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Molecular Detection of *Anaplasma phagocytophilum* in Ixodid Ticks Infesting White Fulani Cattle in Zaria and its Environs, Kaduna State, Nigeria

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ABSTRACT

Anaplasmosis, a disease caused by various species of *Anaplasma*, poses important economic constraints to animal breeders. In Zaria, molecular detection of tick-borne pathogens infecting cattle is very sketchy. Being hematophagous, ticks are capable of transmitting disease-causing agents such as viruses, bacteria and protozoa. In this investigation, a Polymerase Chain Reaction was employed to detect the presence of *Anaplasma* pathogen in the various species of tick infesting white Fulani breed of cattle in Zaria and environs. Three hundred and eighty-four (n=384) white Fulani breed of cattle of varying age and sex were sampled from 32 herds in 4 villages (Bomo, Tofu, Ungwan Dabosa and Majeru). The DNA was extracted using Qiagen commercial kit following manufacturer's instructions. Genomic DNA of the species of ticks was amplified in a semi-nested PCR targeting the 16S rRNA fragment of the *Anaplasma* spp at expected amplicon size of 711bp. Following manufacturer's directions, the amplicon was purified using a Gel Extraction Kit. To ascertain their identities and evaluate their homologues and similarity to those in the GenBank, all sequences were subject to a Basic Local Alignment Search Tool (BLAST) to determine their identities and assess their homologues and similarities to those in the GenBank. Using the Molecular Engineering Genetic Analysis, a neighbor-joining tree was created to determine the close relationship between the isolates. The findings of this study imply that all five species of ticks infesting cattle in Zaria, Kaduna State, Nigeria harbor *Anaplasma phagocytophilum* (MN044909), which poses a serious threat to both human and animal health.

Keywords: Molecular, *Anaplasma*, PCR, ticks, cattle, Zaria

INTRODUCTION

Ticks are obligate ectoparasites of mammals, reptiles and birds and are of medical and veterinary importance (Melhorn and Armstrong, 2010). Three families have been identified, but two of them are well known and of veterinary importance, *Ixodidae* (hard ticks) and *Argasidae* (soft ticks) (Sonenshine, 2005; Luqman *et al.*, 2007). The tick's bite causes discomfort and can lead to secondary infections, some species are capable of causing paralysis in animals and humans, and they also serve as vectors of a number of diseases affecting both animals and humans (Shaw, 2001; Ghosh and Najar, 2014).

Most investigations of tick-borne pathogens in cattle from Nigeria have been based on cytological examinations of blood smears and lymph node biopsies (Leefflang and Ilemobade, 1977; Saidu *et al.*, 1984; Akinboade and Dipeolu, 1984; Kamani *et al.*, 2010; Okubanjo *et al.*, 2013)

and/or on serological methods (Obi, 1978; Ajayi *et al.*, 1982; Saidu *et al.*, 1984; Akinboade and Dipeolu, 1984; Kamani *et al.*, 2010). Several molecular surveys have evaluated the existence of multiple vectors borne pathogens in specific regions including, Europe (Cardoso *et al.*, 2010), Middle East (Peleg *et al.*, 2010), Asia (Suksawat *et al.*, 2001) and Africa (Oyamada *et al.*, 2005; Kamani *et al.*, 2010; Ogo *et al.*, 2012; Lorusso *et al.*, 2013; Elelu *et al.*, 2016). Because of the similarity of clinical symptoms of vector-borne diseases, clinical examination of infected animals is not sufficient (Bilgiç *et al.*, 2013) and a range of laboratory techniques including blood smears, serology, mouse inoculation and molecular techniques are required to make a definitive diagnosis (Berlin *et al.*, 2012). Tick-borne pathogens are readily diagnosed by microscopic examination of stained blood smears, but this technique has a low sensitivity in subclinical or chronic infections (Elhaig *et al.*, 2013; Takeet *et al.*, 2013). Molecular techniques in

addition to being capable of detecting active infections, offer higher sensitivity and specificity than other diagnostic techniques and are increasingly being used (Almeria *et al.*, 2001; Elhaig *et al.*, 2013; Sharma *et al.*, 2013; Takeet *et al.*, 2013; Tran *et al.*, 2014).

