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Article

Genotyping of ESBL-Producing *E. coli* from Food-producing Animals, Animal Food Products and Humans in South-West, Nigeria

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Abstract

Extended-spectrum beta-lactamase- (ESBL-) producing *Escherichia coli* strains are emerging globally in both humans and animals. The use of antibiotics in animal production and treatment has led to this phenomenon. This study aimed at determining the resistance patterns of *E. coli* isolates from humans, food-producing animals, and their food products in South-western Nigeria. The prevalence and distribution of antibiotic-resistant *E. coli* in three categories were evaluated: Animals (goats, pigs, poultry, cattle, sheep), Humans (butchers, meat sellers, animal farm workers, buyers), and Animal food products (milk, cheese, beef, chicken, yogurt) from selected animal farms in South-west Nigeria. Out of a total number of 280 samples that were collected, 216 *E. coli* strains were isolated. The prevalence of isolated *E. coli* from humans (96%) was higher than that from animals (89%) and about 38.8% were isolated from animal food products. Out of the 216 *E. coli* isolates that were obtained from the different sources, 60 (27.8%) were multiple drug-resistant and were also ESBL- positive. Seven resistance genes were amplified in the multi-drug resistant *Escherichia coli* isolates**:** *TEM* (61.7%), *CTX-M-15* (51.7%), *AAC-6-LB* (43.3%), *CTX-M-1* (38.3%), *CTX-M-9* (33.3%), *CTX-M-2* (21.7%) and *SHV* (11.7%). The results suggest the need for continuous surveillance of antibiotic resistance to curtail the spread of resistance bacteria.

Keywords: E. coli, Extended-spectrum beta-lactamase, Antibiotic resistance, Resistance genes, Nigeria.

1. Introduction

Escherichia coli is a commensal bacterium in the intestinal tracts of mammals and certain pathogenic *E. coli* strains are associated with several diseases in animals and humans. Antibiotics are usually used for disease treatment and growth promotion in animal production. Their overuse and misuse can lead to the selection of resistant strains and the proliferation of antibiotic resistance in human and animal *E. coli* strains [1]. The use of third and fourth-generation cephalosporins in food-animal production has led to the further emergence of multidrug resistance among these *E. coli* strains which produce enzymes that hydrolyze the β-lactam ring of such antibiotics [2].

Drug-resistant *E. coli* isolates may constitute a significant reservoir of antibiotic resistance determinants which can spread to other bacteria pathogenic for animals and/or humans [3] and food products.

Reports of surveillance studies conducted in different countries all over the world showed an increase in the level of *E. coli* resistant isolates to major classes of antibiotics used for the treatment of livestock and companion animals [3].

Although there have been several reports on the prevalence and antibiotic resistance of *E. coli* in Nigeria, we seek to characterize the molecular basis of these resistance traits in order to determine the possibility of their transfer from animals to humans and vice versa.

2. Methods

2.1 Study population

Four major animal farm locations (Oyo State: Ogbomoso and Ibadan, Osun State: Osogbo and Ilesa) in South-western Nigeria were sampled between July, 2015 and June, 2016. These States are situated in the Tropical Rainforest belt of the South-western part of Nigeria and lie approximately on latitude 7º 46' 12"N and longitude 4º 34' 54"E. These farms provide most of the animals and animal-related food products to the residents of the towns (approximate population of 1.2 million) in the States. Prior to the commencement of the study, permission was obtained from the Management Boards of the farms in Osun State.

2.2. Ethical Concerns

Ethical approval of this study was obtained from the National Ethical Committee of the Osun State Ministry of Health. Verbal informed consent was obtained from all participating subjects including custodians of animal subjects. The inclusion of participants in all categories listed in this study was based on informed consent given by the participants while individuals who did not give their consent were excluded.

2.3 Chemicals and standards

All solvents and reagents used in this study were of analytical grade and the media and antibiotic sensitivity discs were purchased from Oxoid Ltd. (Oxoid Deutschland GmbH, Germany).

2.4 Sample collection, isolation and identification of E. coli

A total of 280 samples from fecal and food origin were collected from the four different farm locations and three major sources: Animal category (goats, pigs, poultry, cattle and sheep), Human category (butchers, meat sellers, animal farm workers, buyers) and Animal product category (milk, cheese, beef, chicken, yoghurt). Samples were collected in sterile containers on ice and transported into the laboratory. The samples were routinely cultured in buffered peptone water and incubated aerobically overnight at 37 ^{o}C. Subcultures were made on selective plates using CHROMagar orientation medium (Mast Diagnostica GmbH, Reinfeld, Germany), and presumptive *E. coli* colonies selected underwent further identification tests as previously described [4].

2.5 Antibiotic susceptibility testing of the isolates

Antimicrobial susceptibility testing was done using the Kirby-Bauer disc diffusion method on Mueller Hinton agar. This was carried out according to the guidelines of the Clinical and Laboratory Standards Institute [5] using discs containing several different antibiotics. Zone diameters of inhibition to each disc were recorded and strains were classified as resistant or susceptible according to the criteria of CLSI [5]. *Escherichia coli* ATCC 25922 was used as quality control.

