Bartonella bovis in Cattle in Nigeria: Molecular Detection and the Analysis of Risk Factors

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ABSTRACT

Cattle are the most important source of animal protein to humans in Nigeria. They are predominately raised under the extensive system of production. Although, low cost, this management system exposes the animals to several ectoparasites and vector-borne infections, with veterinary and public health consequences. Bartonellosis is an emerging vector-borne infection with veterinary and zoonotic implications. This study examined 462 blood samples from cattle in Nigeria for the presence of *Bartonella* DNA using PCR and sequencing approach. DNA fragments of the citrate synthase gene (*gltA*) and RNA polymerase beta subunit gene (*rpoB*) of *Bartonella bovis* were detected in 43 (9.3%) and 6 (1.3%), respectively, of the samples examined. The *gltA* and *rpoB* sequences from this study had high identities of 97.6% to 99.8% with GenBank deposited sequences of *B. bovis.* Phylogenetic analysis recovered the *gltA* and *rpoB* nucleotide sequences from this study in a monophyletic clade with *B. bovis* sequences from diverse mammals from other countries. Prevalence of *B. bovis* was associated ($p<0.05$) with animals older than two years of age and samples collected from abattoirs. This is the first report of *B. bovis* in cattle in Nigeria. More studies are required to determine the potential public health implications of these findings considering the high rate of detection in animals slaughtered for human consumption and the difficulties in enforcing meat inspection laws.

Keywords: Bartonellosis; Cattle; PCR; *gltA*; *rpoB*; Nigeria.

INTRODUCTION

Cattle are the most common type of large domesticated ungulate found throughout much of the world. They comprise hundreds of breeds that are recognized worldwide (1). In Nigeria, cattle are the single most important livestock species in terms of animal protein supply, value and biomass (2, 3). They are not only the main source of meat, milk, skin, bone, blood and horn products, but they are also used for draught power, transportation of people and loads, as well as to lift water from deep wells (3, 4, 5). About 75 percent of ruminant livestock population in Nigeria are found in the Sahel agro-ecological zone and are managed under the pastoral system (3, 6). Although, this production system is low cost, it exposes the animals to diseases, consequently, affecting productivity and endangering public health.

Bartonellae are considered as emerging pathogens, being increasingly associated with a number of diseases in both humans and animals (7, 8). They are fastidious Gram-negative bacteria that infect and persist in mammalian erythrocytes and endothelial cells and are found in a wide range of wild and domesticated mammals (5). Several species of *Bartonella* have been isolated from blood of ruminants in Africa, Asia,

Europe, and North and South America (4, 9-15). Among cattle, *Bartonella bovis* has been implicated in causing bovine endocarditis (16, 17). Other species such as, *Bartonella chomelii, Bartonella rochalimae, Bartonella schoenbuchensis, Bartonella vinsonii* subsp. *arupensis,* '*Candidatus* Bartonella davousti' have also been isolated from ruminants (13, 15, 18-20). The latter list is likely to increase with more studies and improvements in the detection methods. *Bartonella* spp. are usually transmitted to animals and humans through blood-feeding arthropod vectors such as fleas, lice, ticks and sandflies (12, 21).

The reported prevalence of *B. bovis* in cattle varies widely across studies from different geographic regions, i.e., 6.8% in Poland (22), 11.8% in Greece (15), 24% in Italy (23) and 70% in French Guyana (11). On the African continent, the first report of *B. bovis* infection in cattle was in Cote d'Ivoire, West Africa (4). Subsequently, there were reported prevalence of 27.8% in Senegal (13) and 15.3% in cattle in Algeria (14). *Bartonella* spp. of zoonotic importance have been reported in rodents and bats and their ectoparasites in Nigeria (24, 25). However, to date there is no report on *Bartonella* species infection in cattle in Nigeria. Therefore, this study was aimed at using molecular approach to detect and characterize *Bartonella* spp. in cattle in Nigeria and to determine the risk factors for infection.

MATERIALS AND METHODS

Ethical Approval

Approval for this study was granted by the Institutional Animal Care and Use Committee (IACUC), National Veterinary Research Institute (NVRI), Vom, Nigeria, approval number: AEC/03/108/21. Informed consent was obtained from management of abattoirs and cattle owners before the animals were sampled.

