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Molecular Detection of Extended Spectrum Beta-lactamase Resistance in *Escherichia coli* from Poultry Droppings in Keffi, Nigeria

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Authors' contributions

This work was carried out in collaboration among all authors. Author YBN designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors SCT and IHN managed the analyses of the study. Author RHA managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Objectives: The present study reports extended-spectrum beta-lactamase (ESBL) production in *E. coli* isolates from poultry droppings from selected poultry farms in Keffi, Nigeria.
Methods: Seventy-five (75) samples of poultry droppings were collected, and *E. coli* was isolated using standard microbiological methods. Antibiotic susceptibility testing and minimum inhibitory concentrations were evaluated as described by the Clinical and Laboratory Standards Institute (CLSI). Phenotypic confirmation of ESBL production by the isolates was carried out using double disc synergy test. Molecular detection of ESBL genes was carried out using Polymerase Chain Reaction (PCR) method.
Results: All (100%) samples had *E. coli*. Antimicrobial resistance in the isolates were as follows: imipenem (12.0%), gentamicin (20.0%), cefoxitin (37.3%), cefotaxime (41.3%), ceftazidime (44.0%), ciprofloxacin (48.0%), amoxicillin/clavulanic acid (58.7%), streptomycin (92.0%), sulphamethoxazole/trimethoprim (92.0%) and ampicillin (98.7%). Joint resistance to ampicillin, sulphamethoxazole/trimethoprim-streptomycin was the commonest resistance phenotype at 10.6%.

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Multiple antibiotic resistance (MAR) was observed in 97.3% (73/75) of the isolates; and the most common MAR indices were 0.7 (21.9%), 0.5 (17.8%), 0.4 (16.4%), 0.8 (11.1%) and 0.3 (10.9%). Twenty three (46.9%) of the 49 cefotaxime/ceftazidime isolates were confirmed ESBL producers. Twenty-two of the 23 ESBL positive isolates (95.7%) carried the *bla* genes as follows: 95.5% (21/22) for bla_{SHV}; 68.2% (15/22) for $bla_{TEM;}$ and 50.0% (11/22) for bla_{CTX-M}. Eleven (50%) of the 22 isolates carried two *bla* genes (bla_{SHV} and bla_{CTX-M}, bla_{TEM} and bla_{CTX-M} and bla_{TEM} and bla_{SHV}).
Conclusion: The *E. coli* isolates were less resistant to imipenem, gentamicin and cefoxitin; most isolates were MAR, with resistance to 7 antibiotics being the most predominant. In addition, *the* bla_{SHV} gene was the most common ESBL gene detected in confirmed ESBL-producing *E. coli* isolates.

Keywords: E. coli; poultry; antibiotics; resistance; susceptible; ESBL.

1. INTRODUCTION

Escherichia coli is a common and important pathogen whose infections in poultry and humans (or other animals) is well known and sometimes is non-responsive to treatment [1]. Infections and diseases from *E. coli* could result in economic losses [2].

The poultry industry in Nigeria has become one of the most stable sources of animal protein. Nevertheless, poultry has also been discovered to be a reservoir for extraintestinal pathogenic *E. coli* for humans [3]. *Escherichia coli* is a member of the normal microbiota in poultry intestine but is also said to be the cause of the disease colibacillosis, with effects such as septicemia, pericarditis, and death of birds [4].

Poultry droppings have been reported by many researchers as reservoirs of ESBL-producing *E. coli* [5,6,7]. Though in the study areas, studies of ESBL-producing *E. coli* from poultry droppings have not been done. Hence this study focuses on the detection of ESBL genes in *E. coli* isolates from poultry droppings from selected farms in Keffi metropolis, Nigeria. Detection of ESBL genes is an important form of support to antibiotic resistance and susceptibility surveillance in *E. coli* [8,9].

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Media

Bacteriological media that were used in this study included: MacConkey Agar (MCA), Mueller-Hinton Agar (MHA), Nutrient agar (NA), Luria-Bertani (LB) broth, Eosine Methylene Blue (EMB) Agar, Nutrient Broth (NB), Simmons Citrate Agar (SCA), Methyl red/Voges-Proskauer (MR/VP) medium and Peptone water (PW). All the media were sourced from Oxoid Ltd. (U.K.).