2.6 ESBL Confirmation by Combination Disk Method

The isolates showing reduced susceptibility to ceftazidime or cefotaxime were tested for ESBL production by the combination disk method according to CLSI guidelines [5]. Combination disk method was performed using four disks: cefotaxime (CTX) (30 μg), cefotaxime (30 μg) + clavulanic acid

(10 µg), ceftazidime (CAZ) (30 µg), and ceftazidime (30 µg) + clavulanic acid (10 µg). A 5 mm increase in zone diameter for the tested antimicrobial agent (CAZ or CTX) in combination with clavulanic acid versus its zone when tested alone was considered as ESBLs positive. Quality control for ESBL production was performed using *E. coli* ATCC 25922 as a negative control.

2.7 DNA extraction

The genomic DNA of the *Escherichia coli* isolates was extracted by boiling method. This was done by picking about 20 colonies suspended in 500 µl of sterile distilled water at 100 °C for 15 min. The boiled suspension was rapidly cooled on ice chips and then centrifuged at 16,000 rpm for 3 min after which the supernatant was collected. The DNA lysate was stored at -20 °C for the duration of this work.

2.8 Polymerase Chain Reaction for the detection of genes

Polymerase chain reaction (PCR) for the detection of CTXMs, aac-6-lb, TEM and SHV was carried out on multiple resistant strains of *E. coli* using DNA lysate, specific primers and appropriate PCR conditions specific to the targeted genes. Amplification reactions were performed in a total volume of 25 µL of reaction mixture containing 5 µL of 10 × PCR buffer, 2.5 mM $MgCl_2$, 200 mM dNTP, and 1.25 units of Taq polymerase, 10 pmol of each primer and 1 µL of the sample DNA. The primer details and PCR conditions with expected base pairs are shown in Table 1. Positive and negative controls were used for each of the genes.

Table 1: Primer sequences, their molecular sizes and their different conditions of amplification

Primer	Sequence 5^1 - 3^1	Product size (bp)	PCR conditions	Reference
TEM-2 F TEM-2 R	ATCAGCAATAAACCAGC CCCCGAAGAACGTTTTC	516	94 5′ 1 circle 94 30″ 51 30″ 30 circles 72 30″ 72 7′ 1 circle	Colom *et al.,* [6]
SHV F SHV R	AGGATTGACTGCCTTTTTG ATTTGCTGATTTCGCTCG	392	94 5′ 1 circle 94 30″ 56 30″ 30 circles 72 30″ 72 7′ 1 circle	Colom *et al.,* [6]
CTX-M-15 F CTX-M-15 R	CACCTCATGTTTGAATTCGCC CTCTGTCACATCGAAATCGC	996	94 5′ 1 circle 94 30″ 57 30″ 30 circles 72 30″ 72 7′ 1 circle	Moodley *et al.,* [7]
CTX-M-9 F CTX-M-9 R	GTGACAAAGAGAGTGCAACGG ATGATTCTCGCCGCTGAAGCC	500	94 5′ 1 circle 94 30″ 60 30″ 30 circles 72 30″ 72 7′ 1 circle	Moodley *et al.,* [7]
CTX-M-2-F CTX-M-2-R	GGACGCTACCCCTGCTATT CCAGCGTCAGATTTTTCAGG	400	94 5′ 1 circle 94 30″ 55 30″ 30 circles 72 30″ 72 7′ 1 circle	Moodley *et al.,* [7]
CTX-M-1-F CTX-M-1-R	GACGATGTCACTGGCTGAGC AGCCGCCGACGCTAATACA	585	94 5′ 1 circle 94 30″ 55 30″ 30 circles 72 30″ 72 7′ 1 circle	Moodley *et al.,* [7]
aac-6-lb F aac-6-lb R	TTGCGATGCTCTATGAGTGGCTA CTCGAATGCCTGGCGTGTTT	482	94 5′ 1 circle 94 30″ 55 30″ 30 circles 72 30″ 72 7′ 1 circle	Park *et al.,* [8]

3. Results

3.1 The distribution of E. coli isolates according to the subject categories and study sites

Out of a total number of 280 samples that were collected {fecal samples: animals (100), humans (100); animal food products (80)}, 216 (77.1%) *E. coli* strains were isolated (Table 2). The prevalence of isolated *E. coli* from humans (44.44%) was higher than from animals (41.20%) and about 14.35% were isolated from animal food products. Ibadan had the highest prevalence of *E. coli* (27.31%) followed by Ilesa (25.46%) and then Osogbo (25.00%) greater than Ogbomoso (22.22%).

