in monocytes are generally believed to be either *E. canis* or *E. chaffeensis* and those found in granulocytes to be *E. ewingii* (Blanco and Oteo, 2002; Bowman et al., 2009). Although the infection stage was not classified in the dogs sampled in this study, the three cases in which morulae were detected by microscopy may represent acute infection as suggested by other workers (Aguero-Rosenfeld et al., 1996; Bakken et al., 1996) who posited that morulae are usually detected in blood smears only during acute febrile episodes.

The detection of *E. canis* DNA in 22.9% of the sampled population of dogs was higher than the prevalence of 12.7% reported by Kamani et al. (2013)

in the northern part of Nigeria and those reported by Nazari et al. (2013) and Lasta et al. (2013) in Malaysia and Brazil, respectively. The higher occurrence reported in this study may partly be associated with the predominance of the tick vector (*Rhipicephalus sanguineus*) of the parasite in the study area (Oke et al., 2013) and may be due to optimum environmental conditions that favour its breeding. Since we only amplified with *E. canis* specific primers, there is a need for further studies including other species-specific primers to determine whether mixed infections of these *Ehrlichia* parasites are possible or exist.

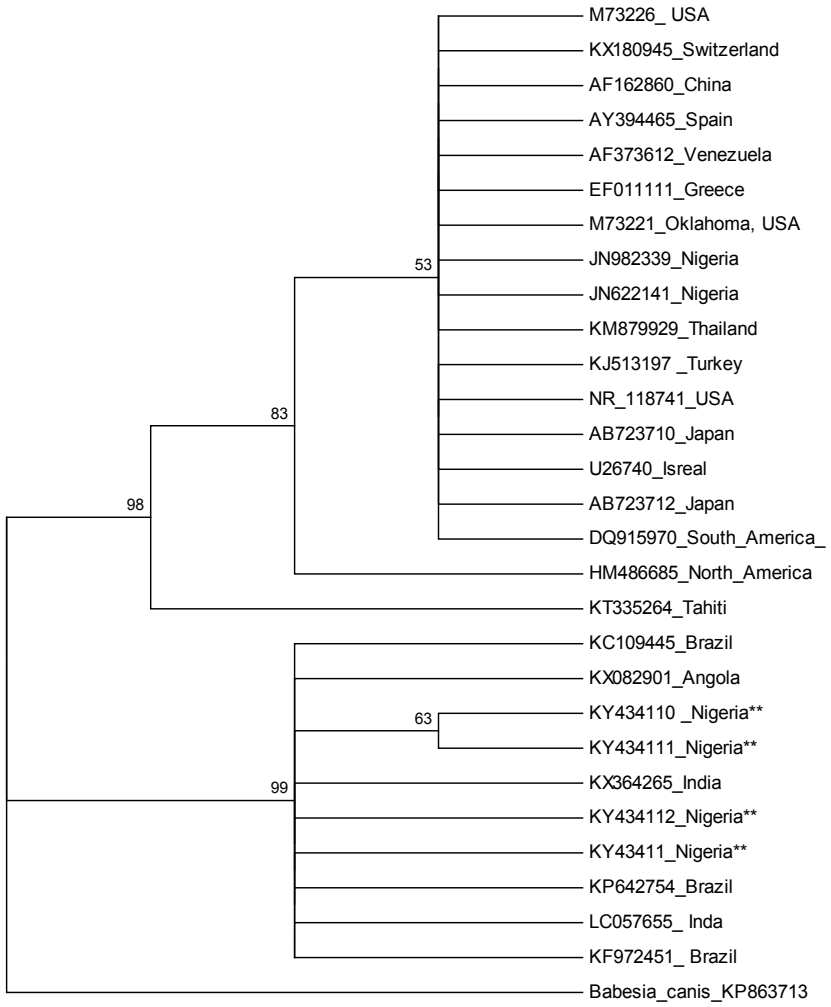

Fig. 2. Phylogenetic analysis of 16S rRNA gene partial sequences of *E. canis* isolates inferred using the Maximum Likelihood method. The percentage (50% and above) of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches

