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PREVALENCE AND PHYLODIVERSITY OF ESBL-PRODUCING COLIFORMS ISOLATED FROM RUMINANT MASTITIS IN NIGERIA

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ABSTRACT

The public health threat posed by Extended-spectrum beta-lactamase producing *E. coli* (ESBL-EC) in food animal production systems has attracted global attention. Data on the prevalence, diversity and genetic characteristics of ESBL-producing coliforms are key to advocacy on promoting responsible antimicrobial stewardship and proper planning of control strategies. The coliforms were isolated from 1052 milk samples of 160 cows, 103 ewes and 103 does with mastitis in Plateau State, Nigeria and analysed for ESBL production by phenotypic, biochemical, antimicrobial sensitivity and genetic characterization. The percentage of occurrence of clinical mastitis in cows, ewes, and does were 0.2 %, 0 %, 1.5 % respectively, while the percentage occurrence of subclinical mastitis in ruminants were 18.1 %, 28.2 % and 38.3 % respectively. From the 677 isolates, 31.3 % (n = 212) were ESBL producing coliforms, with a prevalence of 48.6 %, 18.4 %, 12.7 %, 8.9 %, 5.7 %, 3.8 % and 1.9 % for *E. coli*, *K. pneumoniae, C. freundii, K. aerogenes, S. marcescens, K. oxytoca* and *E. cloacae*, respectively. The genetic characterization revealed a higher prevalence of bla_{CTX-M} than bla_{TEM} in the samples analysed (24.39 % *vs.* 12.19 %). High pairwise identity was observed among the bla_{CTX-M} and bla_{TEM} gene sequences obtained in this study, but they displayed high phylodiversity with sequences from ruminants and humans from other climes. The bla_{SHV} gene was not detected. Multidrug resistances especially to the commonly used antimicrobials; ofloxacin, gentamycin and streptomycin in veterinary practice in Nigeria were observed. This has public health implications considering the fact that consumption of raw unpasteurized milk is a common practice in some cultures in Nigeria. Such practise will facilitate the transfer of multidrug resistant coliforms to humans resulting in the complications of treatment outcomes. To the best of our knowledge this is the first genetic characterization of ESBL-producing agents from ruminant mastitis in Nigeria.

Key words: antibiogram; β-lactamases; coliform; mastitis; phylogeny; ruminants

INTRODUCTION

Antibiotics have been used successfully over the years in the treatment and prevention of diseases, or as growth promoters in livestock, thereby, promoting animal production and safeguarding public health [1, 36]. However, the development of resistance poses a serious challenge to the continued use with desirable outcomes [28]. More so, the inordinate use of antimicrobials especially in veterinary practice in developing nations where the enforcement of regulations is weak or non-existent, which makes the situation worse [20, 37].

Most Gram-negative bacteria possess naturally occurring chromosomally mediated β-lactamases brought about by selective pressure exerted by β-lactam producing soil organisms found in the environment [18]. These β-lactamases provide an extended resistance to β-lactam antibiotics and are usually produced by a variety of coliforms such as *Escherichia coli*, *Klebsiella* spp. and *Citrobacter* spp., that are commonly called Extended-spectrum β-lactamase (ESBL) bacteria [15, 40]. ESBL-producing Enterobacteriaceae are now considered among the "priority pathogens" of emerging global health threat because of their increasing prevalence in livestock [6, 13, 34].

The first β-lactamase resistance gene in *E. coli* was isolated from a patience called Temoniera in Greece, hence was named TEM [10]. TEM is among the most frequently isolated ESBL genes usually in association with CTX-M genes [3]. *E. coli* and other Enterobacteriaceae species producing CTX-M-type extended-spectrum-lactamases have been more frequently isolated from humans, their environment and their companion animals [22], from food-producing animals [9], and from retail meats, including chicken, beef, and pork [32], globally.

Mastitis is a common disease of ruminants, especially cows [7]. Coliforms like *E. coli* and *K. pneumoniae* have been incriminated as life-threatening cause of clinical mastitis [9]. Other Gram-negative commonly isolated bacteria from mastitis in cows include *Enterobacter* spp., *Citrobacter* spp., *Klebsiella* spp., *Serratia* spp., and *Proteus* spp. [29]. Therefore, food-animals have been incriminated as reservoirs of ESBL-producing *E. coli*, with zoonotic potential [12, 17, 27]. In Nigeria, several studies have reported the isolation of different bacteria from mastitis in ruminants with varying prevalence. However, most of these studies relied on the classical methods for the identification of the bacterial causes of mastitis [2].

To the best of our knowledge there is no reported study on the genetic characterization of coliforms associated with mastitis in ruminants in Nigeria. Therefore, the objective of this study was to determine the prevalence and characterize by amplification and sequence analyses the bla_{TEM} and bla_{CTX-M} genes in ESBLs producing coliforms from milk of ruminants suffering from mastitis in Nigeria.