Table 2: The distribution of *E. coli* isolates according to the subject categories and study sites

Category	Presence of *E. coli*: *E. coli* positive samples/number of samples				
	Ibadan	**Ogbomoso**	**Osogbo**	**Ilesa**	**Total**
Animal					
Cow	5/5	3/5	5/5	5/5	18/20
Goat	5/5	3/5	3/5	5/5	16/20
Pig	5/5	4/5	5/5	5/5	19/20
Poultry	5/5	4/5	4/5	5/5	18/20
Sheep	5/5	3/5	5/5	5/5	18/20
Total	**25/25**	**17/25**	**22/25**	**25/25**	**89/100(41.2%)**
Human					
Meat seller	5/5	4/5	5/5	4/5	18/20
Butcher	5/5	5/5	5/5	5/5	20/20
Animal farm worker	5/5	5/5	5/5	5/5	20/20
Non-farm worker	5/5	5/5	5/5	4/5	19/20
Buyer	5/5	5/5	5/5	4/5	19/20
Total	**25/25**	**24/25**	**25/25**	**22/25**	**96/100(44.4%)**
Food product					
Milk	2/4	2/4	0/4	1/4	5/16
Beef	4/4	2/4	¾	3/4	12/16
Chicken	1/4	1/4	2/4	3/4	7/16
Cheese	1/4	2/4	2/4	1/4	6/16
Yoghurt	1/4	0/4	0/4	0/4	1/16
Total	**9/20**	**7/20**	**7/20**	**8/20**	**31/80(14.4%)**
All samples	**59(27.3%)**	**48(22.2%)**	**54(25.0)%**	**55(22.5%)**	**216/280(77.1%)**

3.2. Prevalence of multi-drug resistant ESBL-producing strains among the E. coli isolates

Out of the 216 *E. coli* isolates, 60 (27.8%) were multiple drug-resistant and were also ESBL- positive. The sources included humans 23(38.3%), goats 8(13.3%), sheep 1(1.7%), pigs 8(13.3%), poultry 7(11.7%), cows 6(10.0%) and beef 7(11.7%) (Table 3). The prevalence of isolating the 60 ESBL-producing strains was highest in the animal category (33.7%) followed by the human category (24.0%) and the animal food products (22.6%). These multidrug-resistant strains were most prevalent in Ibadan (38.9%) > Ilesa (30.9%) > Osogbo (20.4%) > Ogbomosho (18.8%)

3.3 Antibiotic resistance patterns of the multi-drug resistant ESBL E. coli isolates

All 60 *Escherichia coli* isolates (100%) were phenotypically resistant to clindamycin and penicillin. In contrast, the isolates exhibited 100% susceptibility to imipenem (Figure 1). High rates of resistance were also experienced for ceftazidime, tobramycin, cefazolin, enrofloxacin, levofloxacin, sulfamethoxazole/ trimethoprim, kanamycin, cefuroxime, piperacillin/tazobactam, ampicillin, cefalexin, streptomycin, doxycycline, neomycin, spectinomycin, amoxycillin/ clavulanate, sulfamethoxazole, ampicillin/sulbactam, cefotaxime, ticarcillin, ciprofloxacin, trimethoprim and tetracycline (50% -

98.3%). Low levels of resistance rates were recorded for amikacin, cefoxitin, chloramphenicol, gentamicin and meropenem (25% - 41.7%).

Table 3: Prevalence of multi-drug resistant ESBL-producing strains among the *E. coli* isolates

Category	Number of ESBL *E. coli*/ total number of *E. coli*				
	Ibadan	**Ogbomoso**	**Osogbo**	**Ilesa**	**Total**
Animal					
Cow	2/5	1/3	1/5	1/5	5/18
Goat	4/5	0/3	1/3	2/5	7/16
Pig	3/5	2/4	1/5	2/5	8/19
Poultry	2/5	2/4	1/4	4/5	9/18
Sheep	0/5	0/3	0/5	1/5	1/18
Total	**11/25**	**5/17**	**4/22**	**10/25**	**30/89 (33.7%)**
Human					
Meat seller	3/5	1/4	2/5	2/4	8/18
Butcher	1/5	1/5	1/5	0/5	3/20
Animal farm worker	2/5	1/5	2/5	1/5	6/20
Non-farm worker	2/5	1/5	1/5	1/4	5/19
Buyer	1/5	0/5	0/5	0/4	1/19
Total	**9/25**	**4/24**	**6/25**	**4/22**	**23/96 (24.0%)**
Food product					
Milk	0/2	0/2	0/0	0/1	0/5
Beef	3/4	0/2	1/3	3/3	7/12
Chicken	0/1	0/1	0/2	0/3	0/7
Cheese	0/1	0/2	0/2	0/1	0/6
Yoghurt	0/1	0/0	0/0	0/0	0/1
Total	**3/9**	**0/7**	**1/7**	**3/8**	**7/31 (22.6%)**
All samples	**23/59(38.9%)**	**9/48(18.8%)**	**11/54(20.4%)**	**17/55(30.9%)**	**60/216(27.8%)**