Study Area and Sample Collection

The study was conducted on blood samples collected from cattle from the three agro-ecological zones (AEZs) of Nigeria between July to December 2021. Samples were collected from two, five and three states in the Sahel, Savanna and Guinea AEZs, respectively (Fig.1). Blood samples were collected from 50 cattle slaughtered in each abattoir located in Jalingo, Maiduguri and Katsina, 35 in Lafiya abattoir and 54 in Jos abattoir. The remainder of the tested samples were obtained from sedentary herds in Ekiti (n=4), Kaduna (n=8), Nasarawa (n=15), Jos (n=26), Akwa Ibom (n=29), Ogun (n=41) and Kwara (n=100).

Before sampling, visual examination was conducted to assess the body condition of each cattle. Data on the age, gender and breed of the animals were recorded for each study site. Trained personnel properly restrained animals and 5 mL of blood was drawn from the jugular vein into ethylene diamine tetra-acetic acid (EDTA) tubes. Samples were kept in cold boxes packed with ice and transported to the Molecular Biology laboratory, Parasitology Division, NVRI, Vom, where they were kept at -20°C until analysis.

DNA extraction

DNA was extracted from anticoagulated blood using the Quick-DNA™ Miniprep Plus kit (Zymo Research, USA) with slight modification of the manufacturer's protocol. Briefly, 20 µL of proteinase K was added to 200 µL of anticoagulated blood and equal volumes of BioFluid in a 1.5 mL micro centrifuge tube. The mixture was vortexed for 15 seconds and incubated at 55°C for three hours. 420 uL of DNA binding buffer was added to the lysate, vortexed briefly and centrifuged at 16,000 xg for 3 minutes. The mixture was then transferred to a Spin column and the procedure was continued according to the manufacturer's instructions. The DNA was eluted in 80 µL of elution buffer and stored at -20°C until analysis.

Amplification of *Bartonella* spp. DNA from cattle blood by conventional PCR

All the DNA samples extracted from cattle blood in this study were initially screened for the presence of *Bartonella* spp. 350 bp citrate synthase (*gltA*) gene using the primers CSH1F (5' GCG AAT GAA GCG TGC CTA AA-3') and BhCS1137 (5'-AAT GCA AAA AGA ACA GTA AAC A-3') (26). Positive samples in the *gltA* amplification were tested for the amplification of the 850 bp RNA polymerase beta subunit gene (*rpoB*) using the primers 1400F (5'-CGC ATT GGC TTA CTT CGT ATG-3') and 2300R (5'-GTA GAC TGA TTA GAA CGC TG-3') (27).

The PCR mix consisted of 16 µL of 2X Master Mix with standard buffer (New England Biolabs Inc.), 0.6 µL of each primer (10 mM), 5 µL of template DNA and 8.8 µL of DNA/RNA-free water (BioConcept, Switzerland) in a final volume of 31 µL. Amplification for the *gltA* gene