MATERIALS AND METHODS

Study location and demography

The study was conducted on milk samples obtained from ruminants in Plateau State, Nigeria from March 2018 to October 2019. Plateau State (9°00′—10°30′ N and Latitude 8°30—09°30′ E) occupies an area of approximately 26,899 km^2 in the central part of Nigeria. Located at an altitude of 1217 m above sea level, the state enjoys a more temperate climate than other parts of Nigeria. The temperature in most of the state ranges from 13 and 22 °C and the mean annual rainfall of 131.75 cm (52 in) in the southern part and 146 cm (57 in) on the Plateau. Plateau State has an estimated population of about 5.5 million inhabitants, most of them are engaged in agricultural activities. Milk from ruminants is consumed as raw or pasteurized or processed into milk products such as cheese, yoghurt or ice cream.

Ethical clearance

In line with the guidelines of the Animal Use and Care Committee (AUCC) of National Veterinary Research Institute (NVRI), Vom, Nigeria, the procedure involved in the milk sampling was non-invasive and does not inflict pain or discomfort to the animals, Therefore, no ethical approval was required.

Milk Sampling

This is a cross-sectional survey on lactating cows, ewes and does in Plateau State, Nigeria. By Snowball Random Sampling, herds who met the inclusion criteria were identified, and those identified recommended other farms within the LGA who meet the inclusion criteria. Due to different strata in each herd, such as meat ruminants, lactating but dry ruminants, and lactating ruminants, the Stratified

Random Sampling was used to select ruminants be included in the study. The lactating ruminants comprising of 160 cows, 103 ewes and 103 does were assessed both clinically and using the California Mastitis Test (CMT) for evidence of subclinical mastitis. About 10 ml of milk was aseptically collected from all the affected quarters and halves into labelled sterile universal bottles as described earlier [44]. The samples were kept on ice and transported to the Microbiology Laboratory of Federal College of Animal Health and Production Technology Vom for further processing.

Culture and biotyping

Bacteriological examination was done according to Geser et al. [17]. Briefly, one ml of each milk sample was inoculated into 9 ml of sterile Peptone Water for enrichment and incubated overnight at 37 °C. A loopful of broth culture was streaked on sterile MacConkey Agar (Oxoid, UK) and Eosin Methylene Blue (EMB) Agar (Oxoid, UK) plates using the quadrant streaking method and incubated at 37 °C under aerobic condition. The plates were checked for bacterial growth after 24, 48 and 72 hours to rule out slow growing bacteria. The colonies were examined for morphological features such as size, shape, and colour. Pink colonies on MacConkey Agar, and greenish metallic sheen, purple, pink, blue-black, and orange colonies on EMB were sub-cultured respectively on freshly prepared MacConkey Agar and EMB Agar plates and incubated at 37 °C for 24 hours to obtain pure culture of coliform isolates. Representative colonies were stored on slant of Nutrient Agar and kept 4 °C until required for further work [11].

Presumptive coliforms by Gram stain were subjected to conventional biochemical tests, namely gelatine liquefaction, nitrate reduction, urease production, oxidase, indole-methyl-red-Voges-Proskauer (IMVP), catalase, citrate agar, and sugar fermentation tests [31].

Identities of the presumptive coliforms were further confirmed using Oxoid™ Microbact™ GNB 24E according to the manufacturers' instructions. Briefly, 1—3 distinct colonies were picked from an 18—24 hours old culture and emulsified in 5.0 ml sterile saline and mixed thoroughly to obtain a homogeneous suspension. The plate containing the substrates was placed in a holding tray and using a sterile Pasteur pipette 4 drops (approximately 100 µl) of the bacterial suspension were added. The substrates underlined in the black wells were overlaid with sterile mineral oil except for well 20 which was used for the detection of oxidase-positive and miscellaneous Gram-negative bacteria. Incubation was done at 37 °C for 18—24 hours [5]. The results were read and interpreted according to the manufacturers' instructions.

Antimicrobial susceptibility test

An antibiotic sensitivity test was carried out on the coliforms isolated using the disc diffusion method. The antibiotics employed were ofloxacin (5 µg), pefloxacin (5 µg), ciprofloxacin (5 µg), amoxicillin/clavulanic acid (30 µg), gentamycin (10 µg), streptomycin (10 µg). Colonies of the isolated coliforms were suspended in bottles of 9ml sterile peptone water, standardized to 0.5 McFarland and incubated for 4 hours. A sterile swab stick was dipped into the standardized inoculum and excess fluid removed from the swab by pressing it on the side of the bottle. The swab was used to spread on the entire surface of dried Mueller Hinton agar plate and allowed to stand for 30 minutes. The antibiotic discs were placed on the surface of the seeded plates aseptically 15 mm apart. The plates were then incubated at 37 °C for 24 hours. The diameter of the zone of inhibition around each disc was measured in millimetres (mm) and compared against a reference standard [8].

Phenotypic assay for ESBL production

The phenotypic assay for ESBL production was assessed as earlier described [14]. Inocula of the isolated coliforms that have already been standardized to 0.5 McFarland standards were inoculated on Brilliance ESBL Chromogenic Culture Medium (Oxoid, UK). Inoculated plates were incubated at 37 °C under aerobic condition for 24 hours and 48 hours. Change in the colour of colonies was observed and interpreted according to the guidelines provided by Oxoid, UK. *E. coli* ATCC 25922 and *Klebsiella pneumoniae* ATCC 700603 were used as negative and positive controls, respectively.