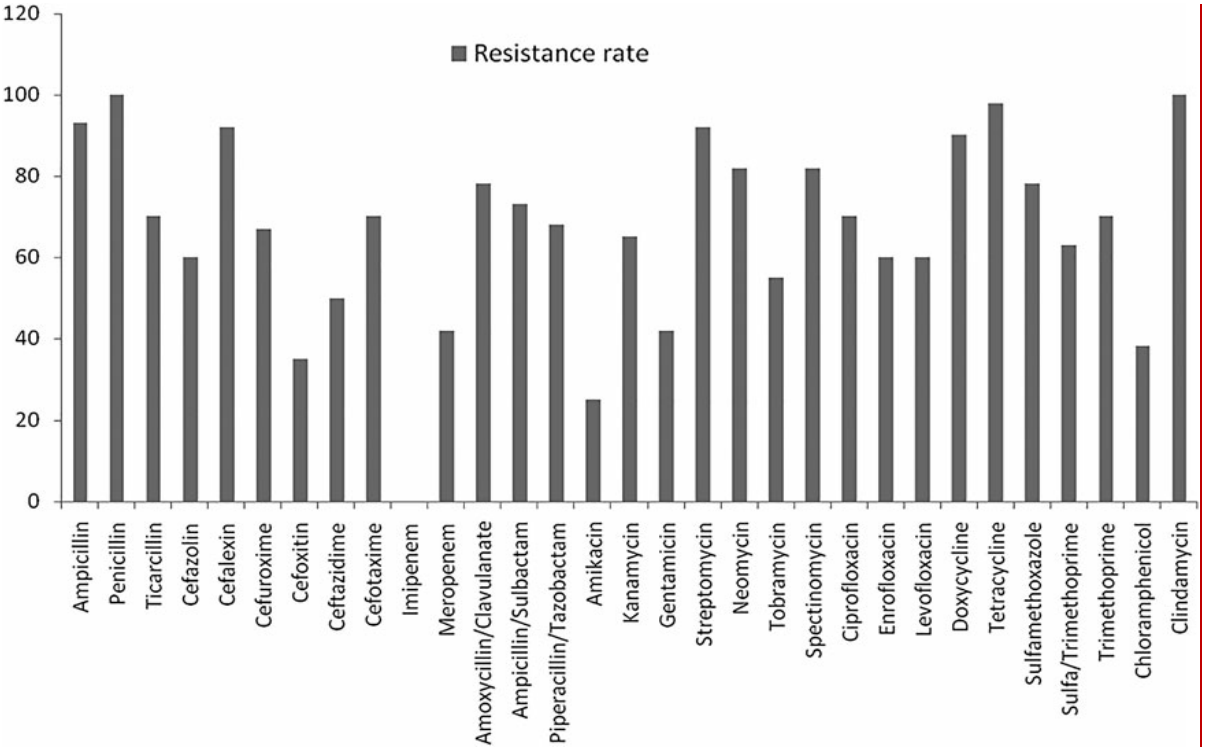

Figure 1: Antibiotic resistance patterns of the 60 ESBL-producing *E. coli* isolates

3.4 The distribution of the antibiotic resistance genes among the multi-drug resistant ESBL E. coli isolates by subject categories

Seven resistance genes were amplified in the multi-drug resistant *Escherichia coli* isolates: TEM (61.7%), CTX-M-15 (51.7%), aac-6-lb (43.3%), CTX-M-1 (38.3%), CTX-M-9 (33.3%), CTX-M-2 (21.7%) and SHV (11.7%) (Table 4). The TEM gene was most prevalent (28.3%) in isolates from humans and was the only gene produced by the isolate from sheep. The CTX-M-15 was the most prevalent gene in isolates from pigs and poultry (3.3% each) followed by those from humans (10%), goats and beef (6.7% each). The CTX-M-9 gene was found in isolates from humans, pigs, poultry, cows (6.7% each), goats and beef (3.3% each). CTX-M-2 gene was predominant in human and pig isolates (5% each) while the CTX-M-1 was mostly amplified in human and poultry isolates (10% each). The aac-6-lb gene was prevalent in isolates from humans (13.3%) followed by those from beef (10%), pigs (6.7%), poultry and cows (5% each). The electrophoresis gel pictures of the antibiotic resistance genes are presented in Figures 2 – 6.

Table 4: The distribution of the antibiotic resistance genes among the multiple-drug resistant ESBL *E. coli* isolates by subject categories

SOURCES	CTX 1	CTX 2	CTX 9	CTX 15	TEM	SHV	aac- 6-lb
Humans (23)	6	3	4	6	17	2	8
Goats (8)	2	1	2	4	3	0	2
Pigs (8)	4	3	4	7	5	2	4
Poultry (7)	6	2	4	7	4	1	3
Cows (6)	2	2	4	3	4	2	3
Sheep (1)	0	0	0	0	1	0	0
Beef (7)	3	2	2	4	3	0	6
Total (60)	23	13	20	31	37	7	26
% of total	**38.3**	**21.7**	**33.3**	**51.7**	**61.7**	**11.7**	**43.3**

KEYS: CTX-1: cefoxitin resistance gene 1; CTX-2: cefoxitin resistance gene 2; CTX-9: cefoxitin resistance gene 9; CTX-15: cefoxitin resistance gene 15; TEM: beta-lactamase resistance gene (Temniora); SHV: beta-lactamase resistance gene (sulphydril variable); aac-6-lb: aminoglycoside resistance gene.