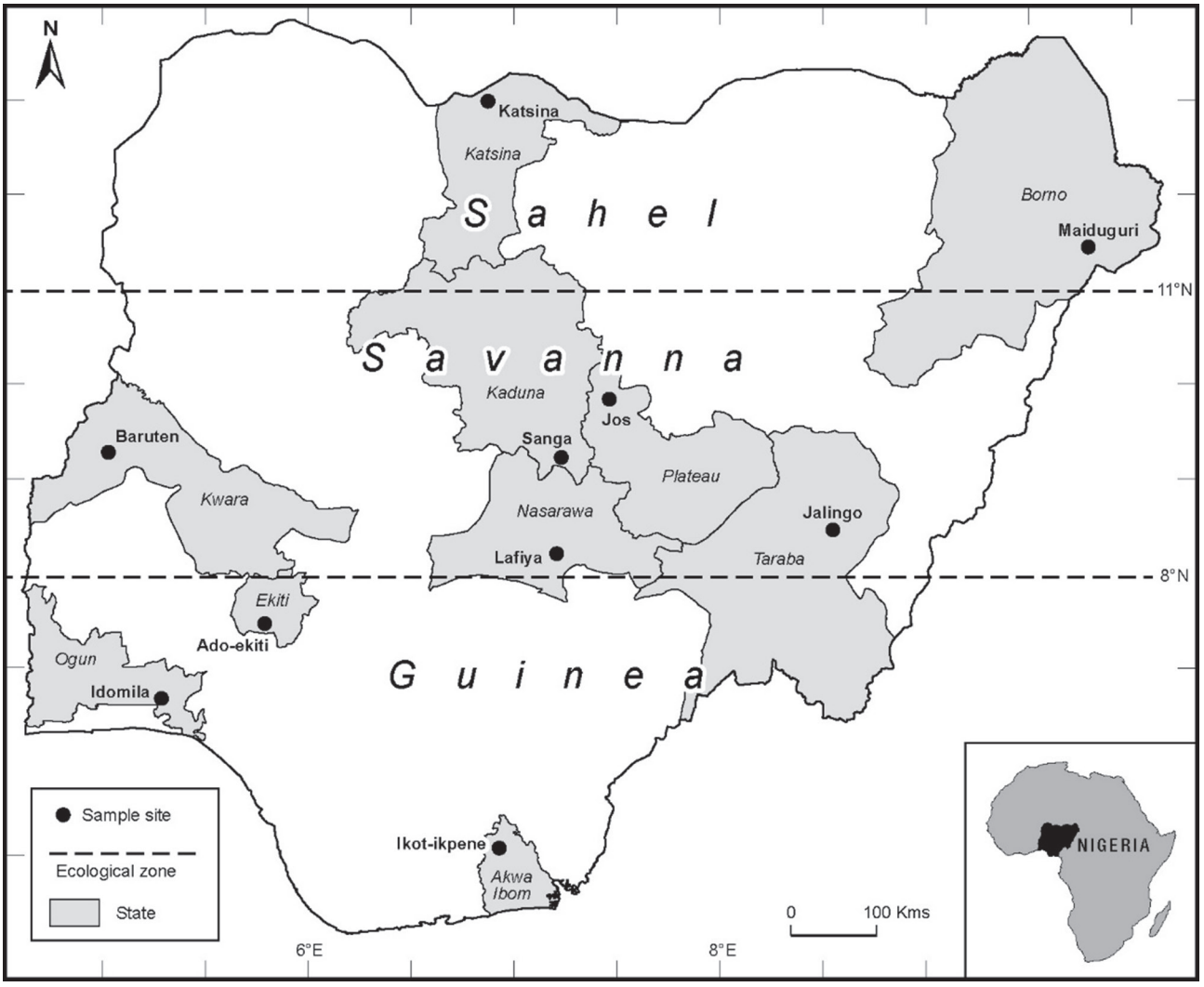

Fig.1. Map of Nigeria, West Africa showing the study sites.

involved 30 seconds at 94°C initial denaturation followed by 35 cycles of 30 seconds at 94°C, 1 minute at 51°C (annealing), and 1 minute at 68°C followed by a final elongation at 68°C for 8 minute. The amplification of the *rpoB* gene was similar except annealing which was done at 53°C for 30 seconds. The conventional PCR was conducted on a GenAMP 7400 (Applied Biosystems, Foster City, CA), in the Molecular Biology Laboratory, Parasitology Division, NVRI Vom, Nigeria. The DNA of *Bartonella elizabethae* obtained from a commensal rodent in Nigeria was used as a positive control. A non-template control (NTC) containing all the reaction mix except DNA was included in each PCR run. The PCR products were electrophoresed in a 1.2% agarose gel stained with SafeView™ Classic (Applied Biological Materials, Canada) and were visualized under a Blue light Transilluminator (Cleaver Scientific, UK) for the size of amplified fragments by comparison to a 100-bp DNA molecular weight marker. Positive amplicons were sequenced at the Center for Genomic Technologies, Hebrew University of Jerusalem, Israel using the PCR primers.