2.1.2 Equipment

The equipment that was used in this study included: Autoclave (Certoclav, Model SM280E, Surgifriend Medicals, England), Oven (Hotbox Size One, Galengkamp, U.K.), Incubator (Model 12-140E, Quincy Lab Inc), Refrigerator/Freezer (Model PRN 1313 HCA, BEKO, Germany), Thermocycler (Model TC-312, Techne, England), Gel electrophoresis machine (Max Fill Scie-plas Model HU10 serial no5237), Laminar air flow cabinet (PCR-8 re-circulating laminar flow pre station, Labcaire product 220/240v), Microscope (Model CME 1349522X, Leica, USA), Spectrophotometer (Eppendorf Biophotometer 8.5 mm, Lichtstrahihohe), UV illuminator (VilberbLourmat TFX-35-M serial no NoV02 8104), Centrifuge (Model 5417R: Touch plate Super Mixer, CAT No 1291, Lab-line Instrument Inc USA), Microwave oven (HINARI Life Style 800watts model MX310TCSL), Electronic weighing balance (Model QT 600: Touch plate Super Mixer, CAT No 1291, Lab-line Instrument Inc USA),Vortex machine (Touch plate Super Mixer, CAT No 1291, Lab-line Instrument Inc USA), and Gel Doc system (Biorad, U.K.).

2.1.3 Chemicals and reagents

The chemicals and reagents used in this study included: Acridine orange, Carbolfuschin, Crystal violet, Ethanol, Xylene solution, Creatinine, Potassium hydroxide and Kovac's reagents, obtained from BDH chemical Ltd, England; Ethidium bromide, Iodine solution, EDTA and Glycerol obtained from Sigma Chemical Ltd, England; and Agarose gel from Schwarz/ Mann Biotech.

2.1.4 Bacteria isolates

Confirmed *E. coli* isolates from the poultry droppings were obtained and used for this study. The antibiotic resistance profiles of the isolates are as shown in Table 1.

2.2 Methods

2.2.1 Antibiotic susceptibility testing

The antibiotic susceptibility test for *E. coli* isolates from poultry droppings was carried out using the Kirby-Bauer disc diffusion method as modified by the Clinical and Laboratory Standards Institute - CLSI (2011). Briefly, 5 colonies of *E. coli* isolates were inoculated into 5 ml of Mueller-Hinton broth (MHB) and incubated at 37ºC for 24 hours after which the 24-hour MHB was standardized to the turbidity equivalent to 0.5 McFarland standards. The 0.5 McFarland standard was prepared as follows: 99.5 ml of 1% ($\frac{V}{V}$) H_2SO_4 + 0.5ml of 1.172% ($\frac{W}{V}$) $BaCl_2.2H_20$. A sterile cotton swab stick was dipped into the standardized *E. coli* suspension and streaked on MHA plates.

Antibiotics discs were gently placed on the MHA plates using a pair of sterile forceps and the plates were allowed to incubate at room temperature for 1 hour before re-incubating at 37ºC for 17 hours. After incubation, the diameters of the zones of inhibition were measured to the nearest millimetre (mm) using a ruler and the result of the susceptibility test was interpreted using susceptibility breakpoint earlier described by CLSI (2015).

2.2.2 Minimum inhibitory concentration

The Minimum Inhibitory Concentration (MIC) of cefotaxime and ceftazidime antibiotics against cefotaxime and ceftazidime resistant *E. coli* was carried out using the Clinical and Laboratory Standards Institute method (CLSI, 2015). Briefly, MHA plates containing different concentrations (64-0.25 µg/ml), each of cefotaxime and ceftazidime was prepared. 0.2 mg of cefotaxime and ceftazidime was dissolved in 10 ml of water according to the Manufacturer's instructions to give stock solution while dilution was done to obtain the concentration ranges. 10 µl of standardized *E. coli* suspension was spotted and the plates were incubated at 37ºC for 24 h.