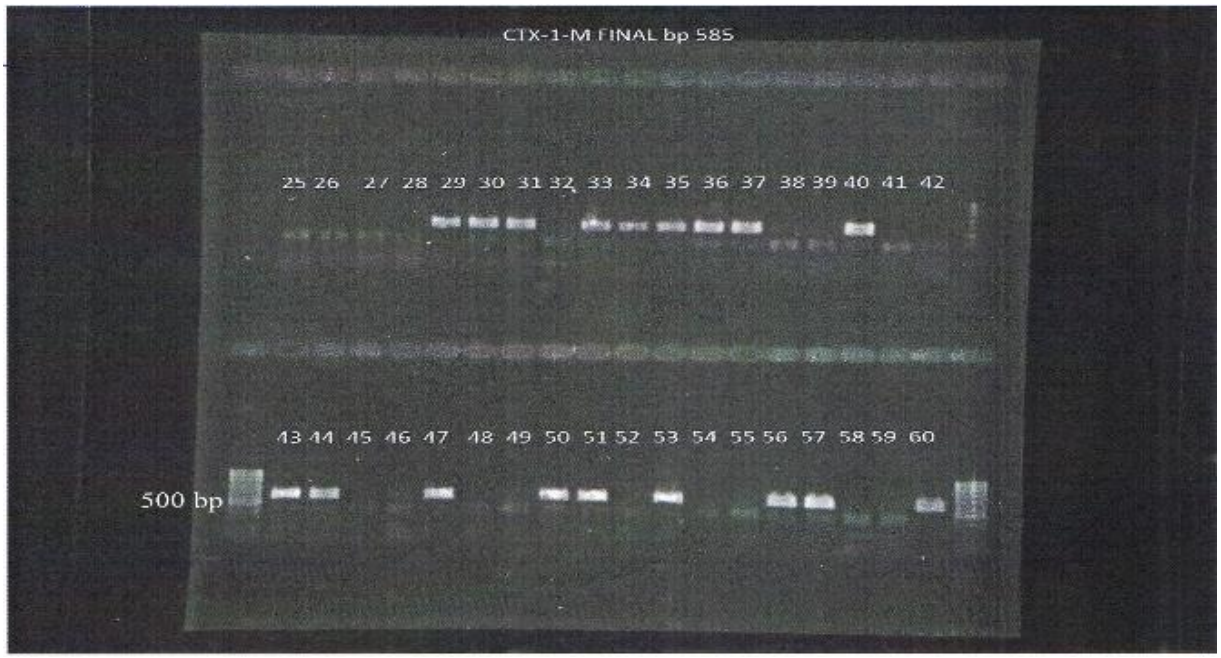

Figure 2: Electrophoresis gel picture of the CTX-M-1 gene (585 bp)

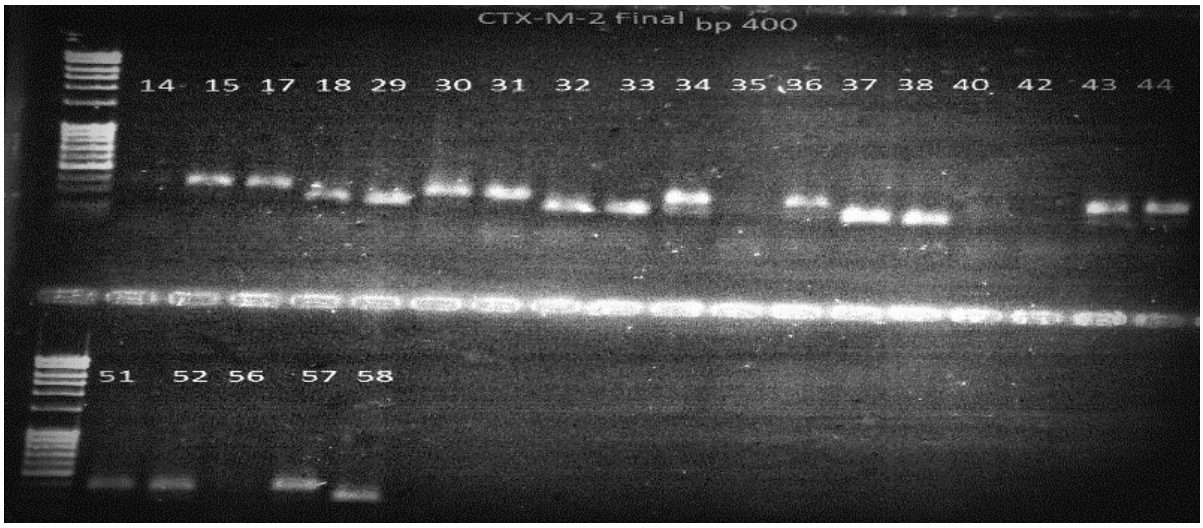

Figure 3: Electrophoresis gel picture of the CTX-M-2 gene.

