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PHENOTYPIC AND MOLECULAR CHARACTERIZATION OF EXTENDED-SPECTRUM BETA-LACTAMASE PRODUCING *Escherichia coli* OBTAINED FROM ANIMAL FECAL MATERIALS IN OVIA NORTH-EAST, EDO STATE, NIGERIA

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Abstract
Antibiotic resistance has continued to constitute serious problems not only in clinical medicine but also in livestock management and veterinary medicine. The present study was carried out to determine the occurrence of Extended-Spectrum Beta-lactamase (ESBL) producing *Escherichia coli* (*E. coli*) from fecal samples in Poultry and Cattle farming in Ovia North East local government area, Benin City, Edo State.
A total of 100 fecal samples were collected randomly from apparently healthy cattle and poultry birds. Heterotrophic bacteria, Coliforms and *E. coli* were cultured using standard procedures. Antibiotic susceptibility profile of the *E. coli* isolates were carried out using Kirby Bauer disc diffusion method. ESBL phenotypic detection was carried out using ETEST® ESBL strips. Molecular detection of *tem, shv, ctx-m* and *oxa* genes were carried out using standard molecular methods.
The mean heterotrophic bacteria count from chicken fecal samples was $5.56\pm0.21x10^6$cfu/g, while that of cattle fecal samples was $4.91\pm0.33x10^6$cfu/g. The mean total coliform count from chicken fecal samples was $3.73\pm0.14x10^6$cfu/g while that of cattle fecal samples was $3.28\pm0.22x10^6$cfu/g respectively. Sixty eight *E. coli* isolates were recovered from the 100 samples, out of which 13 (19.1%) suspected isolates for phenotypic ESBL production testing were selected based on a $\geq$0.6 multiple resistance index criteria to the 10 broad-spectrum antibiotics used during antibiotic susceptibility test. Five of these isolates were positive for ESBL by ETEST® ESBL strips. Consequently, out of the 5, *tem*, *oxa* and *shv* genes were detected in 2(40%), 3(60%) and 4(80%) of the isolates respectively. Only the *ctx-m* genes (100%) was detected in all isolates. Cattle fecal samples had more carriage of extended spectrum beta lactamase producing *E. coli* than chicken fecal samples, suggesting that *E. coli* isolates from cattle possessed more beta-lactamase genes than *E. coli* isolates from chicken. This study showed high resistance of *E. coli* isolates (15-42.50 %) to antibiotics and the presence of beta-lactamase genes in these isolates from animals within the studied locale. These findings are of significant concern to public health because of the inevitable interaction between man, microorganisms and environment. Regular monitoring and regulated use of antibiotics in livestock is recommended.

Keywords: *Escherichia coli*, extended spectrum beta lactamases, ESBL phenotypic detection, antibiotic resistance, cattle, chicken

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Introduction

Production of extended-spectrum β (beta)-lactamases (ESBLs) is the most typical mechanism of resistance to third-generation cephalosporins among the family Enterobacteriaceae particularly *E. coli* and others like *Klebsiella pneumonia* [1, 2]. Extended-spectrum β-lactamase determinants have been detected not only in clinical isolates but also in commensal bacteria (*E. coli*) from humans and animals and in isolates from products of the food and sewage, revealing distribution and suggesting the presence of environmental reservoirs for these resistance determinants [3, 4]. Extended-spectrum *β*-lactamase producing *E coli* which are ubiquitous, represent a major problem in human and veterinary medicine, particular in nosocomial infections.

The increase in antimicrobial-resistant bacteria of animal origin resembles the process in humans, decades ago [5]. Since the late 1990s, extended-spectrum β-lactamase producing Enterobacteriaceae, in particular *E. coli*, has emerged globally. Initially, extended-spectrum β-lactamase producing bacteria were only observed in human medical practice [6] but the recent observation of these bacteria, first in companion animals, increasingly in livestock and even in wildlife has initiated monitoring studies focused on livestock [3]. Extended-spectrum β-lactamase producing *E. coli* isolates are now being found in increasing numbers in food-producing animals leading to the hypothesis that animals might become infection sources or even reservoirs contributing to the spread of these bacteria [7].