Nucleotide and phylogenetic analysis

Sequences were edited manually in the software Geneious Prime 2022.0.1 (https://www.geneious.com) and were compared to reference sequences available in the GenBank using the BLASTn algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Nucleotide sequences were quality checked and ambiguous base calls or missing data were coded with N´s or the corresponding ambiguity code. Double nucleotide peaks in otherwise high-quality sequence parts

Table 1. Prevalence of *Bartonella bovis*-DNA in cattle in Nigeria

Study location	No. positive/no. tested (%)	
	gltA	*rpoB*
Katsina	5/50 (10.0)	1/50 (2.0)
Jalingo	16/50 (32.0)	4/50 (8.0)
Borno	2/50 (4.0)	0 (0)
Ekiti	0/4 (0)	0 (0)
Kwara	0/100 (0)	0 (0)
Kaduna	0/8 (0)	0 (0)
Akwa Ibom	0/29 (0)	0 (0)
Ogun	6/41 (14.6)	0 (0)
Plateau	7/80 (8.8)	1/80 (1.3)
Nasarawa	7/50 (14.0)	0 (0)
Total (%)	43/462 (9.3)	6/462 (1.3)

were scored as mixed haplotype infection and haplotype diversity was assessed in Geneious Prime. Sequences were aligned using the MAFFT algorithm (28, 29). Reference sequences were retrieved from GenBank, mainly based on the dataset of Goncalves *et al.* (30) and added to alignments of the sequences of the study (all accession numbers are listed in the phylogenetic trees, see Figs 2 & 3). The program ModelTest-NG was used to test different DNA substitution models (31). The phylogenetic analyses of the partial *rpoB* gene consisting of a total of 792 nucleotides (nt) comprising 38 sequences including two new sequences of this study and the substitution model TIM3+G+I was used. The phylogenetic analyses of the partial *gltA* gene of a total of 378 nt comprised 37 sequences including five new sequences of this study and the substitution model TIM3+G was used. For Maximum likelihood (ML) analysis, we used raxmlGUI version 2.0.6 (32). Nodal support was evaluated using 1000 thorough bootstrap and consensus. Phylogenetic trees were displayed in FigTree (http://tree.bio.ed.ac.uk/software/figtree/).

Sequences obtained in this study were deposited in the GenBank under the following accession numbers: *gltA* (OM317751– OM317755); *rpoB* (OM317756 & OM317757).

Statistical analysis

The prevalence of *B. bovis* DNA was calculated as percentage of the total samples examined. The association between the detection of *B. bovis* DNA based on the *gltA* gene and the variables: age (≤ 2 years old versus > 2 years old), body condition score (good, medium or poor), source of sample (abattoir or herds), agro-ecological zones (Sahel, Savanna or Guinea), sex and breed of animals were analyzed. Univariate analysis was performed for each risk factor using the Chi-square test. The analysis was performed using the R Statistical Software (33). The level of significance was set at $p \leq 0.05$.

RESULTS

Blood samples were collected from 462 cattle from ten states located in the three agro-ecological zones of Nigeria (Table 1, Fig. 1). From this number, 239 of the samples (51.7%) were obtained from cattle slaughtered in abattoirs in four cities, while 223 (48.3%) where from cattle in herds located in different parts of Nigeria. The majority of the cattle were females; (278/462, 60.2%) and 72.3% (334/462) were older than two years of age. Eight breeds of cattle were sampled, although the bulk of them, 360 (77.9%) were of the White Fulani breed (Table 2). 288 of the 462 (62.3%) of the cattle sampled were from the Savanna agro-ecological zone, followed by 100 and 74 from the Sahel and the Guinea zones, respectively (Table 2, Fig. 1). Overall, 43 (9.3%) and 6 (1.3%) out of the 462 of the cattle examined were positive for *B. bovis* DNA based on the *gltA* and *rpoB* genes, respectively (Table 1). The highest prevalence (32.0%) of *Bartonella bovis* DNA was detected in cattle from an abattoir in Jalingo, followed by 14.6% in cattle from a sedentary herd in Ogun State. Although the prevalence

Fig. 2. Maximum likelihood phylogenetic analysis of 387 bp *gltA* sequences of *Bartonella* species. Bootstrap values are indicated. The sequences of this study are highlighted in bold and accession numbers are given in parentheses. The geographic origin (country) and the vertebrate host of each sequence are provided. The taxon *Brucella melitensis* was used as an outgroup.

of *B. bovis* DNA varied among the different categories of cattle examined, significant association ($p<0.05$) was found with older cattle (>2 years) and those slaughtered in the abattoirs, but not with the gender, breed, body condition or ecological zones (Table 2).

