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RESEARCH ARTICLE
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Genetic characterization of *Coxiella burnetii* in *Amblyomma varigatum* ticks from North-central Nigeria: public health importance

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Abstract

Aim: The purpose of this pilot study was to genetically identify and characterize *Coxiella burnetii* from *Amblyomma varigatum* ticks collected on cattle in North central Nigeria.

Materials and Methods: A total of 40 partially fed ticks morphologically identified as adult *A. variegatum* ticks collected from cattle owned by Fulani pastoralists were evaluated for the presence of *C. burnetii* using PCR, cloning, and sequencing of the heat shock polypeptide gene *htpB*.

Results: *C. burnetii* DNA was detected in 10 (25%) of the ticks analyzed. Sequences for the *C. burnetii* gene *htpB* detected in our samples had 99-100% identity to all other *C. burnetii* that have been described and that are deposited in the GenBank database. Phylogenetic analysis using neighbor-joining method indicates the clustering of *C. burnetii* sequences from our study areas with those collected from Oyo state, South-western Nigeria and Spain.

Conclusion: This study shows a high infection rate of *C. burnetii* in *A. variegatum* ticks in the study areas. Phylogenetic inferences indicates that the strain of *C. burnetii* found in the North central states of Plateau and Nasarawa were same as those previously reported in the South western state of Oyo. The presence of this pathogen in naturally occurring *A. variegatum* tick populations could present an additional risk of Q-fever disease to humans, especially to the pastoralists that are closely associated with their animals and are easily exposed to tick bites. Therefore, further studies are needed to assess the competence of *A. variegatum* ticks as vectors of *C. burnetii* pathogens.

Keywords: *Amblyomma variegatum*, *Coxiella burnetii*, Nigeria, ticks, zoonosis

Introduction

Ticks are important vectors of various pathogenic agents that cause disease in humans and animals; some of these agents, such as *Coxiella burnetii*, are considered as emerging vector-borne pathogens as well as agents of bioterrorism [1]. *C. burnetii* is an obligate intracellular, gram-negative, gamma proteobacteria that infect and cause a worldwide zoonoses, Q fever, in humans and animals. Ticks act as reservoirs and responsible for the transmission of the pathogen to animals through their bite or fecal contamination [2, 3], and the major source of dissemination of the pathogen in the environment as a result of the high concentration of the pathogen in tick feces, saliva, and coxal fluid [4]. The infected domestic animals and pets through their milk, urine, feces, placental, and birth fluids are the main source of human infection [5,6].

Although Inhalation of aerosolized *C. burnetii* organisms is the most important route of infection in humans, ingestion of raw milk or fresh dairy products can also cause infection [7]. Tick transmitted livestock coxiellosis have been associated with about 40 different tick species [6]. Even though a few of these tick species have been shown to be able to harbor and maintain *C. burnetii*, they are not the primary vectors of this pathogen for transmission to animal or humans since their role in the natural cycle of *C. burnetii* remains to be defined till date [4]. However, they are thought to acquire the pathogen during a blood meal on infected animals and may transmit the bacterium to other mammals during the next blood meal or by aerogenic spread of dried tick faecal excretions [3,8].

Amongst the tick genera that serve as reservoir for this pathogen, the genus *Amblyomma* seem to have a high prevalence and capacity to maintain the *Coxiella* and *Coxiella*-like symbionts as observed in studies involving *A. americanum* and *A. cajennense* [9,10,11]. The importance of this genus in the maintenance of tick-borne infectious pathogens and as vector of several zoonotic agents requires that species in the genus be properly studied.

In Nigeria, *Amblyomma variegatum* ticks are the largest, most abundant, and destructive on domestic animals, and pets, and are associated with the transmission of diseases such as anaplasmosis, babesiosis, heartwater, and rickettsiosis, which are of