Ixodid ticks play an important role in maintaining *Anaplasma* species in nature. It is evidenced that various species of *Ixodes*, *Dermacentor*, *Rhipicephalus* and *Amblyomma* genera are the main vectors of the *Anaplasma* bacteria in different regions of the world (Stafford, 2007). Anaplasmosis, a disease caused by various species of *Anaplasma*, poses important economic constraints to animal breeders (Suarez and Noh, 2011). Besides the cost of the additional veterinary care anaplasmosis causes abortion in animals, reduction of milk production, body weight and frequently leads to death (Stuen *et al.*, 2003). PCR is an efficient, simple and but expensive tool used to amplify the desired sequence or sequences of DNA into billions of identical copies (Mohammed *et al.*, 2016). To the best of our knowledge, there are few studies on the presence of *Anaplasma* pathogen affecting cattle production in Zaria. The alarming misdiagnosis of tick-borne pathogens i.e haemoparasites via microscopic detection in Nigeria is a limitation towards achieving a correct diagnosis. In the present study, a molecular detection of *Anaplasma* spp based on PCR amplification was carried out to detect for the presence of *Anaplasma* spp in tick species found on exclusively white Fulani breed of cattle in Zaria, Kaduna State, Nigeria.

MATERIALS AND METHODS

Study area

Zaria is an old commercial, administrative and academic town in Northern Nigeria. The study was carried out in four villages namely; Bomo, Tofu, Majeru and Ungwan Dabosa in Zaria environs. Zaria (11° 3' N; 7° 42' E) is located about 83km North of Kaduna, along the Kaduna-Kano highway (Mortimore, 1970; Obadiah and Shekaro, 2012). It comprises of two Local Government Areas namely; Zaria and Sabon Gari. By the existing pattern of settlement; it is made up of a natural and stable ecosystem in the Northern Guinea Savannah zone, with a discontinuous layer of sparsely distributed short trees followed by relatively continuous layers of tall, medium and short grasses (Obadiah and Shekaro, 2012). The mean annual rainfall in the area is 1100 mm lasting from May to October (816 mm/month). Mean daily temperatures during the wet season are 25°C and mean relative humidity of 72%. The dry season lasts from November to April, the mean daily temperature ranging from 14 to 36 °C and the relative humidity of 20-30% (Natala *et al.*, 2009; Obadiah and Shekaro, 2012).

Study design

Ticks were collected from Tofu and Bomo districts from Sabon Gari Local Government, Majeru and Ungwan Dabosa districts in Zaria Local Government. In each district, 8 herds were selected, and all visible adult ticks were collected from 12 randomly selected cattle varying in age and sex, all belonging to the indigenous (*Bos indicus*) White Fulani breed.

Sample size determination

In estimating the sample size for this study, 50% prevalence obtained by Obadiah and Shekaro (2012) for tick infestation on cattle in Zaria was adopted. The formula of Thrusfield (2007) was adopted for determining the sample size. The actual sample size so derived was 384.

Collection of Ticks

The entire body surface of three hundred and eighty-four white Fulani breed of cattle (n=384) was thoroughly examined. The collection was performed using blunt steel forceps, ticks were collected from different parts of the body including the neck/dewlap, eyes, ear, udder and external genitalia, inner thighs, under the tail/perineum and legs/interdigital spaces by using forceps and hand gloves. Ticks from each animal were stored separately in vials containing 70% ethanol, labeled with information of the host (i.e., sample number, age, and sex), village, and date of sampling as well as the predilection site (s) collected.

Morphological identification

Ticks identification was carried out in the Entomology Teaching and Research laboratory of the Department of Veterinary Parasitology and Entomology, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria, Nigeria. Once in the laboratory, all collected ticks were counted and identified to the genus and species level using a stereomicroscope (up to 100× magnification) and following the morphological key by Walker *et al.* (2003). Two other separate keys were also used in identifying tick species belonging to the genus *Rhipicephalus*, keys by Walker *et al.* (2000) and Madder *et al.* (2012).