Table. 2: Prevalence and relative risks of *Bartonella bovis* in cattle in Nigeria

Variables	Number of animals tested			Prevalence (%)	95% CI	χ2	P
	Positive	Negative	Total				
Source of sample							
Abattoir	36	203	239	15.1	0.108-0.202	18.04	0.00002*
Herds/Farms	7	216	223	3.1	0.013-0.064		
Gender							
Male	14	170	184	7.6	0.042-0.124	0.73	0.39
Female	29	249	278	10.4	0.071-0.146		
Breed							
White Fulani	34	326	360	9.4	0.066-0.130	2.32	0.94
Bokoloji	2	15	17	11.8	0.015-0.364		
Sokoto Gudali	1	23	24	4.2	0.001-0.211		
Keteku	0	1	1	0.0	0.000-0.975		
N'dama	0	1	1	0.0	0.000-0.975		
Red Fulani	3	27	30	10.0	0.021-0.265		
Ambala	1	3	4	25.0	0.006-0.806		
Wadara	2	23	25	8.0	0.010-0.260		
Age							
Adult (>2years)	40	294	334	12.0	0.087-0.160	11.47	0.0007*
Young (≤2years)	3	125	128	2.3	0.005-0.067		
Body condition							
Good	20	153	173	11.6	0.072-0.173	1.70	0.42
Medium	17	191	208	8.2	0.048-0.128		
Poor	6	75	81	7.4	0.028-0.154		
Ecological zone							
Sahel	7	93	100	7.0	0.029-0.139	1.18	0.55
Savanna	30	258	288	10.4	0.071-0.145		
Guinea	6	68	74	8.1	0.030-0.168		

χ2 = Chi-square, P= significant value

Nucleotide and phylogenetic analysis

Partial sequences of the *gltA* gene were amplified and sequenced for five samples. Sequences of four samples matched *B. bovis,* which share sequence identities of 97.6– 99.8%. One sample (JA53) did not feature any ambiguity bases and therefore represents a single haplotype, whereas the other three sequences (OG11, OG40, T33) were mixed haplotype infections, featuring several double nucleotide peaks in the electropherograms. The phylogenetic analysis grouped all four sequences with other reference sequences of *B. bovis* in one monophyletic clade with high support (bootstrap value of 94). The fifth sequence (sample T1, mixed haplotype infection) was closer related to the species *Bartonella grahamii* according to the phylogenetic analysis with low bootstrap value of 36 (Fig. 2). The reference sequence with highest sequence identity of 97.38% was an uncultured *Bartonella* sp. sample from a spleen of a small mammal in Equatorial Africa (Fig. 2).

The two partial *rpoB* gene sequences of the Nigerian *Bartonella* samples of the study shared a sequence identity of 98.8%. Both sequences contained ambiguous base calls which indicate mixed haplotype infections. The phylogenetic