The increase in non-intrinsic antimicrobial resistance in morbific microorganisms started with the introduction of antibiotics in medication some sixty years ago suggesting a correlation between antimicrobial pressure and also the emergence of resistance in pathogens [8]. Although the detection of multidrug resistant pathogens like extended-spectrum beta-lactamases producing Gram-negatives in wildlife is considered a new phenomenon, it should have been anticipated, as antimicrobial resistant bacteria other than intrinsically resistant ones were already found in environmental samples apparently free from any antimicrobial pressure decades ago [2].

While various bacterial species are important in terms of being multi-drug resistant and agents of nosocomial infections in humans and veterinary medicine, extended-spectrum beta-lactamases producing Gram-negative bacteria, *E. coli* (ESBL-*E. coli*) is considered as being key indicator pathogen to trace the evolution of multiresistant bacteria in the environment and wildlife[9, 10] Although the majority of ESBLs are still reported from human clinical isolates, they are also increasingly recorded in community-acquired bacterial infections[1]. This indicates that ESBL-*E.coli* have made their way out of the clinics, have been successfully transmitted and now persist in the community [10].This study was therefore, conducted to identify phenotypically and molecularly ESBL producing *E. coli* from the feces of cattle and poultry birds in Ovia North East local government area, Benin City, Nigeria.

Materials and Methods

Sample Collection

A total of one hundred fecal samples were collected from cattle (n = 50) and chicken (n

= 50). Freshly passed feces from apparently healthy cattle and poultry birds were randomly collected into sterile capped universal bottles with sterile disposable spatula and were transported to the laboratory in ice packs immediately and analyzed within 4-5hrs after collection. The sample sites were Cattle Market/Abattoir, cattle ranch, Parliament Chicken farm and Runamin Chicken farm in Ovia North East, Benin City, Edo State.

Enumeration, Isolation and Identification

Five grams of each sample was homogenized with 45 ml sterile normal saline. Further ten-fold serial dilutions of the resultant homogenates were made to obtain 10^{-2}, 10^{-3} and 10^{-4} dilutions respectively. One mililitre (1ml) from 10^{-4} dilutions were placed in the center of sterile petri dish with a sterile pipette. Molten cooled Nutrient agar and Eosin Methylene Blue (approx.15ml) were separately poured into each of the petri dishes containing the inoculum (pour plate technique). The plates were inverted and incubated at 37ºC for about 18-24hrs. At the end of the incubation period, growths were observed on the various petri dishes. Colonies formed were counted using illuminated colony counter. The counts for each plate (petri dish) were expressed as colony forming unit per gram of sample homogenate (cfu/g). Cultural and Morphological attributes of the colonies were observed as presumptive biochemical tests confirmed the probable bacterial isolates in the fecal samples of cattle and chicken (data not shown). Colonies of *E. coli* formed on Eosin Methylene Blue agar were isolated by sub-culturing to get discrete colonies. Pure cultures were stored on nutrient agar slants.

Antibiotic Susceptibility Tests

Antibacterial susceptibility testing was carried out using Kirby-Bauer disc diffusion method. The susceptibility of *E. coli* isolates to Ceftazidime (CAZ) (30 μg), Ciprofloxacin (CIP) (5 μg), Gentamycin (GEN) (10 μg), Streptomycin (STR) (10 μg), Augmentin (AUG) (10 μg), Cefixime (CFX) (5µg), Tetracycline (TET) (30µg), Chloramphenicol (CHL) (30µg), Ampicillin (AMP) (10µg) and Ofloxacin (OFL) (5µg) was determined. The results were interpreted based on resistance, intermediate and sensitive/susceptible according to standard disc diffusion method and adopting the breakpoints of Clinical and Laboratory Standard Institute [11, 12].

Multiple antibiotic resistance (MAR) index

In this study, the MAR index was obtained using the formula:

$$MAR\ index = \frac{y}{nx}$$

$$where\ y = number\ of\ resistance\ scored,$$

$$n = number\ of\ isolates\ and$$

$$x = total\ number\ of\ antibioitics$$

According to Clinical and Laboratory Standard Institute [12], an index of ≥ 0.2 and above is indicative of a 'high-risk' contamination source.