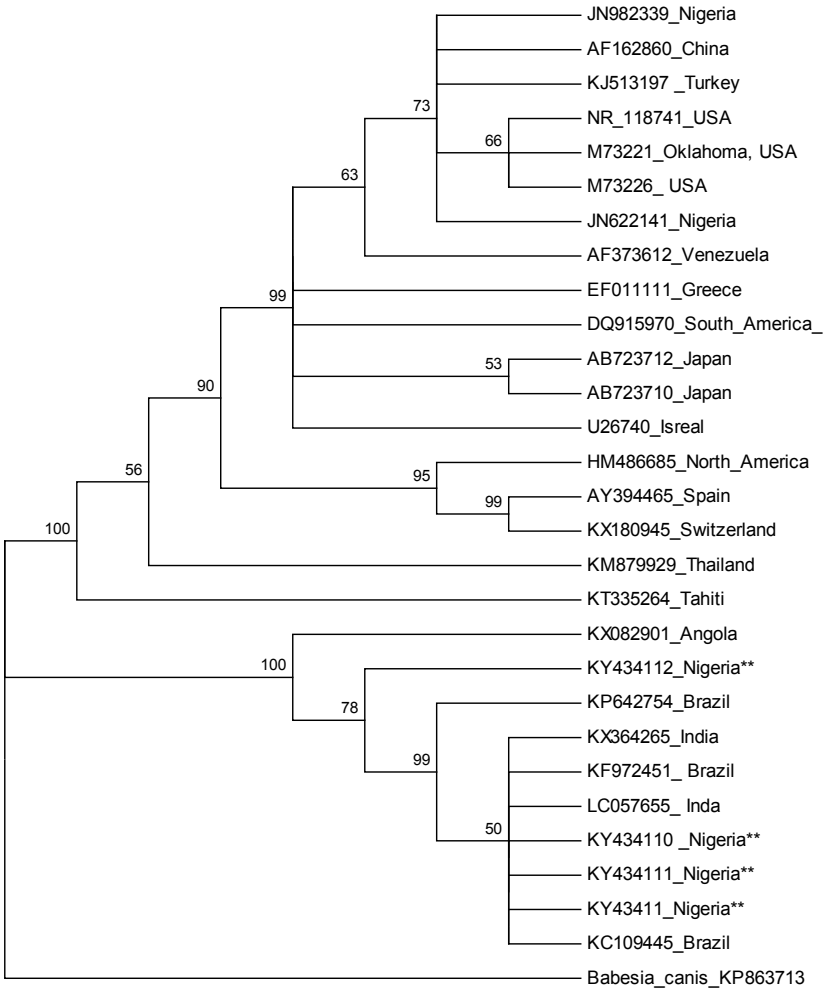

Fig. 3. Phylogenetic analysis of 16S rRNA gene partial sequences of *E. canis* isolates inferred using the Neighbour-Joining method. The percentage (50% and above) of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches

The *E. canis* sequences that were used to construct the phylogenetic trees exhibit diphyletic clades, separating the autochthonous sequences from those previously described in Nigeria. The separation may be an indication that more than one strain of *E. canis* are in circulation among the dog population in Nigeria. Clinical manifestation was not reported either in our study or in the previous study on ehrlichial infection of dogs in Nigeria; hence, further investigations on the pathogenicity of these strains in infected dogs are needed. The diphyletic clades exhibited by the autochthonous 16S rRNA partial sequences and those re-

ported previously in Nigeria are not supported by some of the previous works on the phylogenetics of *E. canis* (Vinasco et al., 2007; Vargas-Hernández et al., 2012). This could be an indication that the 16S rRNA gene of *E. canis* strains in Nigeria is more polymorphic than those reported elsewhere and this may have potential implications on the clinical outcome and management of canine ehrlichiosis in Nigeria. Generally, *E. canis* (Dawson et al., 1993; Unver et al., 2001; Perez et al., 2006), *E. chaffeensis* (Dawson et al., 1993; Chen et al., 1997; Rojas et al., 2015) and *E. ewingii* (Buller et al., 1999; Allen et al., 2014) have been reported in human ehrlichiosis around the world but not in Nigeria. This does not necessarily preclude the presence of ehrlichial infection in humans in Nigeria and, as such, the screening for such parasites should be considered in human febrile conditions.

In conclusion, this study provides molecular evidence that shows the presence of an *E. canis* strain in naturally infected dogs in Abeokuta (South West Nigeria), which is genetically different from the strain previously reported in the northern part of Nigeria and more closely related to sequences from Brazil and India.

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