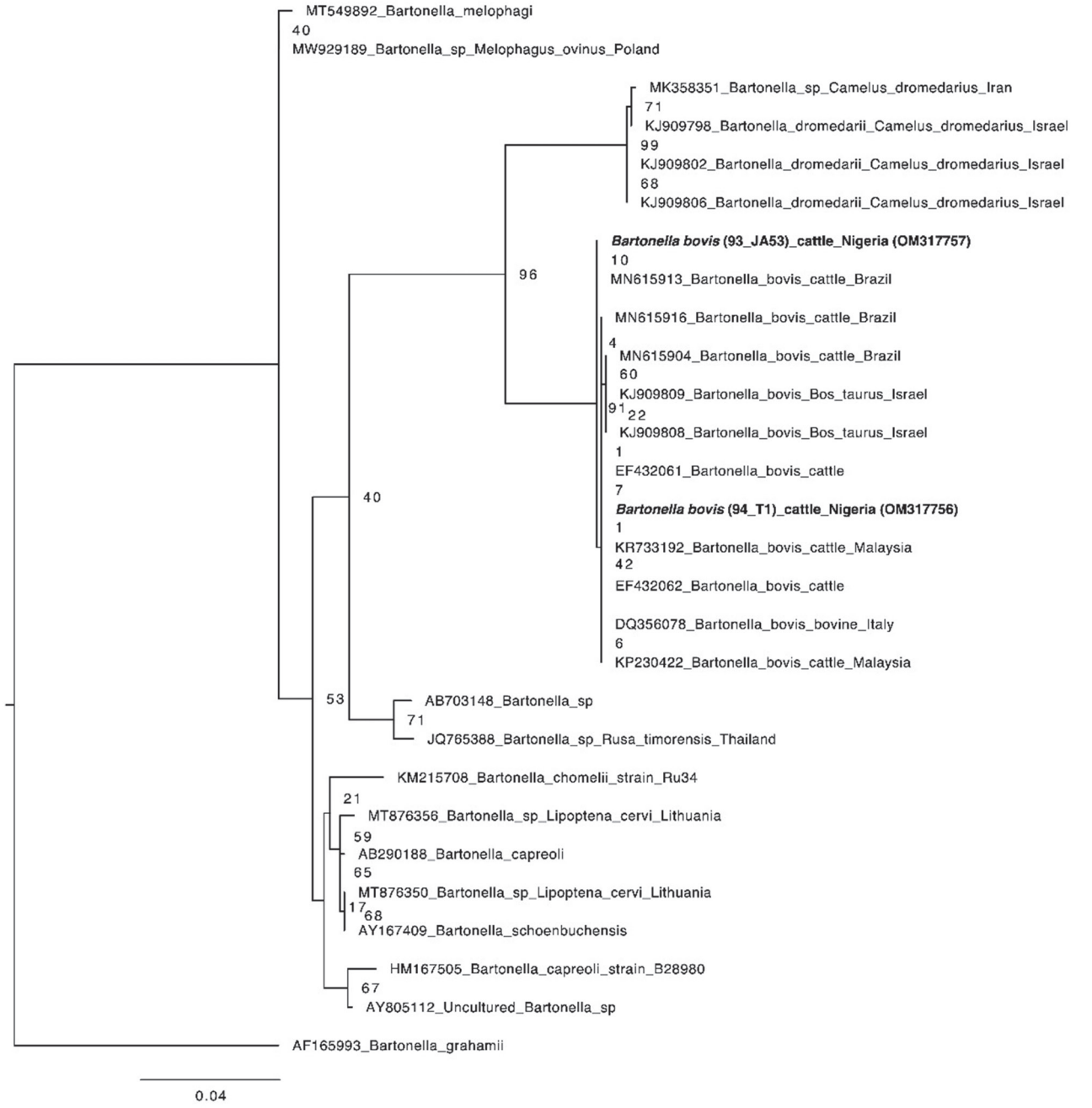

Fig. 3. Maximum likelihood phylogenetic analysis of 792 bp *rpoB* sequences of *Bartonella* species. Bootstrap values are indicated. The sequences of this study are highlighted in bold and accession numbers are given in parentheses. The geographic origin (country) and the vertebrate host of each sequence are provided. The taxon *Bartonella grahamii* was used as and outgroup. The phylogenetic analysis of *rpoB* recovered both Nigerian sequences within the monophyletic group of *Bartonella bovis* sequences from cattle from e.g., Brazil, Israel, Malaysia, and Italy with high support (bootstrap value of 91) as sister clade to the species *Candidatus* Bartonella dromedarii (named *Bartonella dromedarii* in the tree) (bootstrap value of=96).

analysis of the *rpoB* sequences placed both Nigerian sequences within the monophyletic group of *B. bovis* sequences from cattle from Brazil, Israel, Malaysia, and Italy with high support (bootstrap value of 91) as sister clade to the species *Candidatus* Bartonella dromedarii (bootstrap value of = 96). The nucleotide sequence of the sample T1 that clustered with sequences *B. grahamii* in the *gltA* dendrogram was clustered with *B. bovis* in the *rpoB* phylogenetic tree with high bootstrap (Fig 3).