Molecular identification

Generally, three ticks each from the same species were pooled to make one sample separately according to their respective species. First, each tick was washed three times in sterile phosphate-buffered saline and then stored at -20°C. The frozen ticks were mechanically crushed using pestle and mortar and transferred into 1.5ml Eppendorf tube. Digestion with proteinase K (200 µg/ml; Roche, Mannheim, Germany) was performed by incubation at 56°C overnight. Total genomic DNA was extracted from the homogenized ticks. Briefly, DNA was extracted from all the organs using TriReagent (Sigma-Aldrich, St. Louis, Minnesota, United States) according to the manufacturer's instructions. The concentration of each DNA sample was determined in a NanoDrop 2000c spectrophotometer (Thermo Scientific, San Jose, CA, USA). The efficiency of the nucleic acid extraction was evaluated by electrophoresis in 1.5% (w/v) Agarose gels containing Ethidium bromide and visualized under ultraviolet light. The DNA was then stored at -20°C until further use.

PCR amplification

A Nested PCR (polymerase chain reaction) for genomic DNA extracted from five (5) different species of ticks was performed as described (Rar *et al.*, 2005), with slight modification. Amplification was aimed at targeting the 16S rRNA gene using the forward primer F1 5'-GAACGAACGCTGGCGCAAGC-3' and R1 5'-CACGCTTCGCACCTCAGTGTC-3' as reverse primer

for the outer reactions and inner reactions with forward primer F2 5'-TGCATAGGAATCTACCTAGTAG-3' and reverse primer R2 5'-CACGCTTTCGCACCTCAGTGTC-3', at expected amplicon size of 711bp (Rar *et al.*, 2005). Five μ l DNA extract from each tick sample was amplified in a 25 μ l reaction mixture containing; 13.65 μ l nuclease free water, 2.5 μ l of 10X PCR buffer, 1 μ l of 50mM MgCl₂, 0.5 μ l of 10mM Deoxynucleoside Triphosphate (dNTP), 0.25 μ l of DMSO, 1 μ l each of the forward and reverse primers (Rar *et al.*, 2005), and 0.1 μ l of Taq polymerase following conditions by the manufacturer. Amplicons were visualized with Ethidium bromide after electrophoresis in 1.5% agarose gels. Bands were detected from the gel by viewing and capturing under UV light with the aid of a Gel documentation system. Expected amplicon size was 711 bp.

Amplicon sequencing

Before sending positive samples to be sequenced, the Amplicons were purified following the protocol of Wizard SV Gel and PCR Clean-Up System (Promega, USA). To the PCR amplification 10 μ l of membrane binding solution (Promega, USA) was added. Each PCR amplicon to be sequenced was transferred to respective SV mini column (Promega, USA) placed in collection tubes. Centrifugation was performed at 16,000 x g for one minute and the flow-through was discarded. Thereafter 700 μ l Membrane Wash Solution containing ethanol was added and the mini-columns were centrifuged at 16,000 x g for one minute. The flow-through was discarded and the mini-columns reinserted into the collection tubes. The same procedure was repeated but with 500 μ l Membrane Wash Solution and the mini-columns were centrifuged at 16,000 x g for five minutes. The collection tube was emptied thoroughly and the mini-columns reinserted and centrifuged at 16,000 x g for one minute without the centrifuge lid on to evaporate any remaining ethanol. The purified PCR amplification was eluted from the Mini-column with 30 μ l of Nuclease-free water.

Phylogenetic analysis

BLASTN search sequence deposits that are identical to the sequences in this study was done using the NCBI database (<http://www.ncbi.nlm.nih.gov/BLASTn>). Phylogenetic tree was constructed using the Molecular Evolutionary Genetic Analysis (MEGA 7.0) software program (Kumar *et al.*, 2016). The evolutionary distances were computed using the Maximum Composite Likelihood method and Neighbor-joining (NJ) algorithm was used to construct a phylogenetic tree (Saitou and Nei, 1987).