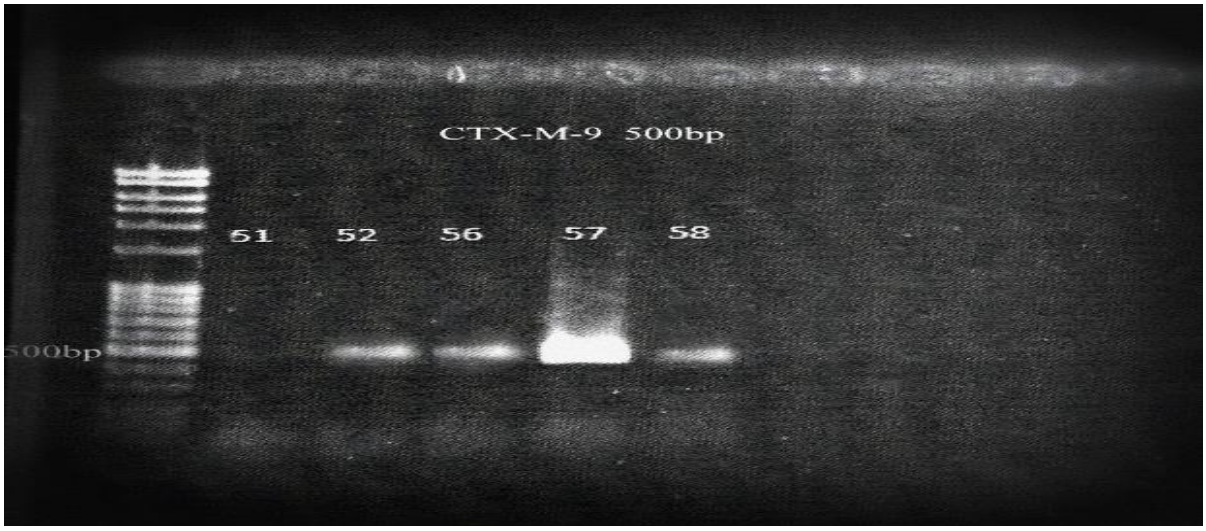

Figure 4: Electrophoresis gel picture of the CTX-M-9 gene

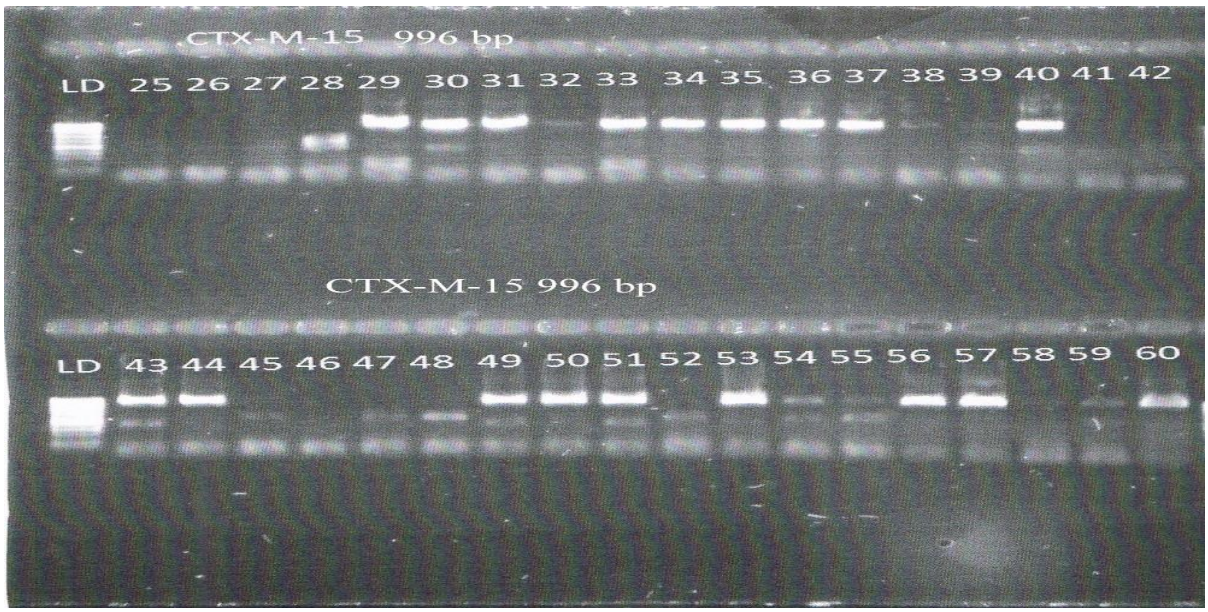

Figure 5: Electrophoresis gel picture of the CTX-M-9 gene.