PCR assays for TEM and CTX-M beta-lactamase genes

Conventional PCR was used for the amplification and detection of $bla_{\text{CTX-M}}$, bla_{SHV}, bla_{TEM} genes as described previously [24, 38, 42]. The primers (Table 1) and expected product sizes are listed in table S1. The reaction was performed on a GeneAmp PCR System 9700 thermocycler

Table 1. Primers used for amplification of ESBL genes

Target	Primer name	Sequence (5'—3')	Product size	References
bla_{TEM}	TEM-F	TCCGCTCATGAGACAATAACC	931 bp	[22]
	TEM-R	TTGGTCTGACAGTTACCAATGC		
bla_{CTX-M}	CTX-F	CGC TTT GCG ATG TGC AG	550 bp	[34]
	CTX-R	ACC GCG ATA TCG TTG GT		
bla_{SHV}	SHV-F	TGGTTATGCGTTATATTCGCC	868 bp	[22]
	SHV-R	GGTTAGCGTTGCCAGTGCT		

(Applied Biosystems, USA) under the following conditions: Initial denaturation at 95 °C for 4 minutes followed by 35 cycles of denaturation were at 95 °C for 1 minute, annealing at 48 °C for bla_{TEM} and 60 °C for bla_{SHV} and bla_{CTX-M} for 1 minute. Primer extension was at 72 °C for 1 minute and the final extension step at 72 °C for 5 minutes for all the genes. Amplified genes were separated by gel electrophoresis in 1% agarose gel in TBE 0.5X (Tris/borate/EDTA) buffer. *Klebsiella pneumoniae* ATCC 700603 containing bla_{SHV}, bla_{CTX-M} and bla_{TEM} genes was used as positive control while *Escherichia coli* ATCC 25922 not containing the bla_{SHV}, bla_{CTX-M} and bla_{TEM} genes was used as negative control. PCR products were detected with ethidium bromide fluorescence using the Bio-imaging system (VWR-Syngene, USA).

Sequencing and sequence analyses

Amplicons of the appropriate sizes were sequenced in both directions at a commercial sequencing company (Macrogen, Netherlands) using the PCR primers. The raw sequences were manually edited using the BioEdit v.7.2.3 program [21] and were compared and matched to annotated sequences in the GenBank™ database using the BLASTn search algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequences generated in this study were submitted to GenBank under the accession numbers; bla_{TEM} (MZ310390—MZ310395); bla_{CTX-M} (MZ310396—MZ310406).

Pairwise identities and phylogeny

The bla_{TEM} and bla_{CTX-M} gene sequences obtained in this study and those within the database were aligned using Clustal W [41]. The analysis of pairwise similarities between the sequences from Nigeria was performed using SDT v.1.2 [30] with pairwise gap deletions. Phylogenetic relationships were inferred using the maximum likelihood method based on the Jukes-Cantor model implemented in MEGA6 with bootstrap replicate values set at 1,000 [39].

Analyses of population genetics

The genetic structure and diversity of the two genes within the populations were estimated via nucleotide and haplotype diversity, the number of polymorphic sites, number of mutations, population mutation estimates based on the number of segregating sites and mutations, and the number of nucleotide differences between sequences. These were estimated in DnaSP v5.10.01 [26]. The NAsp v5.10.01 was used to calculate the Tajima's D, Fu and Li's F* and D*, and Fu's Fs to determine the deviation of the two antimicrobial genes from neutrality assuming a constant population size, with zero recombination and migration [33].

Data analysis

The Statistical Package for Social Science (SPSS) version 23 software was used to analyse data collected. Chi-square test (χ^2), and P values < 0.05 were considered statistically significant.

RESULTS

Isolation and phenotypic identification of coliforms

The percentage occurrence of clinical mastitis in cows, ewes, and does were 0.2%, 0%, 1.5% respectively, while the percentage occurrence of subclinical mastitis in ruminants were 18.1%, 28.2% and 38.3% respectively. A total of 677 coliforms were isolated from 1052 milk samples

collected from ruminants in Plateau State of Nigeria. The high number of coliforms isolated may due to the type of housing system practiced by the pastoralists in this part of the world. Table 2 showed that *Escherichia coli* was the most commonly (44.0%) isolated coliform from the milk samples analysed. This was followed by *K. pneumoniae* (23.6%), *C. freundii* (11.2%), *K. aerogenes* (10.1%), *K. oxytoca* (7.1%), *E. cloacae* (2.2%) and *S. marcescens* (1.8%). Based on the study location, 183 (72.9%) of the positive samples were isolated from Plateau North, 160 (20.5%) from Plateau Central and 334 (82.3%) from Plateau South (Table 3).

Beta-lactamase production

From the total of 677 coliforms isolated, 212 (31.3%) were ESBL producers. *E. coli* had the highest prevalence of 48.58%, followed by *K. pneumoniae* (18.40%), *C. freundii* (12.74%), *K. aerogenes* (8.96%), *S. marcescens* (5.66%), *K. oxytoca* (3.77%) and *E. cloacae* (1.89%). The milk samples obtained from Plateau South had the highest number (n = 132; 62.3%) of ESBL-producing coliforms while Plateau Central had the least (n = 38; 17.9%) (Table 7).