The lowest concentration of cefotaxime or ceftazidime that inhibited visible growth was recorded as the MICs.

2.2.3 Extended spectrum β-Lactamase production test

The phenotypic confirmatory test for ESBL production by isolates resistant to cefotaxime and ceftazidime was carried out using Double-Disc Synergy Test (DDST) method earlier described by Giriyapur et al. [10]. Briefly, 10^5cfu/ml bacterial suspension was streaked on sterile Mueller-Hinton agar plates and amoxicillin-clavulanic acid (30 µg) disc was placed at the centre of the plate. Cefotaxime (30 µg) and ceftazidime (30 µg) discs were then placed 15mm (edge-to-edge) from the centre disc. Enhancement of zone of inhibition in the area between the amoxicillin-clavulanic acid disc and any one of the β-lactam discs compared with the zone of inhibition on the far side of the drug disc was interpreted as indicative of the presence of an ESBL in the tested strain.

2.2.4 Molecular detection of extended-spectrum β-Lactamase genes

Isolates that were confirmed ESBL producers were screened to detect the presence of some ESBL resistance genes namely: bla_{SHV}, bla_{TEM} and bla_{CTX-M}.

2.2.5 DNA extraction

The DNA extraction was performed by the boiling method as described previously [11]. Following purification on MacConkey agar, bacterial DNA was isolated from a 24-h culture in Luria-Bertani broth prepared according to the manufacturers' protocol.

The bacterial cells were harvested by centrifugation at 3200 rpm in a microcentrifuge for 2 min at room temperature and the supernatant was discarded. The harvested cells were re-suspended in 1 ml of sterile normal saline and the micro-centrifuge tubes were placed in the vortex for 5 sec. Centrifugation was carried out at 3200 rpm for 1 min and the supernatant was discarded. 0.5 ml of sterile normal saline was added to the pellets and the tubes were vortexed for 5 sec after which they were heated in the block heater at 90°C for 10 min. immediately after heating, rapid cooling was done by transferring the tubes into the freezer for 10 min. Cell debris was removed after centrifugation was done at 3200 rpm for 1 min and 300 µl of the supernatant was transferred into a sterile 2 ml Eppendorf tube as DNA and stored at -10°C until use.

Table 1. Antimicrobial resistance profile of *Escherichia coli* isolates from poultry droppings from selected poultry farms in Keffi, Nasarawa State, Nigeria

Antibiotics	Disc content (µg)	No. (%) resistance in *E. coli* (n=75)
Ampicillin (AMP)	10	74 (98.7)
Gentamicin (CN)	30	15 (20.0)
Amoxicillin/Clavulanic acid (AMC)	30	44 (58.7)
Sulphamethoxazole/Trimethoprim (SXT)	25	69 (92.0)
Cefotaxime (CTX)	30	31 (41.3)
Streptomycin (S)	10	69 (92.0)
Ceftazidime (CAZ)	30	33 (44.0)
Ciprofloxacin (CIP)	5	36 (48.0)
Cefoxitin (FOX)	30	28 (37.3)
Imipenem (IPM)	10	9 (12.0)

Estimation of the concentration, purity and yield of the DNA sample was accessed using the absorbance method (the measurement of absorbance) with the spectrophotometer (Nanodrop 1000, InqabaBiotec^{tm}, South Africa). For DNA concentration, absorbance readings were performed at 260 nm (A_{260}) and the readings were observed to be within the instrument's linear range (0.1 – 1.0). DNA purity was estimated by calculating the A_{260}/A_{280} ratio and this was done by the spectrophotometer's computer software (where A_{260}/A_{280} ratio ranges from 1.7 – 1.9).

2.2.6 DNA amplification of extended-spectrum β-Lactamase genes

Simplex Polymerase Chain Reaction (PCR) was performed in order to amplify the ESBL genes being assessed in the isolates. The presence of *bla_{CTX-M}, bla_{SHV} and bla_{TEM}* genes were tested for using previously published primer sets and conditions. The primer sequences and expected amplicon sizes for each gene are listed in Table 2.