Phenotypic Detection of Extended-Spectrum Beta-Lactamase (ESBL)

ESBL Resistance Detection

The ETEST® ESBL ceftazidime/ceftazidime with clavulanic acid (ESBL TZ/TZL 32/4) strips were used to confirm the presence of ESBL enzymes in the *E. coli* isolates and were performed according to manufacturer's instruction. The strip was placed on surface of inoculated agar plate with an instantaneous release of the antimicrobial gradient from the plastic carrier to agar

forming a stable and continuous gradient beneath and in the immediate vicinity of strip. Incubation was within 18-24 hrs at 37ºC. After incubation the minimum inhibitory concentrations (MICs) on both ends were read on the intersection of the inhibition ellipse and E test-strip edge. An isolate is considered as ESBL-positive when the ratio of TZ/TZL ≥8 µg/ml and also the outcome of the result were indeterminate when both MICs were outside the test range of the test strip (rare), [13, 14].

Polymerase Chain Reaction to Detect *bla* Genes

The DNA of the ESBL confirmed isolates were extracted separately using a DNA extraction kit (Biospin plasmid extraction). Genomic DNA used for PCR was purified using the UltraClean Microbial DNA Kit following the manufacturer's instructions. The presence of resistance genes *blaOXA, blaTEM, blaSHV* and *blaCTX-M,* were determined by multiplex PCR kit using specific primers that have been described by authors according to previously published work [15. 16]. The primers were developed based on conserved sequences that were available in the Gene Bank database for resistance genes. All PCR amplifications were performed in total reaction volumes of 50 mL consisting of 0.1 mg of template DNA, 0.25mM concentration of deoxynucleotide triphosphate, 1.5mM $MgCl_2$, 50 pmol of each primer, and 0.2U of AmpliTaq Gold. PCR cycling conditions included an initial denaturation at 94ºC for 10 minutes followed by 30 cycles of 94ºC for 30 seconds, annealing at primer-specific pair for 30 seconds, followed by extension at 72ºC for 20 seconds. A final extension step was done at 74ºC for 6 minutes. The final PCR products were run on 1.5% agarose gel mixed with ethidium bromide. The results were visualized under UV transilluminator and photographed.

Statistical analysis: Descriptive statistics; mean and standard error were calculated using the Statistical Package for Social Sciences software (SPSS version 20) for bacteria and coliform enumeration from chicken and cattle fecal samples [17]. Unpaired students' distribution test was used to evaluate differences between chicken and cattle fecal samples.

Results

The bacterial enumeration of chicken and cattle fecal samples, which showed the mean value (average) of the total heterotrophic bacteria count and total coliform count, is shown in Fig.1.

The prevalence of *E. coli* and phenotypic ESBL-*E. coli* from animal feces in the study revealed that 68% of *E. coli* isolates were recovered and out of which 13 (19.12%) were suspected for phenotypic ESBL production (Table 1). Following testing with ETEST® ESBL Strip, 7.35% of the isolates were confirmed to be ESBL-producing *E. coli.* The selection of isolates for testing and confirmation of ESBL, were on the strength of the antibiotic susceptibility of all isolates (Table 2) where it was observed that varying resistance were shown by isolates to the used antibiotics in the study. Furthermore, the multiple antibiotic resistance distribution of *E. coli* isolates from chicken and cattle feces were evaluated (Table 3) and isolates with $\geq$0.6 multiple resistance index were selected for ESBL screening. Five out of thirteen *E. coli* isolates were positive for ESBL, which were later taken to the laboratory for molecular detection of beta-lactamase genes (Table 4). Cattle fecal