The sequences were assembled in notepad using BankIt method and then the corrected ticks' sequences were deposited into the GenBank and their ascension numbers were obtained.

Statistical Analysis

Data obtained were organized, edited and analyzed using SPSS Version 20. Results generated from the investigation were expressed using descriptive statistics (mean \pm standard error of mean, percentage, and graph).

Ethical Statement

All applicable international, national, and/or institutional guidelines for the collection of tick samples from cattle were correctly followed. Informed consent was obtained from all the Fulani herders and their respective traditional authorities.

RESULTS

Tick species collected

In this study, out of 384 white Fulani breeds of cattle screened 336 (87.5%) were tick infested. A total of 1074 ticks were collected and of three genera *Amblyomma*, *Hyalomma*, *Rhipicephalus* and including the sub-genus *Rhipicephalus* (*Boophilus*). Five species of ticks were identified: *Amblyomma variegatum*, *Rhipicephalus* (*Boophilus*) *decoloratus*, *Rhipicephalus simusmuhsamae*, *Rhipicephalus sanguineus*, and *Hyalomma impeltatum*. The frequency of occurrence in the study showed that *Rhipicephalus simus* Group 669 (62.3%) was the commonest ticks observed in cattle in all the herds followed by *Rhipicephalus* (*Boophilus*) *decoloratus*, 158 (14.7%), *Hyalomma impeltatum* 96 (8.9%), *Amblyomma variegatum* 82 (7.6%), and *Rhipicephalus sanguineus* 14 (1.3%) respectively. A relatively high number (n = 699) of adult *Rh. simus* group ticks were collected from cattle of all age groups.

Tick distribution in relation to predilection sites

The distribution (%) of tick infestation in different body parts of cattle examined revealed that; tail (46.5%), axillary (17.9%), neck/dewlap (14.1%), udder/scrotum (9.5%), prepuce/perineum (8.2%), head (1.9%), legs/interdigital space (1.1%) and dorsum (0.8%). This shows that the tail region/area (46.5%) is the most infested followed by the axillary (17.9%), the neck (14.1%), while the dorsum (0.8%) is the least infested area on the sampled animals.

Molecular identification of tick species

DNA was isolated from five (5) tick samples (as in materials and methods), three (3) from each species of ticks. Following amplification of the 16S rRNA and gel electrophoresis of the PCR products, all the five (5) tick species were found to be positive and yielded products of approximately 711bp (Stafford, 2007).

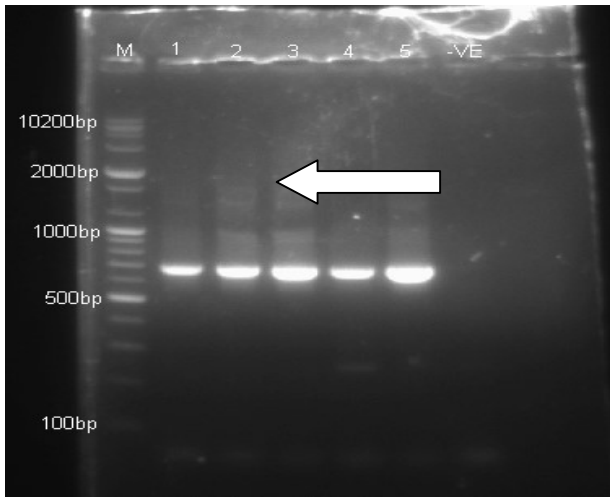


Figure 1: Gel image of *Anaplasma* spp DNA in ticks found on cattle. Lanes: M; 1 kb⁺ ladder, 1. *Amblyomma variegatum*, 2. *Rhipicephalus decoloratus*, 3. *Rhipicephalus sanguineus* 4. *Hyalomma impeltatum* 5. *Rhipicephalus simus* Group. -VE; Non-template control (NTC). The amplified 711 bp product was subjected to electrophoresis in 1.5% agarose gel and run at a current of 130 Ampere, 6V for 45 minutes.