The reactions were carried out in 20 µl reaction volume made up of 10 µl of Mastermix (InqabaBiotec^{tm}, South Africa), 0.32 µl of primers (0.16 µl each of forward and reverse primers), 3 µl of DNA and 6.68 µl of nuclease-free water. The primer concentration stood at 0.2 M. The reaction tubes were placed in the holes of the thermal cycler and the door of the machine was closed.

Conditions for amplification of all the genes during the reactions were set as 3 min of initial denaturation at 95°C, followed by 35 amplification cycles of denaturation at 95°C for 30 sec, annealing at 56°C for 40 sec, initial extension at 72°C for 50 sec, final extension at 72°C for 3 min and a hold at 4°C infinitely.

2.2.7 Agarose gel electrophoresis

Exactly 7 µl of the amplified DNA was transferred into the wells of a 1.5% Agarose gel by stabbing the wells using a micropipette and this was done carefully to ensure that each well had only one sample. Each gel had one well which contained a DNA ladder (1500 bp, Thermo Scientific, InqabaBiotec^{tm}, South Africa) in order to estimate the size of the DNA amplicons. Electrophoresis was run at 125 volts for 20 min, after which the gels were viewed using ultra-violet trans-illuminator.

3. RESULTS AND DISCUSSION

3.1 Antimicrobial Resistance Profile

The antimicrobial resistance in the *E. coli* isolates from poultry droppings are shown in Table 1. The resistance in the isolates were as follows: imipenem (12.0%), gentamicin (20.0%), cefoxitin (37.3%), cefotaxime (41.3%), ceftazidime (44.0%), ciprofloxacin (48.0%), amoxicillin/clavulanic acid (58.7%), streptomycin (92.0%), sulphamethoxazole / trimethoprim (92.0%) and ampicillin (98.7%).

3.2 Antimicrobial Resistance Phenotypes

Resistance was observed to the antibiotics tested in 73 (97.3%) of the 75 isolates. The resistant isolates were distributed into various phenotypes as shown in Table 2. The commonest phenotypes were the AMP-SXT-S having 8 isolates (10.6%), followed by AMP-SXT-S- CIP-AMC and AMP-SXT-S- IPM, and AMP-SXT-S-FOX-CAZ-CIP-CTX-AMC combinations, each being exhibited by 5 isolates (6.66%).

Table 2. Antimicrobial resistance phenotypes of *Escherichia coli* isolated from poultry droppings from selected poultry farms in Keffi, Nasarawa State, Nigeria

S/N	Antibiotics	No. (%) isolates (n=75)
1	CIP	2(2.7)
2	AMP, IPM	1(1.3)
3	AMP, SXT, S	8(10.7
4	AMP, SXT, S, CIP	2(2.7)
5	AMP, SXT, S, IPM	5(6.7)
6	AMP, SXT, S, AMC	2(2.7)
7	AMP, SXT, S, CAZ	1(1.3)
8	AMP, FOX, S, AMC	2(2.7)
9	AMP, SXT, S, CAZ, CTX	1(1.3)
10	AMP, SXT, S, CIP, AMC	5(6.7)
11	AMP, SXT, S, CN, AMC	1(1.3)
12	AMP, SXT, S, CN, CAZ	2(2.7)
13	AMP, SXT, FOX, CTX, AMC	1(1.3)
14	AMP, SXT, FOX, S, AMC	3(4.0)
15	AMP, SXT, FOX, S, CAZ, CTX	1(1.3)
16	AMP, SXT, S, CAZ, CTX, AMC	1(1.3)
17	AMP, SXT, S, CIP, CN, AMC	2(2.7)
18	AMP, SXT, FOX, S, CAZ, AMC	2(2.7)
19	AMP, SXT, FOX, S, CIP, AMC	1(1.3)
20	AMP, SXT, S, CAZ, CIP, CTX, AMC	3(4.0)
21	AMP, SXT, FOX, S, CIP, CTX, IPM	1(1.3)
22	AMP, SXT, CAZ, CIP, CTX, CN, AMC	2(2.7)
23	AMP, SXT, S, FOX, CAZ, CIP, AMC	3(4.0)
24	AMP, SXT, S, FOX, CAZ, CTX, AMC	2(2.7)
25	AMP, SXT, S, CIP, CN, AMC, IPM	1(1.3)
26	AMP, SXT, S, FOX, CAZ, CTX, IPM	2(2.7)
27	AMP, SXT, S, FOX, CAZ, CIP, CTX	2(2.7)
28	AMP, SXT, S, FOX, CAZ, CTX, CN, AMC	2(2.7)
29	AMP, SXT, S, FOX, CAZ, CIP, CTX, AMC	5(6.7)
30	AMP, SXT, S, CAZ, CIP, CTX, CN, AMC	2(2.7)
31	AMP, SXT, S, FOX, CAZ, CIP, CTX, CN	2(2.7)
32	AMP, SXT, S, FOX, CAZ, CIP, CTX, AMC, IPM	3(4.0)
33	AMP, SXT, S, FOX, CAZ, CIP, CTX, CN, AMC	2(2.7)