Antibiotic susceptibility testing of ESBL producing coliforms

Four out of the seven coliforms, *E. coli*, *C. freundii*, *K. aerogenes* and *S. marcescens* isolated from ruminant mastitis in this study showed the highest resistance to gentamycin (Table 4). *K. pneumonia* showed the highest resistance equally to amoxicillin-clavulanic acid and streptomycin. These are antibiotics commonly used in veterinary practice in Nigeria.

Occurrence of ESBL variant genes in ruminant mastitis

ESBL producing genes were identified in 30 coliforms isolated from milk samples obtained from ruminants with mastitis, by conventional PCR. The bla_{CTX-M} gene had a higher prevalence of 24.39% than bla_{TEM} with 12.19% in the samples screened in this study. However, the bla_{SHV} gene was not detected (Table 8).

Clonal diversity of CTX-M and TEM genes of isolated coliforms

The pairwise identities within the bla_{TEM} gene sequences in this study ranged from 93.3% to 97.6% (Fig. 1). However, the bla_{CTX-M} gene sequence similarities ranged from 86.0% between to 99.5% (Fig. 2). Phylogenetic analyses showed that the bla_{TEM} and bla_{CTX-M} gene sequences in this study formed four clusters along with sequences in GenBank indicating putative taxonomic and evolutionary inferences within the populations (Figs. 3 and 4).

Further evaluation of the genetic diversity within the bla_{TEM} and bla_{CTX-M} genes form ruminants in Plateau State show that 6 haplotypes were identified within the bla_{TEM} isolates while 11 were present within the bla_{CTX-M} sequences. This shows that each isolate represented individual haplotypes. The numbers of segregating sites within the bla_{TEM} isolates (86) and bla_{CTX-M} (88) were almost similar while analogous trends were also observed for the total number of mutations and nucleotide diversity (Table 5). Both sets of genes had haplotype diversity at exactly 1.000.

Table 2. Frequency of coliforms from milk samples of ruminants

Bacteria	n = 677	
	Number	[%]
Escherichia coli	298	44.0
Klebsiella pneumoniae	160	23.6
Klebsiella oxytoca	48	7.1
Citrobacter freundii	76	11.2
Klebsiella aerogenes	68	10.1
Enterobacter cloacae	15	2.2
Serratia marcescens	12	1.8

χ^2 = 747.925; P < 0.000—statistically significant

Table 3. Distribution of coliform isolates from different locations in the study area

Zone	Number samples	Number of coliforms	[%]
Plateau North	251	183	72.9
Plateau Central	395	160	20.5
Plateau South	406	334	82.3
Total	**1,052**	**677**	**64.4**

χ^2 = 747.925; P < 0.000—statistically significant

Table 4. Percentage of resistant ESBL coliforms isolated from ruminant mastitis to some antibiotics

Isolate	No. of isolate	OFX [%]	PEF [%]	CPX [%]	AMC [%]	CN [%]	S [%]
E. coli	124	69 (55.64)	53 (42.74)	46 (37.09)	59 (47.58)	89 (71.77)	80 (64.51)
K. pneumoniae	66	36 (54.54)	31 (46.97)	33 (50.00)	44 (66.67)	34 (51.51)	44 (66.67)
K. oxytoca	8	5 (62.50)	3 (37.50)	4 (50.00)	2 (25.00)	5 (62.50)	6 (75.00)
C. freundii	45	28 (62.22)	29 (64.44)	22 (48.89)	27 (60.00)	33 (73.33)	29 (64.44)
K. aerogenes	14	11 (78.57)	7 (50.00)	7 (50.00)	5 (35.71)	12 (85.71)	11 (78.57)
E. cloacae	4	3 (75.00)	1 (25.00)	2 (50.00)	1 (25.00)	2 (50.00)	3 (75.00)
S. marcescens	11	3 (27.27)	1 (9.09)	3 (27.27)	3 (27.27)	9 (81.82)	7 (63.63)

OFX—Ofloxacin; PEF—Pefloxacine; CPX—Ciprofloxacin; AMC—Amoxicillin-Clavulanic acid; CN—Gentamycin; S—Streptomycin

Table 5. Genetic variability determinants of two antimicrobial resistance genes amplified from bacteria isolates obtained from ruminant animals in Plateau State, Nigeria

Population	N	H	S	Hd	Eta	Π	K	θ-W	θ-Eta
***bla*$_{TEM}$ gene** (n = 6)	935	6	86	1.000	90	0.04501	37.0000	0.04795	39.4161
***bla*$_{CTX-M}$ gene** (n = 11)	551	11	88	1.000	98	0.05131	28.2182	0.06083	33.4589

N—Number of nucleotide sites, h—Number of haplotypes, S—Number of variable sites, Hd—Haplotype diversity, Eta—Total number of mutations, π—Nucleotide diversity, k—Average number of nucleotide differences between sequences, θ-W—Waterson's estimate of population mutation rate based on the total number of segregating sites, θ-Eta—Waterson's estimate of population mutation rate based on the total number of mutations

Table 6. Neutrality tests on the two antimicrobial resistance genes amplified from bacteria isolates obtained from ruminant animals on Plateau State, Nigeria