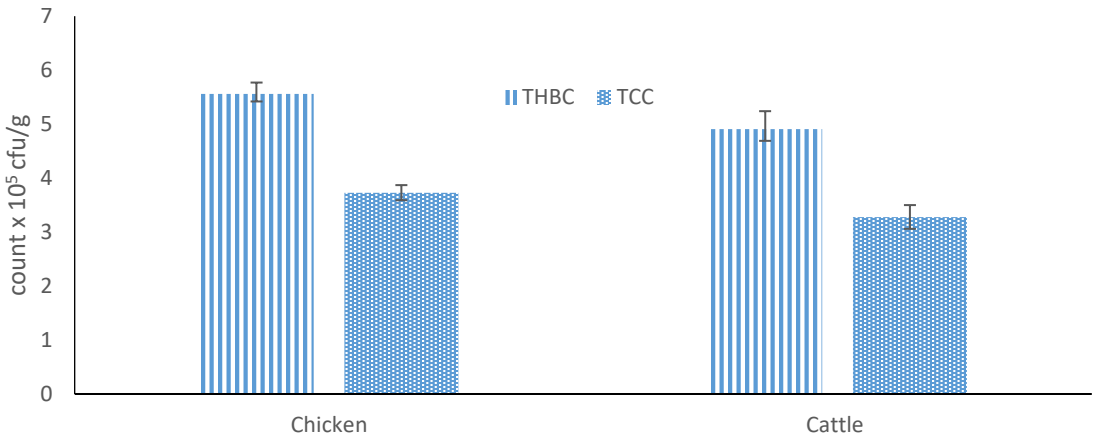

Table 1: Prevalence of *E. coli* and phenotypic ESBL-*E. coli* from animal feces

	Chicken feces	Cattle feces	Study prevalence
E. coli (68)	28 (56.00)	40 (80.00)	68 (68.00)
Tentative ESBL-*E. coli* (13)	4 (5.88)	9 (13.23)	13 (19.12)
ETEST® ESBL Strip (confirmation)	2 (2.94)	3 (4.41)	5 (7.35)

samples had more carriage of extended beta lactamase producing *E. coli* than chicken fecal samples. PCR was used to detect the beta-lactam genes in the ESBL positive *E. coli* isolates. Plate 1 describes Multiplex PCR amplification products. Lane M-Molecular weight marker; Lane N-Distilled water (negative control); lane 21 and 30 are *E. coli* isolates from chicken; Lane 4, 24, and 26 are *E. coli* isolates from cattle (Plate 1). The band shows the presence of the beta-lactamase genes in each lane having the isolates. TEM, SHV and OXA genes were detected in 2, 4 and 3 isolates respectively. Only the CTX-M genes were detected in all 5 isolates. Most prevalent genes were the SHV (80%) and CTX-M (100%) genes (Table 5).

Table 2: Percentage antibiotic resistance of *E. coli* isolated from cattle and chicken fecal samples

Antimicrobial Agent	Chicken (%)	Cattle (%)
Augmentin (5µg)	5(17.50)	17(42.50)
Gentamicin (10µg)	5(17.50)	10(25.00)
Cefixime (5µg)	7(25.00)	7(17.50)
Tetracycline (30µg)	6(21.42)	11(27.50)
Streptomycin (10µg)	7(25.00)	10(25.00)
Ofloxacin (5µg)	6(21.42)	6(15.00)
Chloramphenicol (30µg)	8(28.57)	11(27.50)
Ampicillin (10µg)	7(25.00)	6(15.00)
Ciprofloxacin (5µg)	6(21.42)	13(32.50)
Ceftazidime (30µg)	6(21.42)	6(15.00)

Table 3: Multiple antibiotic resistance distribution of *E. coli* isolates from chicken and cattle feces

Isolate code	Number of Antibiotics	Resistant antibiotics	Percentage (%) resistant phenotypes	MAR Index
ChiF50, ChiF24, ChiF25, ChiF12, ChiF22, ChiF10, ChiF11, ChiF23, ChiF16, ChiF01, ChiF08, ChiF09, ChiF22, CatF02, CatF01, CatF31, CatF33, CatF32, CatF08, CatF19, CatF29, CatF43, CatF44, CatF45, CatF46,	10	AUG^R, AMP^R, CIP^R, CAZ^R	25(36.76)	0.40
ChiF20, ChiF14, ChiF05, ChiF12, ChiF02, CatF32, CatF11, CatF01, CatF23, CatF07, CatF43, CatF04, CatF09	10	AUG^R, GEN^R, CFX^R, TET^R, STR^R, CAZ^R,	13(18.84)	0.60
ChiF29, ChiF18, ChiF15, ChiF42, ChiF32, ChiF33, ChiF34, ChiF35 CatF38, CatF39,	10	CFX^R, TET^R, STR^R,	10(14.71)	0.30
ChiF29, ChiF18, ChiF15, ChiF42, ChiF32, ChiF33, ChiF34, ChiF35	10	CHL^R, AMP^R, CIP^R, CAZ^R,	8(11.76)	0.40
ChiF29, ChiF18, ChiF15, ChiF42, ChiF32, ChiF33,	10	TET^R, CHL^R,	6(8.82)	0.20
ChiF50, CatF49, ChiF36 CatF48	10	STR^R, OFL^R, CHL^R, AMP^R, CIP^R,	4(5.88)	0.50
CatF04, CatF09	10	CHL^R, AMP^R , TET^R,	1(11.11)	0.30