Key: Amp: Ampicillin, CN: Gentamicin, AMC: Amoxycillin/Clavulanic Acid, SXT: Sulphamethoxazole/Trimethoprim, CTX: Cefotaxime, S: Streptomycin, CAZ: Ceftazidime, CIP: Ciprofloxacin, FOX: Cefoxitin, IPM: Imipenem

Table 3. Multiple Antibiotic Resistance (MAR) index of resistant *Escherichia coli* isolated from poultry droppings from selected poultry farms in Keffi, Nasarawa State, Nigeria

No. of antibiotics isolate is resistant to (a)	No. of antibiotics tested (b)	MAR index $(\frac{a}{b})$	No. (%) MAR isolates (n=73)
10	10	1.0	0(0.0)
9	10	0.9	5(6.8)
8	10	0.8	11(15.1)
7	10	0.7	16(21.9)
6	10	0.6	7(9.6)
5	10	0.5	13(17.8)
4	10	0.4	12(16.4)
3	10	0.3	8(10.9)
2	10	0.2	1(1.4)

3.3 Multiple Antibiotic Resistance (MAR) Index

Multiple antibiotic resistance (MAR), defined here as resistance to at least two (2) antibiotics, was observed in 73 (97.3%) of the 75 isolates. Two (2.66%) isolates had to MAR index of < 0.20 suggesting that most of the isolates originated from environments where antibiotics were freely abused or misused [12]. The most common indices were 0.7 (21.9%), 0.5 (17.8%), 0.4 (16.4%), 0.8 (11.1%) and 0.3 (10.9%).

3.4 Minimum Inhibitory Concentration

The MICs of ceftazidime and cefotaxime antibiotics against *E. coli* isolates resistant to ceftazidime and cefotaxime is as given in Table 4. [The *E. coli* isolates were more resistant to ceftazidime (33%) than cefotaxime (32%)].

3.5 Phenotypic Confirmation of Beta-lactamase Production

Twenty three (46.9%) of the 49 cefotaxime/ceftazidime isolates tested revealed enhanced zones of clearing and exhibited the expected "keyhole effect" towards the amoxicillin-clavulanic acid disc when examined by this method.

3.6 Molecular Detection of Extended Spectrum Beta-lamase Genes

Twenty-two of the 23 ESBL positive *E. coli* isolates (95.7%) carried the *bla* genes at the following frequencies: 95.5% (21/22) for bla_{SHV}; 68.2% (15/22) for $bla_{TEM;}$ and 50.0% (11/22) for bla_{CTX-M}. Eleven (50%) of the 22 isolates carried two *bla* genes (bla_{SHV} and bla_{CTX-M}, bla_{TEM} and bla_{CTX-M} and bla_{TEM} and bla_{SHV}).