Population	Tajima's D	Fu and Li's D	Fu and Li's F
***bla*$_{TEM}$ gene** [n = 6]	–0.3958	–0.2485	–0.3076
***bla*$_{CTX-M}$ gene** [n = 11]	–0.7504	0.2167	–0.0365

Table 8. Distribution of PCR-identified ESBL genes from ruminants with mastitis in Nigeria

Coliform	No. examined	No. of ESBL genes [%]	
		***bla*$_{TEM}$**	***bla*$_{CTX-M}$**
E. coli	30	4 (13.33)	4 (13.33)
K. pneumoniae	22	1 (4.55)	9 (40.90)
K. oxytoca	4	1 (25.00)	2 (50.00)
K. aerogenes	14	2 (14.28)	1 (7.14)
C. freundii	8	1 (12.50)	2 (25.00)
S. marcescens	4	1 (25.00)	2 (50.00)
Total	**82**	**10 (12.19)**	**20 (24.39)**

Table 7. Occurrence of ESBL-producing coliforms isolated from ruminant mastitis in Plateau State, Nigeria

Sampling location	No. of positive samples [%]	Isolate [%]						
		E. coli	*K. pneumoniae*	*K. oxytoca*	*C. freundii*	*K. aerogenes*	*E. cloacae*	*S. marcescens*
P. North	42 (19.8)	21 (50)	9 (21.43)	1 (2.38)	4 (9.52)	6 (14.28)	0 (0.0)	1 (2.38)
P. Central	38 (17.9)	28 (73.68)	6 (15.79)	0 (0.0)	2 (5.26)	2 (5.26)	0 (0.0)	0 (0.0)
P. South	132 (62.3)	54 (40.90)	24 (18.18)	7 (5.30)	21 (15.90)	11 (8.33)	4 (3.03)	11 (8.33)
Total	**212**	**103 (48.58)**	**39 (18.40)**	**8 (3.77)**	**27 (12.74)**	**19 (8.96)**	**4 (1.89)**	**12 (5.66)**

P. North—Plateau North; P. Central—Plateau Central; P. South —Plateau South

Table 9. Properties of bla_{TEM} and bla_{CTX-M} gene sequences obtained from bacterial species isolated from ruminants with mastitis in Plateau State, Nigeria

Gene	Organism	Study location	Sequence ID	Host	Accession Number	Number of nucleotides
bla_{TEM}	*K. pneumoniae*	North	NG-JN-sh1	Sheep	MZ310390	870
bla_{TEM}	*K. aerogenes*	North	NG-JS-cw1	Cow	MZ310391	870
bla_{TEM}	*E. coli*	South.	NG-LS-cw	Cow	MZ310392	877
bla_{TEM}	*C. freundii*	South	NG-QP-shp	Sheep	MZ310393	861
bla_{TEM}	*K. aerogenes*	South	NG-QP-sh1	Sheep	MZ310394	837
bla_{TEM}	*K. pneumoniae*	North	NG-JN-gt	Goat	MZ310395	935
bla_{CTX-M}	*E. coli*	South	NG-LS-cwa	Cow	MZ310396	551
bla_{CTX-M}	*K. pneumoniae*	North	NG-JS-cw	Cow	MZ310397	550
bla_{CTX-M}	*E. coli*	South	NG-LS-cwb	Cow	MZ310398	551
bla_{CTX-M}	*E. coli*	South	NG-QP-gt	Goat	MZ310399	551
bla_{CTX-M}	*K. pneumoniae*	South	NG-QP-gt	Goat	MZ310400	550
bla_{CTX-M}	*K. pneumoniae*	North	NG-JN-gt	Goat	MZ310401	550
bla_{CTX-M}	*E. coli*	Central	NG-KN-cwa	Cow	MZ310402	551
bla_{CTX-M}	*E. coli*	South	NG-LS-cw	Cow	MZ310403	551
bla_{CTX-M}	*K. pneumoniae*	North	NG-JN-cw	Cow	MZ310404	551
bla_{CTX-M}	*C. freundii*	South	NG-QP-cw	Cow	MZ310405	551
bla_{CTX-M}	*E. coli*	Central	NG-KN-cw	Cow	MZ310406	551