CAZ = Ceftazidime, CIP = Ciprofloxacin, GEN= Gentamycin, STR = Streptomycin, AUG= Augmentin, CFX = Cefixime, TET= Tetracycline, CHL = Chloramphenicol, AMP = Ampicillin and OFL = Ofloxacin

Table 4: Amplicon sizes and specific primers of resistant genes

Genes	Primers sequence	Amplicon size (bp)	References
Shv	F: 5´- TCGCCTGTGTATTATCTCCC- 3´ R: 5´-CGCAGATAAATCACCACAATG- 3´	768	16
Tem	F: 5´- TTCGGCATTCTGAATCTCAC- 3´ R: 5´-ATGATCTAACCCTCGGTCTC- 3´	822	16
ctx-m	F: 5´- TGGCCAGAACTGACAGGCAAA-3´ R: 5´-TTTCTCCTGAACGTGGCTGGC- 3´	462	16
Oxa	F: 5´- GGGTATGGATATTATTGATAAAG- 3´ R: 5´-CTAATCCGGCAGCACTATTTA- 3´	670	15

Lane M-Molecular weight marker (100 bp); Lane N-Distilled water (negative control); lane 21 and 30 are *E. coli* from chicken; Lane 4, 24, and 26 are *E. coli* from cattle. The bands across these lanes are the amplified genes present in the template DNA of *E. coli* isolates using different specific primer pairs in the reaction mixture (Table 4 above).

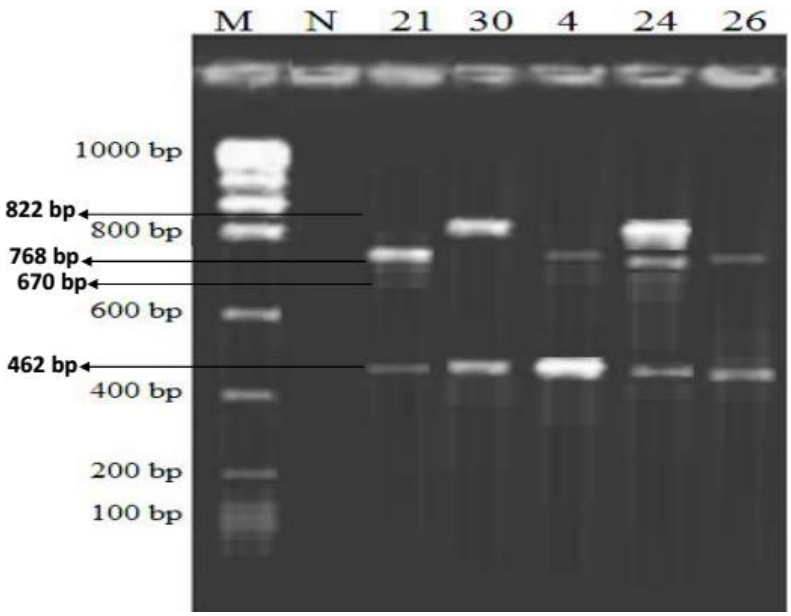

Plate 1: Multiplex PCR amplification products.

Table 5: Identified gene percentage occurrence in *E. coli* isolates from chicken and cattle fecal materials

ESBL Resistant genes	Amount (%)
shv	80
tem	40
ctx-m	100
oxa	60

Discussion

The increased frequency of ESBL *E. coli* morbidity and mortality have become a public health threat and therefore, need to be monitored in the environment [18]. The present study investigated the phenotypic and genotypic characteristics of ESBL *E. coli* from apparent healthy Cattles and birds. A total of hundred fecal samples were collected of which sixty eight *E. coli* isolates were recovered. Thirteen 13 (19.1%) of these isolates were suspected for phenotypic ESBL production testing based on a ≥0.6 multiple resistance index criteria to the 10 broad-spectrum antibiotics used. Subsequently, five isolates were positive for ESBL by ETEST® ESBL strips and out of the 5, *tem*, *oxa* and *shv* genes were detected in 2(40%), 3(60%) and 4(80%) of the isolates respectively while *ctx-m* genes (100%) was detected in all isolates.