3.7 Discussion

Extended-spectrum β-lactamase (ESBL) producing *E. coli* is a serious problem in both community and hospital set up [13]. From this study, it was observed that *E. coli* isolates were more susceptible to imipenem, gentamicin, cefoxitin, cefotaxime, ceftazidime and ciprofloxacin. The high susceptibility of *E. coli* isolates to imipenem observed in this study is similar to the 100% earlier reported by Hiroi et al. [14] and Carissa et al. [15]. This high susceptibility justifies the use of imipenem as the last β-lactam antibiotic of choice for treatment of infections caused by Gram-negative Enterobacteriaceae.

Table 4. Minimum Inhibitory Concentration (MIC) of cefotaxime and ceftazidime against some extended spectrum Beta-lactamase producing *Escherichia coli* isolated from poultry droppings from selected poultry farms in Keffi, Nasarawa State, Nigeria

Antibiotics	MIC ranges (µg/ml)	No. (%) cefotaxime/ceftazidime resistant isolates (n=49)
Cefotaxime	0.25-64	24(49.0)
Ceftazidime	10	25(51.0)

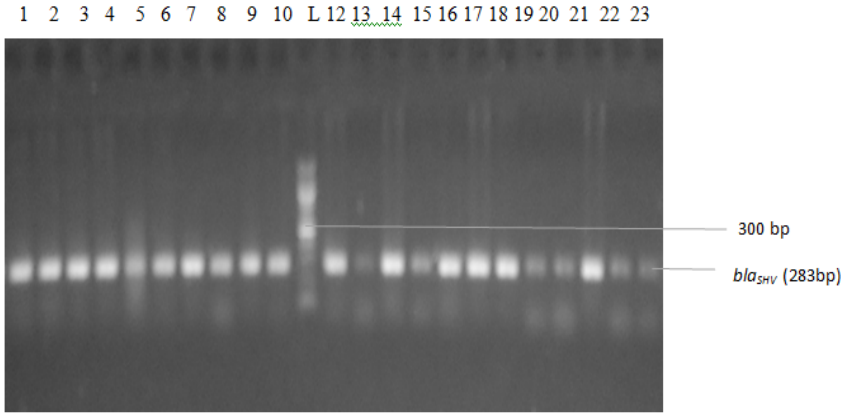

Plate 1: Agarose gel electrophoresis showing the bla_{SHV} bands, lane 1, to 10 and lane 12 to 23 represent bla_{SHV} gene bands while L represents the 100bp molecular ladder.

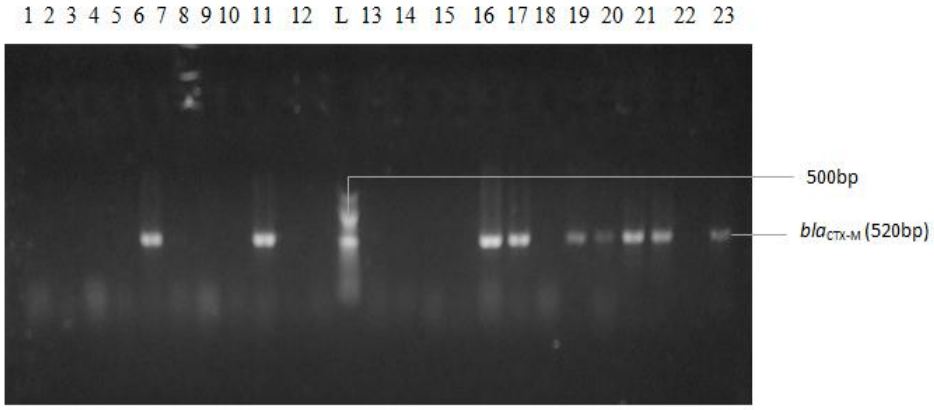

Plate 2: Agarose gel electrophoresis showing the bla_{CTX-M} bands, lanes 6, 11, 16, 17 19, 20, 21, 23 represent bla_{CTX-M} gene bands while L represents the 100bp molecular ladder.