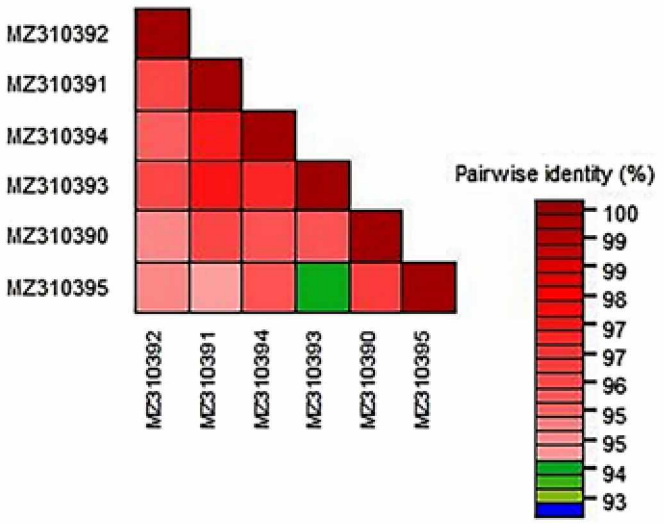

Fig. 1. Pairwise of all *bla*$_{TEM}$

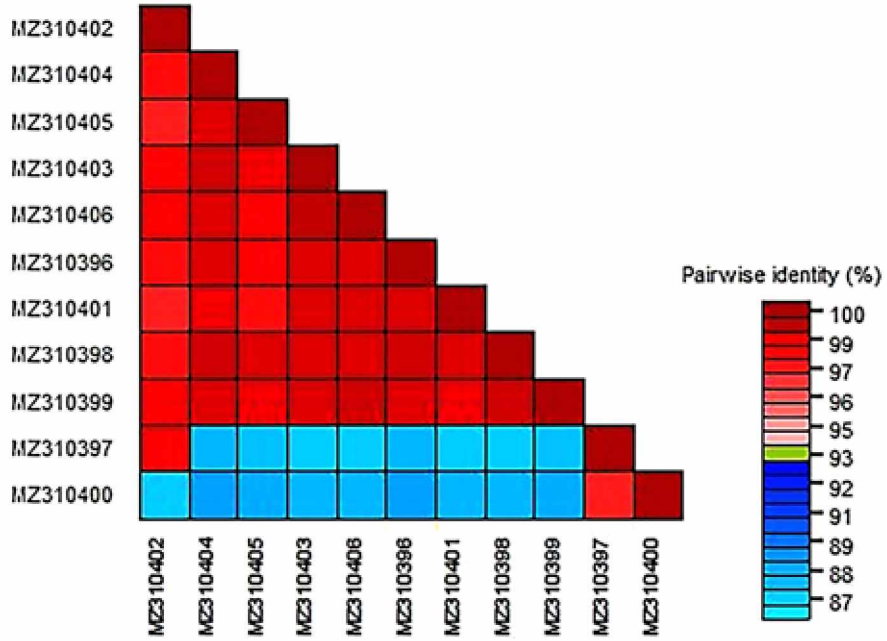

Fig. 2. Pairwise of all *bla*$_{CTX-M}$

Nucleotide polymorphisms within the two gene population, as showing via the test of neutrality, show negative Tajima's D (–0.3958), Fu and Li's D (–0.2485) and Fu and Li's F (–0. 3076) statistic values for the *bla*$_{TEM}$ isolates (Table 6). Similar observations were obtained from the *bla*$_{CTX-M}$ genes, except for Fu and Li's D with positive values of 0.2167. Neutrality tests revealed negative values for the worldwide isolates, which did not statistically deviate from zero ($P > 0.10$). Negative Tajima's D statistic indicates superfluous low-frequency polymorphism triggered by background selection, genetic hitchhiking, or population expansions.

DISCUSSION

In this study, coliforms of veterinary and public health importance were isolated from milk samples of ruminants