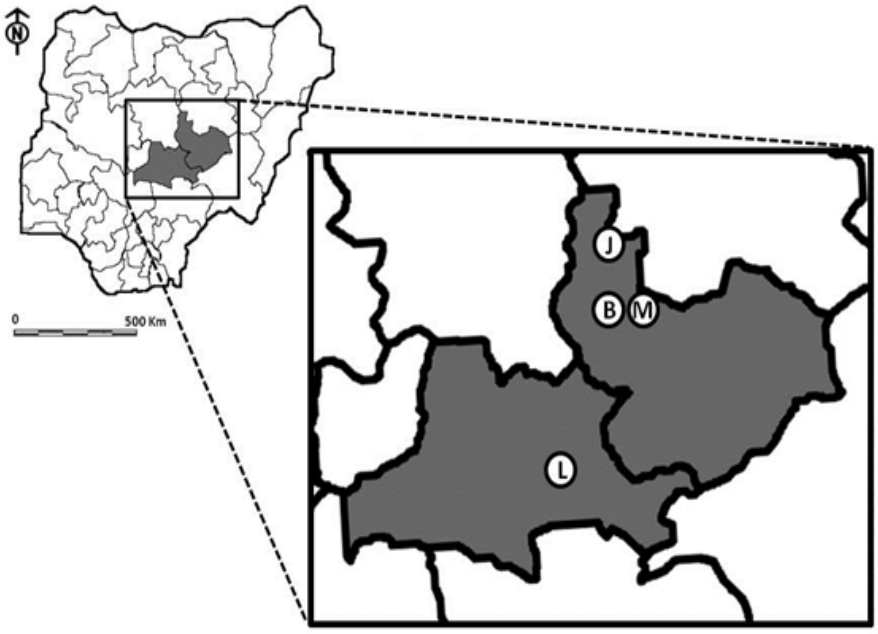

Figure-1. Map of Nigeria highlighting Nigerian regional borders. The amplification shows the Nigerian states (Plateau and Nassarawa) where sampling sites were located (J: Jos; B: Barkin Ladi; M: Mangu; L: Lafia).

great importance to the livestock [12,13]. The notoriety of this tick in terms of damage to the skin of animals during disease transmission and as pest has earned it the name "koti" (´dangerous tick´) amongst the Fulani herdsmen of Nigeria, and thus is accorded much attention during acaricidal treatment and the routine hand de-ticking exercise [14].

Studies have shown that the collection of ticks from hosts or vegetation and analysis by genus-specific and/or species specific PCR is an efficient method for the assessment of tick-borne pathogens since data from such analysis are useful in the risk assessment of emerging tick-borne diseases in a geographic area [15,16].

The presence of *C. burnetti* in other tick species have been reported [3,4]. However, the importance of *A. variegatum* ticks in the Nigerian livestock industry in terms of damage to hides and skin, and their penchant to readily feed on man (especially the pastoralists) and increase their risk of exposure to diseases of public health significance [17,18] influenced our study.

In this paper, we present the result of a survey in which the purpose was to use molecular methods and phylogenetics to identify and characterize the zoonotic pathogen *C. burnetii* in *A. variegatum* ticks collected from pastoralist herds in North central Nigeria.

Materials and Methods

Study area: The study was conducted in three Local Government Areas of Plateau State, Nigeria: Barkin Ladi (9°32´-8°53´), Mangu (9°18´09´-9°11´34´) and Jos-South (9°47´-8°51´), and one local government area in Nasarawa State, Nigeria: Lafia (9°28´-8°32´), Figure-1. The study sites are positioned within the Northern Guinea savanna region; with plain lands and hills measuring up to 3000ft above sea level at some points, daily temperature range of 23°C-27°C, and have a tropical climate and a moderate annual rainfall mean of about 1311.75cm [19]. The area is home to some of the normadic Fulani pastoralists who are known to traverse from their origin in the semi-arid (Northern) parts of Nigeria to the sub-humid zones in the South in search of trade and pasture for their animals. Some of these normads are beginning to settle and live in sedentary households for easy access to regular markets in peri-urban areas where they trade their products [20]. It is from animals in several of such sedentary pastoralist communities that the tick samples were collected for the study.

Sample collection: A total of 40 *A. variegatum* tick samples were collected from the body of cattle on the basis of accessibility and convenience as a result of the ethno-religious conflicts inherent in the areas sampled. Each tick was carefully removed by grasping with a blunt curved forceps as close to the skin surface as possible and pulled upward with a steady even pressure, ensuring that the mouth parts were completely removed with the whole tick. Damaged ticks were removed and safely disposed while the intact ticks were cleaned by first sterilizing them in bleach and subsequently rinsing twice in distilled water and then preserved in 70% ethanol.

DNA extraction: The *A. variegatum* ticks were individually dissected with separate scalpel blades to remove the internal organs and DNA extracted using TriReagent (Sigma-Aldrich, Madrid, Spain) according to the manufacturer's instructions. DNA concentration was determined with a Nanodrop ND-1000 spectrophotometer (Wilmington, DE, USA), and then stored at -20°C until used for analysis.