Phylogenetic tree for *Anaplasma* spp

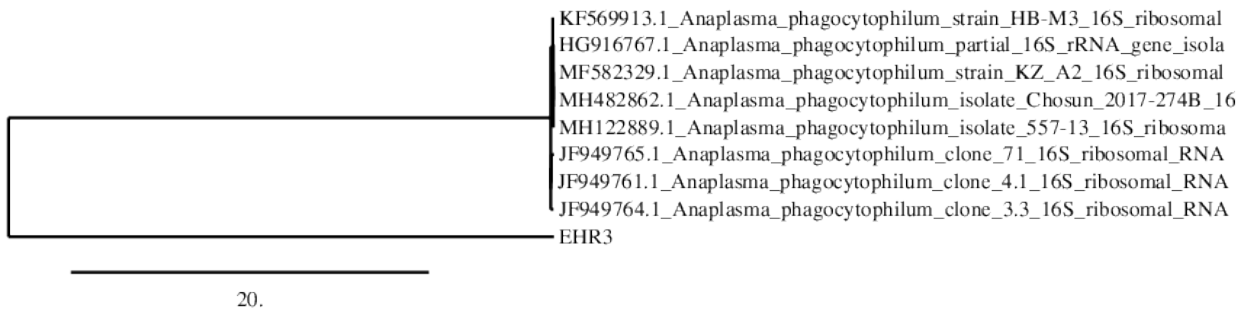


Figure 2: Phylogenetic tree inferred from 711 bp of the 16S rRNA sequences of *A. phagocytophilum* (NCBI accession number: MN044909). Sequence obtained during this study was named EHR3. The number at each branch represents a percentage value of a 1000 bootstrap replicates that support each branch. It shows the repeatability of a branch. The scale bar in the figure shows estimated number of substitutions per site.

DISCUSSION

In this study, out of 384 cattle screened 336 (87.5%) were tick infested. A total of 1074 ticks were collected and of three genera *Amblyomma*, *Hyalomma*, *Rhipicephalus* and including the sub-genus *Rhipicephalus* (*Boophilus*). Six species of ticks were identified: *Amblyomma variegatum*, *Rhipicephalus* (*Boophilus*) *decoloratus*, *Rhipicephalus* (*Boophilus*) *microplus*, *Rhipicephalus simus* Group, *Rhipicephalus sanguineus*, and *Hyalomma impeltatum*. The frequency of occurrence in the study showed that *Rhipicephalus simus* Group 669 (62.3 %) was the commonest ticks observed in cattle in all the herds followed by *Rhipicephalus* (*Boophilus*) *decoloratus* 158 (14.7%), *Hyalomma impeltatum* 96 (8.9 %), *Amblyomma variegatum* 82 (7.6%), *Rhipicephalus* (*Boophilus*) *microplus* (5.1%) and *Rhipicephalus sanguineus* 14 (1.3 %) respectively.

The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2004) and are in the units of the number of base substitutions per site. The analysis involved 9 nucleotide sequences. Codon positions included were 1st+2nd+Noncoding. All ambiguous positions were removed for each sequence pair. Evolutionary analyses were conducted in MEGA7 (Kumar *et al.*, 2016).

A relatively high number (n = 699) of adult *Rh. simus* Group ticks were collected from cattle of all age groups of White Fulani breed of cattle in Zaria, and hence disprove the fact that this species of tick has never been encountered in high loads (Madder *et al.*, 2012).

The distribution (%) of tick infestation in different body parts of cattle examined revealed that tail (46.5%), ventrum (17.9%) and neck (14.1%) were the most tick – infested sites in the body of examined animals and the dorsum (0.8%) was the least part infested. This further confirms that ticks prefer to attach and feed on some parts of the body of animals. This finding is not in agreement with the work by Jajere *et al.* (2014) that recorded prevalence in predilection site on different breeds of cattle in Maiduguri as follows; udder and external genitalia (83.4%), inner thighs (79.0%), under the tail/perineum (69.8%), eyes (26.3%), neck and