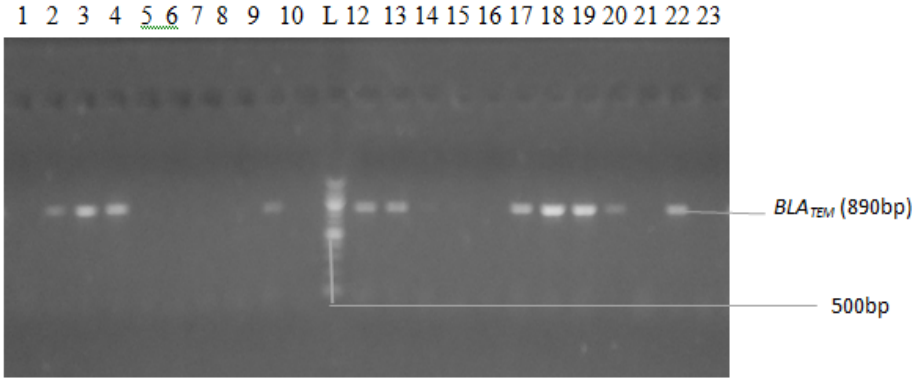

Plate 3: Agarose gel electrophoresis showing the bla_{TEM} bands, lanes 2, 3, 4, 9, 12, 13, 17, 18, 19, 20, and 22 represent bla_{TEM} gene bands while L represents the 100bp molecular ladder.

Also, the high susceptibility of *E. coli* isolates to gentamicin, ceftazidime, cefoxitin, cefotaxime and ciprofloxacin is in agreement with the study reported by Ünal et al. [16], Khoshbakht et al. [17] and Shehu et al. [18]. The high susceptibility of *E. coli* isolates to these antibiotics mentioned above may be due to the fact that the drugs are very expensive, and are likely not abused. Furthermore, antibiotics such as ceftazidime, gentamicin and cefotaxime are in injectable form and because of the pain and discomfort experienced while injecting, such antibiotics are not likely to be abused [19].

The low susceptibility of *E. coli* isolates to antibiotics such as ampicillin, streptomycin and sulphamethoxazole/Trimethoprim observed in this study may be due to misuse, abuse or use of substandard antibiotics in animal husbandry, especially poultry. It is common knowledge that antibiotics and cocktails of antibiotics of different classes, in a single formulation are available in Nigerian markets. The use of such formulations exposes the poultry to different classes of antibiotics at the same time, triggering the microbial flora for multidrug resistance. In the study conducted by Melton [20], 54% of the drugs used by poultry vendors were obtained from open markets without prescription and 68% of these vendors determined the dosages and administered them by experience alone. This could be a reason why no *E. coli* isolate was sensitive to all antibiotics tested.

Seni et al. [21] and Shaikh et al. [13] reported that ESBL producing *E. coli* are well resistant to other classes of antibiotics and this may likely lead to treatment failures, especially in community and hospital setups. An interesting observation in this study was the percentage occurrence of the bla_{SHV}gene in the confirmed *E. coli* isolates which was higher than the bla_{TEM} and bla_{CTX-M} genes. This finding is in variance with earlier reports that show bla_{CTX-M}as the most prevalent of ESBL genes, over others such as bla_{TEM} and bla_{SHV}as reported by Olowe et al. [6], Aliasadi et al. [22], and Chishimba et al. [23]. The presence of ESBL genes observed in this study suggests that the genes may be responsible for the production of ESBL enzymes that is resistant to most β-lactam antibiotics.

The results of this study and other previous studies have demonstrated the widespread distribution of ESBL in Nigeria and other parts of the world. This is worrisome because these poultry birds can serve as a source of meat for humans, and ESBL bacterial pathogens are known to be multidrug resistant and are responsible for a number of nosocomial and community infections.

4. CONCLUSION

The *E. coli* isolates from poultry droppings from selected poultry farms in Keffi were more susceptible to imipenem, gentamicin, ciprofloxacin, ceftazidime and cefotaxime. This implies that the antibiotics are useful in the treatment of infection caused by *E. coli.* Also, most *E. coli* isolates jointly resistant to cefotaxime and ceftazidime were confirmed ESBL producers. In addition, the most ESBL gene detected was bla_{SHV}than bla_{TEM} and $bla_{CTX-M.}$

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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