PCR amplification for *C. burnetii*: The DNA samples from *A. variegatum* ticks were subjected to a nested PCR using oligonucleotide primers synthesized

commercially by Sigma-Aldrich Biotechnology, L.P., Germany. Primary and secondary PCR amplifications were carried out with primer designates; Q5 (5´-GCG GGT GAT GGT ACC ACA ACA- 3´) and Q3 (5´-GGC AAT CAC CAA TAA GGG CCG-3´) ; Q6 (5´-TT GCT GGA ATG AAC CCC A-3´) and Q4 (5´-TC AAG CTC CGC ACT CAT G-3´) respectively. The primers amplifiy 501 and 325bp fragments of the *C. burnetii htpB* (heat shock protein B) gene of a 62kDa antigenic polypeptide [21].

The primary DNA amplification for the nested PCR was carried out in a reaction volume of 50µl containing 10 µl of 10X Tfl buffer (Promega, Madison, WI, USA), 1µl of 10mM dNTPs, 1 µl (10 pmol) each of the primers (forward and reverse), 3 µl of 25mM MgSO4, 1 µl of DNA sample (50 ng/µl), 1 µl of Tfl Polymerase (Promega, Madison, WI, USA) and 32 µl nuclease-free water. The reaction mixture for the secondary nested PCR was the same as the first PCR except that 0.5 µl of the PCR product (amplicons) from the first reaction was used as the DNA template, together with the Q6 and Q4 primers. Conditions for the reactions were an initial denaturation of 94°C for 3 minutes, 30 cycles of denaturation at 94°C for 1 minute, annealing at 56°C for 1 minute, and extension at 72°C for 1 minute, and a final extension of 72°C for 5 minutes. Conditions for the second PCR amplification are as described above with the exception of the annealing temperature which was set at 52°C.

Amplification reactions were carried out in a thermocycler MyCycler 170-9711 (BioRad, Hercules, CA, USA). Amplicons from the reactions were visualized after electrophoresis on a 1% agarose gel stained with SYBR Safe DNA gel stain (Invitrogen, Eugene, OR, USA) and compared to a DNA molecular marker.

Cloning and sequencing of the *htpB* gene: Like in most molecular biology studies where large quantities of DNA molecule can be isolated in pure form for detailed molecular analysis, we ensured that the DNA isolated from *A. variegatum* ticks used in this study were actually that of *C. burnetii* by cloning it into the pGMET vector and purifying before sequencing.

Amplified products from the PCR reactions were purified using a Purelink PCR purification kit (Invitrogen, USA) according to the manufacturer's instructions, then ligated into pGEM®-T Vector System I (Promega, Madison, USA) and transformed into JM109 high efficiency competent *Escherichia coli* cells according to the manufacturer's protocol. The transformed cells were subsequently plated on X-gal/ IPTG plates. The small, colourless positive transformants were selected and cultured in LB-Ampicillin medium at 37 °C overnight. Purification of the plasmid DNA was done with the QIAprep® Spin Miniprep kit (Qiagen, USA) while *Proteus vulgaris* II enzyme (Pvu II) kit (Fermentas®) was used to digest the Plasmid DNA for evaluation of their size on 1% agarose prior to sequencing. For every PCR sample that was cloned, at least two clones from each purified plasmid were submitted for sequence confirmation in an automatic sequencer (3730 DNA analyzer, Applied Biosystem®).

DNA sequence analysis: Comparison of our sequences with sequences previously deposited in the gene bank was done using the basic local alignment search tool (BLAST) at the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST). The sequences were analyzed with the Clustal W Multiple Alignment feature of the BioEdit software. Phylogenetic analysis was done with sequence alignments obtained in this study and those by Reyes et al., [22] from Southern Nigeria and Spain. The GenBank accession numbers of the *C. burnetii* heat shock protein antigenic polypeptide (*htpB*) gene sequences used to construct the neigbour-joining tree [23] includes; JN871859, JN871850, JN871863 and EF547935, while *Ehrlichia* spp., (JF949769) was used as an outgroup. The distance matrix was calculated by use of Kimura-2 parameters, whilst 1000 bootstrap replicates were used to estimate the reliabilities of the branches on the tree. The output of the tree was constructed with the MEGA 4.1 program [24].

Results

Of the 40 partially fed *A. variegatum* ticks screened for *C. burnetii* using a specific nested PCR, we were able to detect the pathogen DNA in 10 ticks samples. This positive results from these *C. burnetii*-specific PCR assays provide molecular evidence for *C. burnetii* infection in the ticks, and with an infection rate of 25% for the areas under investigation. The ticks collected from the various areas had different levels of infection, with ticks from Barkin ladi and Lafia areas having the highest infection rate of 40% each.

BLAST search for the sequenced amplicons using the DDBJ/EMBL/ GenBank databases showed similarity of 99%-100% to the partial sequence of *C. burnetii* heat shock protein B gene (*htpB*) isolated from goat milk (GenBank accession numbers: EU888861-EU888863) and Lions (EF547935). The sequences derived from our study are deposited in the GenBank under accession numbers JQ346185 - JQ346188. Phylogenetic analysis using neighbor-joining indicates the grouping of *C. burnetii* sequencesfrom our study areas with that collected from Oyo state, South-western Nigeria and Spain (Figure-2).