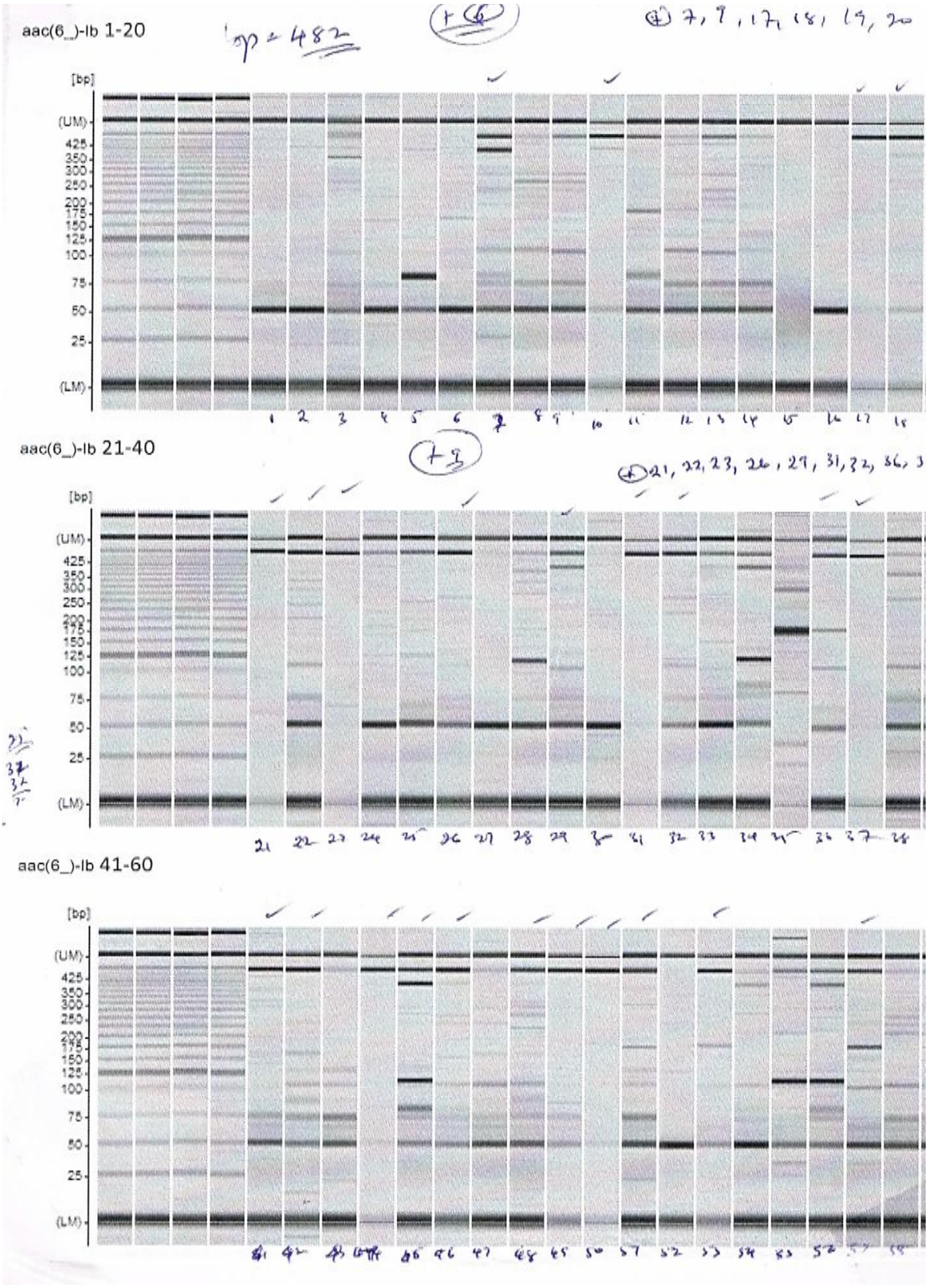

Figure 6: Capillary gel electrophoresis picture of the aac-6-lb gene.

4. Discussion

E. coli strains are usually commensals of the gastrointestinal tracts of mammals but there is also a diversity of intestinal and extra-intestinal pathogenic strains. Antibacterial resistance in these bacteria is growing and is ferociously becoming a source of public health concern in both human and

veterinary medicines. Most of the strains that are usually associated with enteric illnesses in humans originate from animals and can be transmitted directly from animals to humans or indirectly through foods of animal origin, contaminated water, or a common reservoir [9].

In this study, 77.14% of the total samples collected were positive for *E. coli* with the highest prevalence from humans than animals followed by animal food products. In a study conducted by Shittu *et al*., [10] in Abeokuta, Nigeria, *E. coli* strains were isolated from 32.3% of human and animal faecal samples and also with a higher prevalence in humans than in animals. The reason for the lower prevalence of *E. coli* strains isolated from animal faecal samples than from humans in this study may be attributed to a few number of the animal subjects that were just a day old. This was experienced as *E. coli* does not readily colonize the intestines of such neonates. Though Ibadan had the highest prevalence of *E. coli*, there was no significant difference in the prevalence of isolated *E. coli* between the two States. It is therefore safe to infer that geographical locations may not have a role to play in the prevalence of isolation of *E. coli* from humans and animals. Human hygienic practices may however influence the contamination of animal food products from handling to processing.

The result of the antibiotic susceptibility test showed that 60 (27.78%) of the total isolated *E. coli* were ESBL-producers and were multiple-drug resistant strains. In a similar study conducted by Bhoomika *et al*., [11], 21 (10.99%) out of 191 *E. coli* isolates were phenotypically positive for ESBL. Hundred percent of the *E. coli* isolates were resistant to clindamycin and penicillin which was similar to the report of Diarra *et al*., [12] but was above the 25% and 21% sensitivity rates that were recorded for clindamycin and penicillin respectively by Zakaria *et al*., [13]. This high level of resistance to clindamycin is however not surprising as it is usually a drug that is mainly active against the gram-negative anaerobic bacteria coupled with its use as one of the antibiotic growth promoters in livestock which selects for resistance. Penicillin is an antibiotic that has been abused in its usage in both human and veterinary medicines, high-level resistance to this drug agrees with other studies [12, 14, 15, 16, 17, 4].