dewlap (14.6%) and ears (12.2%) respectively. Joseph *et al.*, (2014) in his work suggested that ticks are widely distributed in different parts of the host body such as ventral area, inner thigh, penis, udder, mammary gland, scrotum and vulva, of which inner thigh (26.6%) was most infested, while vulva (10.8%) was the least infested animal body part. Also, Opara and Ezeh (2011) in Borno State, Northeastern Nigeria who found that ticks infesting cattle in this area prefer to attach and feed on inner thighs, dew lap, abdomen, legs, udder, dorsum, ear and hump in this order. Asmaa *et al.*, (2014) also reported that udders and external genitalia were the most tick infested sites (70.7% each) followed by neck and chest (63.0% each), inner thighs (61.1%), perineum (41.7%), ears (14.6%) and around eyes (11.7%). These findings could be attributed to the fact that external genitals, perineum and inguinal/groin region of the body are highly supplied with blood and ticks usually prefer thinner and short hair skin for infestation. This helps in easy penetration of mouthparts of ticks into richly vascular area for feeding (Sajid, 2007). The higher number of *R. simus Group* (46.5%) recorded in the present study could be mainly attributed to the practice of hand-picking of ticks by the Fulanis, carried out up to three times a week during the wet season (Pullan *et al.*, 1980; Lorusso *et al.*, 2013). This control method mainly targets the most conspicuous *Amblyomma* adults, regarded as ‘koti’ (i.e., ‘dangerous ticks’ in Fulfulde language), by the local herdsman, as opposed to the smaller *Rhipicephalus* and boophilid ticks that are consciously left attached, as they are believed to be ‘miri’ (i.e., ‘less harmful’) (Bayer and Maina, 1984; Lorusso *et al.*, 2013).

Members of the genus *Anaplasma* are obligatory intracellular gram-negative bacteria that infect blood cells of mammals. Six *Anaplasma* species are currently recognized (Dumler *et al.*, 2001). Results clearly indicate evidence of *Anaplasma phagocytophilum* in all the five species of ticks sampled. To the best of our knowledge, this is the first molecular detection of *Anaplasma* species infecting cattle in Zaria and its’ environs. *Anaplasma phagocytophilum* causes granulocytic anaplasmosis in humans as well as tick-borne fever in ruminants, equine anaplasmosis in horses, and severe febrile diseases in dogs and cats (Lorusso *et al.*, 2013). A high diversity of the *A. phagocytophilum* biological properties could be connected with its high genetic variability.

After the amplification of 16S rRNA for *Anaplasma* pathogen, all the five tick species were found to be positive and showed their expected amplicons at 711bp. Blasting the sequenced amplicons of *Anaplasma* spp (MN044909) using BLASTN revealed 88% similarity with the *Anaplasma phagocytophilum* detected by Ogoh *et al.* (2012) in Nigeria, while Zhang *et al.* (2013) that isolated *A. phagocytophilum* from patients with febrile diseases of unknown etiology in China; Krakowetz *et al.* (2014) that works on two different strains of *A. phagocytophilum* in *Ixodes scapularis* in Canada; Lee *et al.* (2017) that works on the first clinical case of canine granulocytic anaplasmosis and genotypic analysis of *A. phagocytophilum* in Korea; Kim (2018) reported a case study on the manifestation of anaplasmosis as cerebral infarctions in Korea and Kowalec *et al.* (2019) that worked on Rickettsiales occurrence and co-occurrence

in *Ixodes ricinus* ticks in both natural and urban areas in Poland revealed 100% similarity with that of this study.

Conclusion

This work has shown the presence of *Anaplasma phagocytophilum* in ticks infesting White Fulani breed of cattle in Zaria and its environs. Thus, the increase movement of commodities, animals and humans aid in dissemination of arthropods especially ticks and by extension the pathogens they harboured. This makes transmission of ticks and tick-borne pathogens very easy and a serious problem to combat in Nigeria today.

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Conflict of Interest

The authors have no conflict of interest to declare.

Author’s Contribution

RM drafted this work, NAJand OOO supervised the work, DR participated in the sampling, OFSD was involved in the data analysis, HK and GIA. were involved in the laboratory work, OIA and EN drafted the phylogenetic tree and sequencing of the PCR-product, SD and BAY proof-read the draft, analysis and sampling for this study.

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