DISCUSSION

Molecular detection and nucleotide sequence analysis of *Bartonella* spp. in cattle in Nigeria, confirmed that all the sequences obtained in this study belong to *B. bovis*. The prevalence of 9.3% (*gltA*) and 1.3% (*rpoB*) of *B. bovis* DNA in cattle in this study was lower than the previous reports in cattle of 27.8% in Senegal (13) and 15.3% in Algeria (14). However, a zero prevalence of *Bartonella* spp. has been reported in cattle from Kenya (5). The difference may be due to the study design, the genes targeted and the sensitivities of the assays used in each of the studies. Only the DNA of *B. bovis* was detected in cattle in this study, unlike the studies from Algeria and Senegal, where *B. chomelii* and a novel spp. '*Candidatus* Bartonella davousti' were reported in addition to *B. bovis* (13, 14). Our findings were similar to the first report of *Bartonella* spp. in cattle on the African continent where only *B. bovis* was recovered by both culture and PCR amplification (4). In this study, the prevalence of *B. bovis* was associated with older cattle (>2 years) similar to the report from Spain (20) but, in contrast to the report from France and Algeria (14, 16). Generally, high prevalence of vector-borne diseases have been associated with older animals, especially those raised under the extensive management due to continuous challenge by hematophagous arthropods (34, 35). Furthermore, there was an association between samples obtained from cattle slaughtered in the abattoirs with detection of *B. bovis* DNA.

Likewise, a study in Senegal reported the isolation from cattle blood of a *Bartonella* spp. with unique genetic features, which are different from other species of the *Bartonella* genus. This isolate is being proposed as a potentially novel *Bartonella* spp., highlighting the probable role of cattle as a potential reservoir of *Bartonella* spp. (13).

Beef is the main source of animal protein for humans in most of Nigeria. Each year large numbers of cattle and other livestock are slaughtered at designated abattoirs, slaughterhouses or slaughter slabs to produce meat for public consumption. However, sometimes slaughter may also take place at home or at an outdoor slaughter facility designated for religious and cultural practices (3). Although, veterinary public health officials are mandated to inspect and certify meat targeted for human consumption, enforcement of certain decisions have been hampered due to obsolete or lack of legislation, to back such actions. Worst still, it has been observed that in defiance to directives from veterinary public health officials to trim some infected parts from a carcass before passing the meat for public consumption, butchers cut some of the affected parts and eat it raw to justify the fitness of the meat for human consumption. Hence, butchers and meat processors usually operate without proper public health supervision thereby endangering the health of consumers. A relatively high proportion of the Nigerian populace are immunosuppressed either due to malnutrition or infections with HIV or malaria, thereby predisposing them to several emerging infectious diseases (36-39). Therefore, the role of *Bartonella* spp. in causing disease in immunosuppressed individual deserves attention. For example, a study conducted in South Africa reported that 10% of outpatients attending HIV clinics in an urban center were bacteremic with *B. henselae* (40). As for 2015, Nigeria was rated as having the world's second highest burden of people living with HIV/AIDS (41). Added to this, the predominant cattle production system in Nigeria brings humans into close contact with domestic animals and ectoparasites, which may facilitate the transmission of zoonotic pathogens or even non-pathogenic species to cause disease in immunocompromised people. *Bartonella bovis* may be a potential agent of zoonosis in humans similar to what was demonstrated with *B. rochalimae* that was first detected in a Pulex flea and subsequently isolated from a patient with fever and splenomegaly (42).

In conclusion, the detection of *B. bovis* DNA in cattle in Nigeria, most especially in cattle slaughtered for human consumption in the face of poor sanitation, poor health condition and the inability to strictly enforce meat inspection in most abattoirs is a source of public health concern. More studies in line with the One Health concept should be conducted to elucidate the epidemiology and public health implication of *Bartonella* spp. in Nigeria. Efforts should be intensified to improve the sanitary condition in the abattoirs and to effectively enforce meat inspection policy across the country.

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CONFLICT OF INTEREST / COMPETING INTERESTS

The authors have no conflicts of interest to declare that are relevant to the content of this article.

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