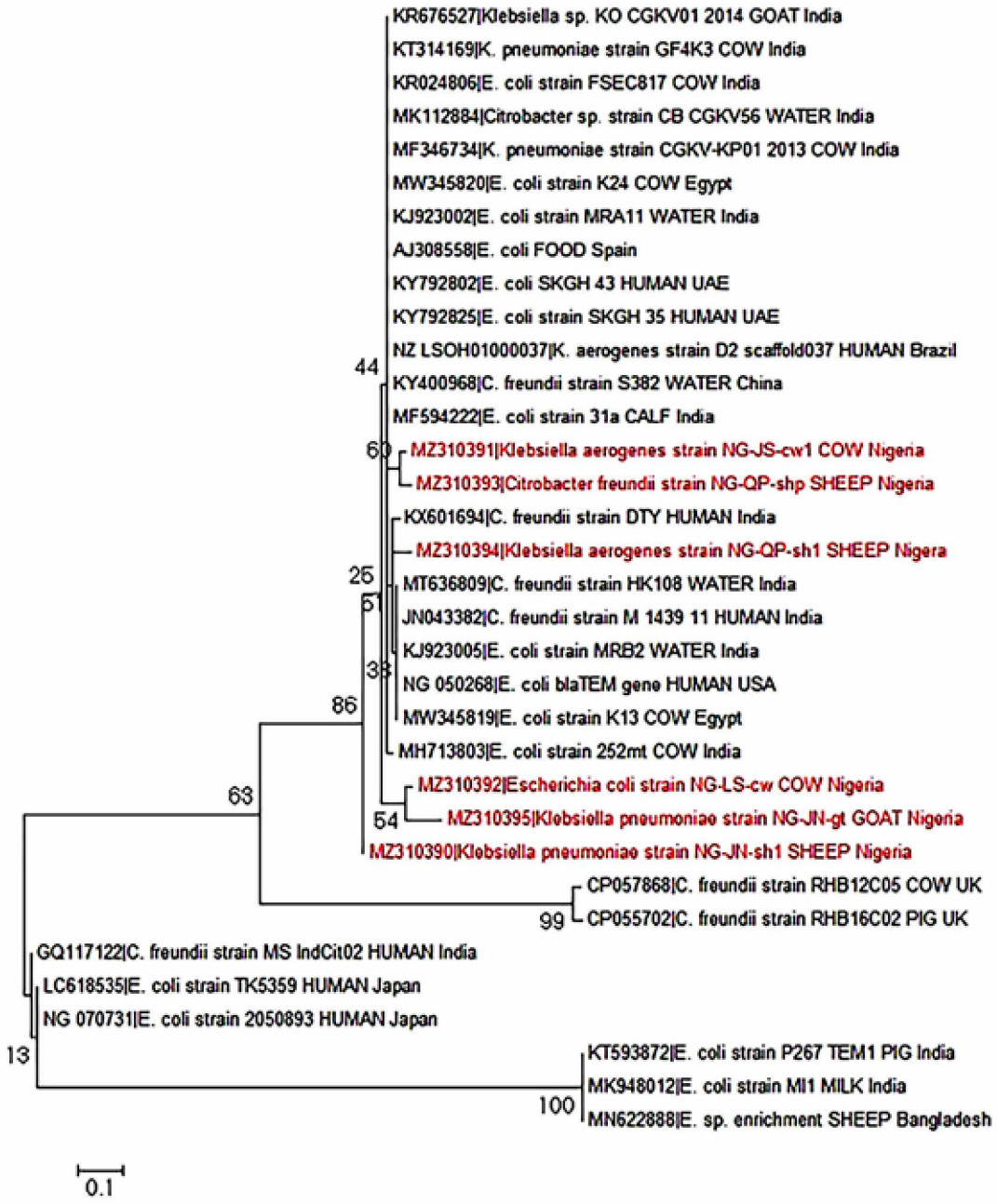

Fig. 3. Phylogenetic analysis of bla_{TEM} genes of coliforms isolated from milk obtained from ruminants with mastitis in Nigeria

with mastitis. Among the ESLB- producing coliforms isolated, *E. coli* had the highest prevalence of 48.6% in all the milk samples from the various ruminants examined in this study. Similar findings have been reported for milk obtained from mastitic cows from different regions of the world [3, 17, 25, 27]. However, the prevalence of *E. coli* in this study is higher than the 32.2% for samples obtained from apparently healthy poultry workers, chickens and environment in Abuja, a neighbouring city to this study area [4]. But, the prevalence reported herein is within the range of 32.2 to 75.5% from previous studies involving different samples from various animal species and man, thus, underscoring the role of *E. coli* as the leading cause of antimicrobial resistance along the food value chain [4, 12, 20].

Additionally, there was an association between the prevalence of *E. coli* and the animal species and study location. The prevalence was 43.1%, 40% and 38% for does, cows and ewes, respectively. Similarly, the preva-