The high mean heterotrophic bacteria count especially from chicken fecal samples could be due to its micronutrients content that could aid the proliferation of bacteria. This was similar to the bacterial count of layer chicken droppings reported by [19]. Diverse and numerous bacterial populations reside in the gut of animals and are vital to the maintenance of animal health [20].Coliforms are normal flora of the gastrointestinal tracts of several domestic animals, including

chicken and can be of particular importance to public health, especially *Salmonella* and *E. coli* [21].

Therefore, the high coliform counts observed in the cattle and poultry feces is not surprising, but they can be sources of enteric microbial contamination within the poultry and ranch.

Scattered across literatures, are reports of resistance of *E. coli* to commonly used antibiotics, thus driving the topic of multiple resistance to antibiotics. Again, presence of high level of antibiotics resistance has been reported from cattle dung and its manure [20]. Similar to the findings with respect to resistance of *E. coli* in this study, it was reported by [23] that multiple resistance isolates of *E. coli* to antibiotics such as tetracycline, ampicillin, and colistin were isolated from vegetables. This also corroborated the findings of [24] who reported that isolates of *E. coli* (suspected to produce ESBL) were found to be resistant to chloramphenicol, ciprofloxacin, ampicillin and gentamicin. In this study, all isolates with MAR index with greater than 0.60 were found to be resistant to six of the ten antibiotics used in the study. Incidentally, all ESBL-producing *E. coli* were found to be amongst the lot. *E. coli* has been reported to be the major ESBL producer in literature [4, 22]. For example, [22] reported that all isolated ESBL-producing bacteria from individuals with livestock contact, cattles and broilers were *E. coli*. Cattle and poultry farms have been shown to be important reservoirs of ESBL producing bacteria especially *E. coli* and *Salmonella* spp [4, 18].Though only five ESBL-*E. coli* were isolated in this study, it is still significant because ESBLs hydrolyse expanded-spectrum Cephalosporins and have variants that can be transferred to other bacteria, even within a distantly related genus through Horizontal Gene Transfer (HGT)[2, 4]. The occurrence of ESBL-*E. coli* in livestock has several implications. Firstly, livestock has the potential to serve as an environmental reservoir contributing to its spread. Secondly, since the ESBL-*E. coli* of livestock origin are basically the same as the ones found in clinical isolates they could disseminate through the food chain and infect human [2, 7, 9].Again, many bird species and cattle, including those that were already identified as carriers of ESBL-*Escherichia coli*, display considerable mobility, often involving the crossing of states, countries and even continents. Bird migration could therefore contribute to the dissemination of resistance over the globe as has previously been observed for human travelers [25].

This study showed that cattle fecal materials had more carriage of extended beta lactamase producing *E. coli* than chicken fecal material. However, it was different from the report of [2] who showed more ESBL-*E. coli* from cattle egrets as compared to cattle in Ibadan, Nigeria. This could be as a result of different sampling size as well as different location.

CTX-M gene was detected in all the five (5) ESBL *E. coli* as compared to *SHV* which is 80%, and *OXA* and *TEM* which were 60 and 40% respectively. This means that the most prevalent beta-lactamase genes from the molecular detection were the *shv* and *ctx-m* genes. Increased incidence of *CTX-M* type of ESBL has been reported in the past decades [4].[18] reported the dominance of *CTX-M* genes in 66.7% of isolates from dairy cattle, milk and farm environments in Peninsular Malaysia. Similarly, [2] showed prevalence of *blaCTX-M* genes from cattle and wild bird fecal samples in Ibadan,

Nigeria. Farm animals including poultry, pigs, beef and dairy cattle have been reported as potential sources of antimicrobial resistant bacteria and resistance genes [18].

Conclusion

This study has shown that cattle and poultry farms are important reservoirs of ESBL producing *E. coli*. Though only five ESBL-*E. coli* was isolated in this study, it is still very significant because they hydrolyse expanded-spectrum Cephalosporins and have variants that can be transferred to other bacteria through Horizontal Gene Transfer (HGT). Thorough and extensive studies of antimicrobial drug resistance should be encouraged in different natural habitats. These will help in tracing potential reservoirs of ESBL genes in humans, animals and the environment.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgment

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