High rates of resistance were also experienced for ceftazidime, tobramycin, cefazolin, enrofloxacin, levofloxacin, sulfamethoxazole/trimethoprim, kanamycin, cefuroxime, piperacillin/tazobactam, ampicillin, cefalexin, streptomycin, doxycycline, neomycin, spectinomycin, amoxycillin/clavulanate, sulfamethoxazole, ampicillin/sulbactam, cefotaxime, ticarcillin, ciprofloxacin and trimethoprim (50% - 98.3%). These high rates of resistance to ampicillin and cephalosporins have also been confirmed by different authors [18, 19, 20]. Resistance to these antibiotics is a result of misuse in animal health as well as abuse in the treatment of human diseases. Most of these drugs are cheaply found over the counter especially in developing countries like Nigeria. The loss of their potency in the treatment of infections is also attributed to the production of beta-lactamases by most *E. coli* strains which hydrolyze the beta-lactam ring found in their core structures. High rates of resistance to the tetracyclines as seen in this study have also been reported by previous studies [12, 21, 22, 23]. The overuse of doxycycline in livestock farming and the unlawful abuse of tetracycline by people in the developing world are the key factors in the development of resistance to these drugs accompanied by their active pump efflux out of the cells of *E. coli* resistant strains [24]. Fluoroquinolones (e.g ciprofloxacin) are used as prophylaxis especially in human medicine before surgery [25] and in the treatment of infections in both humans and animals (enrofloxacin, levofloxacin). Resistance to these antibiotics is usually due to their misuse and also to the co-selection of their genes with other resistance genes on plasmids. High rates of resistance to this class of drugs were also reported by Fortini *et al*. [26], Ashraf *et al*. [27] and Kao *et al*. [28]. Sulfamethoxazole/trimethoprim is always a drug of choice in the treatment of urinary tract infections in women [29], resistance to this antibiotic is however gaining grounds with the high resistance rate that was recorded against it in this study. A similar result was also reported by Moawad *et al*. [29]. This is well explained by the fact that resistance to this drug is attributed to its recalcitrant nature which makes it not easily degraded. Shedding of this drug in the faeces of food-producing animals and in the residues of their feeds is responsible for the spread of their resistance. Most of the aminoglycosides used in this study had high rates of resistance recorded against them except amikacin as was also found by Nalini *et al*., [30]. Spectinomycin, neomycin, gentamicin, and others, except amikacin are used widely in poultry and other animals; which explains the reason for this resistance [3]. Due to its property of being refractory to most aminoglycoside-

modifying enzymes, amikacin has been successfully used to treat aminoglycoside-resistant infections and this makes it the most widely used semi-synthetic aminoglycoside in treating human infections [31]. Resistance to amoxicillin/clavulanate and ampicillin/sulbactam in this study was also high; this can be explained by the fact that these antibiotics are used worldwide to treat all forms of livestock and their use has been largely abused even in human medicine. Low level of resistance was however recorded for chloramphenicol which is in concordance with the findings of Medina *et al*., [32] and Moawad *et al*., [29].

Chloramphenicol is not approved for use in animals [3] and its use is generally restricted, this explains why there is low resistance of *E. coli* to it. A hundred percent sensitivity was recorded for imipenem; very high rates of susceptibility to this drug have also been found by several researchers [33, 34, 35]. Imipenem is usually the last drug of resort, prudent use of it is therefore advocated. Low level of resistance was also observed for meropenem. These antibiotics have a very broad spectrum of antibacterial activity and also have the ability to resist the activity of most beta-lactamases, the high level of sensitivity recorded against them is therefore not surprising.

The CTX-M genes were the most commonly amplified genes in this study and were found in almost all the subject categories. These CTX-M type ESBLs are currently recognized as the most threatening mechanisms of antibiotic resistance in community and clinical settings, cutting across human and animal medicines as well as the environment globally, and are increasingly being pre-dominant in all members of the Enterobacteriaceae family [36, 37, 38]. The predominance of these genes in both human and livestock isolates has also been reported by Dierikx *et al*. [39], Agersø *et al*. [40], Leistner *et al*. [41] and Valentin *et al*. [9]. These common findings and trends point toward linking animal populations and humans, which should be further investigated in more detail [9]. The preponderance of the TEM gene among the human isolates in this study aligns with other studies [42, 43, 44]. A good percentage of the human, animal, and beef isolates also harboured the aminoglycoside acetyltransferase gene (aac-6-lb) which confers resistance to gentamicin [45]. The level of resistance conferred by this resistance gene is now on the increase and its dissemination among both human and animal pathogens is enhanced by co-selection with beta-lactams and other antibiotics [46]. It is therefore of paramount importance to regulate and monitor the prescription of aminoglycosides and other drugs in the treatment of human and animal diseases.

5. Conclusions

This study reports the emergence of CTX-M type ESBLs, TEM, SHV as well as aac-6-lb genes in multidrug resistant *E. coli* isolates from humans, food-producing animals and beef in South - West, Nigeria. This presents a major concern because of the possibility of transfer of these resistance gene-bearing strains by direct contact or through animal product consumption. Adequate hygiene measures must therefore be taken by owners of farms and animal farm workers to curtail the spread of these bacterial strains; this study also advocates that governments at all levels should formulate and strictly execute policies that will lead to law in order to stop the indiscriminate use of antibiotics in animal husbandry and also restrict the easy access to antibiotics by the human populace.

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