Discussion

Previous studies on the presence of *C. burnetii* in Nigeria were centered on the serological analysis of human sera [25]; fresh milk samples from cows; and suckling calves under different management systems [26, 5]. In these studies, the prevalence rate of infection for the human sera was 3%, while prevalence for both the milk and animal samples ranged from 24.3% - 59.8%.

However, Reye et al., [22] recently used PCR to establish the prevalence of 27% for *C. burnetii*

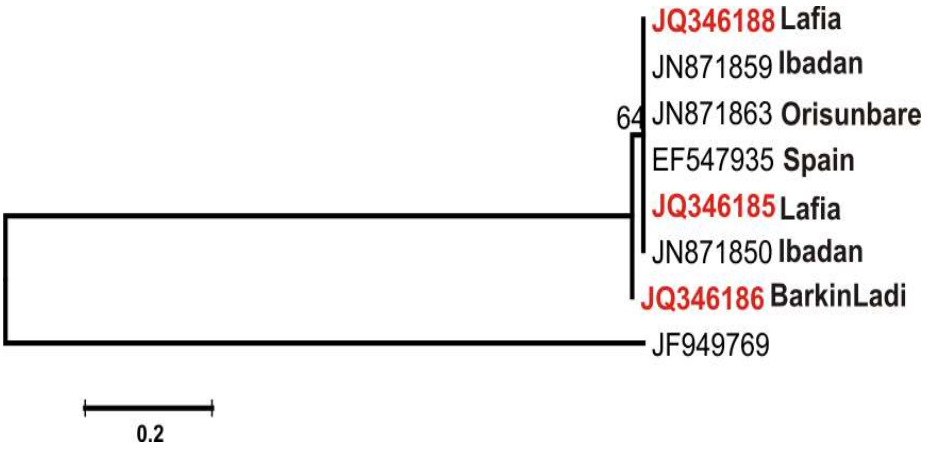

Figure-2. Neighbour-joining tree of 1000 replicates with Kimura 2 parameters from htpB gene sequences for 3 characterized *C. burnetii* isolates (highlighted in red) from North Central Nigeria. The tree illustrates the phylogenetic placement of *C. burnetii* detected in *A. variegatum* ticks from 2 states in North central Nigeria and those in ticks from South west Nigeria and Spain.

infection in ticks collected from the vegetation and livestock in Oyo state, South West, Nigeria. In this study, we sampled regions of the country that have a high concentration of pastoralists living in sedentary households and with high populations of ticks. Results from analyzing cloned fragments of the *C. burnetii htpB* gene obtained from feeding A. variegatum ticks, showed the detection of the organism in 25% of the 40 ticks analyzed. Sequences for the *C. burnetii* (*htpB*) gene detected in our samples had 99-100% identity to all other *C. burnetii* that have been described and were clustered together with those reported in the South western, Nigeria.

In Nigeria, 95% of livestock production is undertaken by the Fulani tribesmen. Their main system of production is pastoralism which involves unrestricted grazing and movement of ruminant livestock (mainly cattle) in response to variation in the availability of water, grazing pasture and the limitation imposed on cattle production by flies and livestock diseases [27]. This close and constant interaction between the pastoralists and their animals increases the chances of disease vectors such as *A. variegatum* attaching and transmitting any pathogen they harbor. Since *A. variegatum* have been involved in the transmission of other zoonotic pathogens [28], a prevalence of 25% *C. burnetii* in ticks collected from pastoralists-owned cattle in various localities in Nigeria increases the risk of Q fever infection and outbreak in this population.

Conclusion

This pilot study indicate that *A. variegatum* ticks are important sources of *C. burnetii* infection, and their presence in a particular locality may be used to assess the risk of human infection, especially in high risk groups like the pastoralists. Phylogenetic inferences indicates that the strain of *C. burnetii* found in the North central states of Plateau and Nasarawa were the same as those previously reported in the South western state of Oyo, we therefore propose that this is the only *Coxiella* spp. present in the country. For future risk assessments, it might be necessary to sample more ticks, increase the study areas of previously *C. burnetii* infected tick collection and possibly include samples from the pastoralists for analysis.

Authors' contributions

NIO and JdF conceived and designed the study, NIO, IGF, and RCG performed the experiments, NIO, OOO, IGF, and JdF analyzed the data, NIO, JdF, HMI, RISA, and OOO drafted and revised the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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