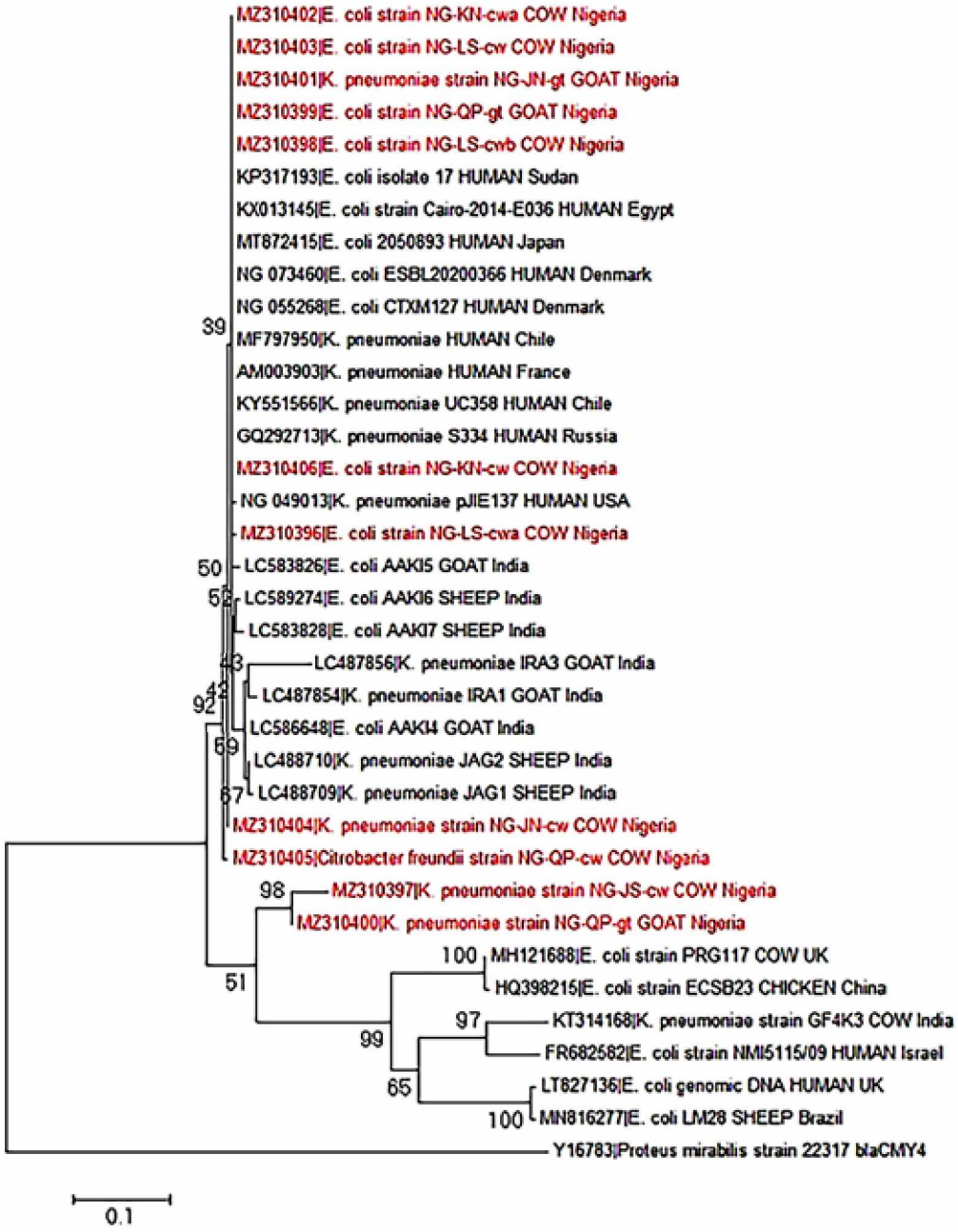

Fig. 4. Phylogenetic analysis of bla_{CTX-M} genes of coliforms isolated from milk obtained from ruminants with mastitis in Nigeria

lence of *E. coli* was higher in the Southern (82.3%), than the Northern (72.9%) and the central (20.5%) regions of Plateau State, Nigeria. These variations can be attributed to environmental conditions, socio-cultural practices, species variation and differences in type and use of antimicrobials. Generally, the profile of coliforms recorded in this study is similar to earlier reports from various regions: Africa [16], Asia [38], and South America [35]. However, the prevalence and diversity of the coliforms differed according to the source of samples analysed and animal host.

Two resistant genes bla_{CTX-M} and bla_{TEM} were characterized from coliforms isolated from milk samples in this study. The overall prevalence of bla_{CTX-M} and bla_{TEM} in ruminant mastitis was 24.39% and 12.19% respectively. Higher prevalence of bla_{CTX-M} reported herein is in agreement with the report of A1i et al. [3], but contrary to that of Yu et al. [43] where bla_{CTX-M} was more prevalent in milk samples from bovine mastitis. On a general note, recent studies have reported the detection of CTX-M genes from different sources [4, 12, 20]. This could be attribut-

ed to the ability of this gene to undergo point mutation in the environment [12]. Phylogenetic analyses showed that some of the bla_{CTX-M} and bla_{TEM} gene sequences from the ruminants in this study have common ancestry with genes from human from different parts of the world. The high-level of phylodiversity observed among ruminants means that there is transfer of coliforms harbouring the CTX-M and TEM genes which might have been transmitted from clones of varying origins [23]. Surprisingly, the bla_{SHV} gene was not detected in any of the samples screened in this study. Similar to our results, a recent study that analysed samples from chickens, humans and the environment did not report the bla_{SHV} gene [4]. Furthermore, lower prevalence of bla_{SHV} (14.2%) than bla_{TEM} (26.1%) and bla_{CTX-M} (73.0%) was reported in isolates from animals in Pakistan [12], a similar trend, 27.54% (bla_{SHV}), 57.97% (bla_{TEM}) and 72.46% (bla_{CTX-M}) was reported for isolates from broiler in Philippines [20]. The reason for the non-detection of the bla_{SHV} gene in isolates in this study is not readily deducible and, is the subject of further studies in this country.

Multidrug resistance was observed in most of the coliforms tested in this study. High resistance by most of the coliforms was particularly recorded for ofloxacin, gentamycin and streptomycin. The majority of the isolates exhibited the highest prevalence (72—85%) of resistance to gentamycin. This is not surprising as gentamycin is one of the commonest antimicrobials used in veterinary and medical practice in Nigeria and may be subject to abuse. However, lower resistance was shown against the drugs that are rarely used in veterinary practice in Nigeria such as pefloxacine, ciprofloxacin and amoxicillin-clavulanic acid. Therefore, veterinarians should consider the use of these compounds whenever handling cases that are unresponsive to routinely used antimicrobials. Of concern is the public health implications of this finding, considering the fact that consumption of raw unpasteurized milk is a common practice in some cultures in Nigeria. Such practise will facilitate the transfer of multidrug resistant coliforms to humans resulting in complications of treatment outcomes.

CONCLUSIONS

A high prevalence of ESBL-producing coliforms was detected in milk samples from ruminants with mastitis in Nigeria. Two resistance genes; bla_{CTX-M} and bla_{TEM} associated ESBL producing agents were characterized revealing a high phylodiversity among the isolated from Nigeria in comparison to sequences from other parts of the world. Future studies should consider other resistance genes associated with agents of ruminant mastitis to provide a holistic picture for effective control strategies. A high resistance to commonly used antibiotics in veterinary practice is worrisome and calls for urgent steps to reverse this trend. Public enlightenment coupled with advocacy on responsible antimicrobial stewardship are needed to sensitize the general public on the dangers associated with the abuse of antimicrobials along the food value chain in Nigeria. Also some socio-cultural practices such as consumption of raw milk should be discouraged.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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