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Research Article

Molecular Characterization of Antibiotic Resistance Genes in *Escherichia coli* from Clinical and Environmental Samples in Ghana

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Abstract

Background and Objective: Throughout the world, antibiotic resistance has been recognized as an emerging major issue of concern. In this study, genes responsible for the production of extended spectrum beta-lactamase (ESBL) in various *E. coli* isolates were characterized. **Materials and Methods:** Isolates of *E. coli* were obtained from clinical and environmental samples. These samples were collected from Cape Coast, Ghana. The isolates were tested for ESBL production. Subsequently, PCR assays of DNA extractions from the isolates were used to determine prevalence of bla_{TEM} and bla_{SHV} genes. **Results:** Findings revealed that more ESBL producers were phenotypically identified in *E. coli* isolates recovered from clinical than those recovered from environmental samples. Very low percentages of environmental and clinical isolates that were phenotypically confirmed as non-ESBL producers were found to carry only bla_{TEM}. There was no detection of either gene in majority of the environmental and clinical isolates that were phenotypically confirmed as non-ESBL producers. **Conclusion:** The environment could be a repository of bla_{TEM} and bla_{SHV} genes thus it is imperative to intensify public health education on the risks associated with the misuse of antibiotics.

Key words: bla_{TEM}, bla_{SHV}, beta-lactamase, *E. coli*, Ghana

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

In recent decades, the rate at which pathogenic micro-organisms have developed resistance to various drugs is on the ascendency worldwide[1,2]. Most often, micro-organisms including bacteria acquire resistance through various mechanisms. The release by bacteria of key enzymes known as β-lactamases is a notable example. Essentially, these enzymes break down β-lactam antibiotics. These antibiotics comprise the penicillins, cephalosporins, cephamycins, monobactams and carbapenems. In Gram-negative enteric bacteria, β-lactamase production is a major contributor to resistance to β-lactam antibiotics[3]. There are over 300 different kinds of β-lactamases that have been well documented. In the past few years, extended spectrum β-lactamases (ESBLs) were identified which can degrade several new β-lactam antibiotics. Examples of such antibiotics with extended spectrum also known as third generation cephalosporins are cefotaxime, ceftriaxone and ceptazidime[3]. Though ESBLs can degrade the monobactam aztreonam, they are ineffective against cephamycins and carbapenems[4]. *Klebsiella pneumonia*, *Escherichia coli* and to a lesser extent *Proteus mirabilis* have been found to be the major ESBL producing bacteria worldwide. The ESBLs can mainly be grouped into three: $\textit{bla}_{\text{TEM}}$, $\textit{bla}_{\text{SHV}}$ and $\textit{bla}_{\text{CTX-M}}$. Currently, most infections caused by ESBL producing organisms are treated using β-lactam antibiotics in combination with clavulanic acid, an inhibitor of β-lactamases. Most strains of other enteric bacteria including *Citrobacter* spp. and *Enterobacter* spp. which are resistant to third-generation cephalosporins produce an AmpC β-lactamase. Typically, AmpC β-lactamases can inactivate cephalosporins and cephamycins, such as Cefotetan and Cefoxitin[3]. However AmpC β-lactamases are not inactivated by clavulanic acid.

The ESBLs are detected either phenotypically by testing the ability of ESBL enzymes to hydrolyze different cephalosporins or genotypically using molecular techniques to screen for genes responsible for the production of these enzymes[5]. Phenotypic and genotypic methods are used in many developed countries for routine clinical diagnosis in laboratories and run alongside other tests in automated systems[6]. In developing countries, such as Ghana however, genotypic tests are yet to be incorporated into antibiotic susceptibility tests that are routinely performed at various hospitals. A few studies however have reported on the prevalence of ESBLs producers among various bacterial isolates recovered from clinical samples in different regions of Ghana[7-10]. Two key ESBL-producing bacteria: *E. coli* and *Klebsiella* spp. have been reported to be multidrug-resistant bacteria to which new treatment options are crucially needed[11].

As at now, little information is available on the prevalence of ESBL-producing bacteria isolated from food, water and other environmental samples[12]. Also, the rising incidence of multi-drug resistance in pathogens such as *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Pseudomonas aeuroginosa* and pathogenic *E. coli* suggests that antibiotic resistance surveillance must be made as often as possible in Ghana[13-16]. The present study is a follow-up to an earlier one, which established the presence of *E. coli* isolates with multidrug-resistance in environmental and clinical samples[17]. In 2006, a study in Ghana showed high resistance of pathogens to various antibiotics used in the 10 socioeconomic regions of the country[18]. The rate of emergence of multi-drug resistance in bacteria such as *E. coli* is on the increase in the Cape Coast Metropolis of Ghana[19]. Though antibiotics have been very beneficial in the treatment of *E. coli* infections, the rapid emergence of resistance to these drugs appear to compromise their efficacy. This is what necessitated the present study to determine the occurrence of two of the genes ($\textit{bla}_{\text{TEM}}$ and $\textit{bla}_{\text{SHV}}$) that confer the extended spectrum β-lactamase-producing trait on *E. coli* isolates.

MATERIALS AND METHODS

Study site and period: The research was carried out in Cape Coast, Ghana and its environs from June, 2010 to May, 2012. Besides being a coastal town, Cape Coast (5°6" 0" N, 1°15' 0" W) is one of the famous tourist destinations in West Africa owing to its numerous sites of attraction.

Ethical considerations: Ethical approval was obtained from the Ghana Health Service Ethical Review Committee on Research Involving Human Subjects, before the entire research began. The consent of all study participants was obtained prior to collection of clinical samples.

Sample collection: Clinical and environmental samples were collected as previously described[17]. The clinical samples comprised wound aspirates, stool, urine, and high vaginal swabs while environmental samples comprised drainage canals surrounding the Abura, Kotokuraba and Anaafo markets, the Kakum river and the Fosu lagoon. Other environmental samples such as cabbage heads, fresh beef, fresh chicken and smoked fish were also included.

Bacteria isolation and identification: Samples collected were subjected to bacteria isolation followed by identification as previously described[17].

Phenotypic detection and confirmation of ESBL producers among *E. coli*: All *E. coli* isolates obtained were screened for their sensitivity to the antibiotics ceftriaxone (30 µg) and cefotaxime (30 µg). Details on antibiotics susceptibility testing methods were earlier described[17]. All *E. coli* that recorded clear zones with diameters $\geq$5 and $\geq$27 mm for ceftriaxone and cefotaxime, respectively, were categorized as potential ESBL producers[20]. The latter were subjected to a phenotypic confirmatory test using Mastdiscs TM ID (Mast Group LTD., Merseyside, UK) following manufacturers' instructions. As a quality control measure, reference bacterial strains: *Escherichia coli* ATCC 25922 (β-lactamase negative) and *Klebsiella pneumonia* ATCC 700603 (β-lactamase positive) were tested once every week.

DNA extraction and confirmation as bacterial DNA: For every *E. coli* isolate, 1 mL of fresh overnight culture of bacterial cells (OD_{600} = 1) growing in Luria-Bertani broth was transferred into a clean 1.5 mL microfuge tube. Using a Wizard Genomic DNA Purification Kit (Promega Corporation, USA) and following manufacturers' instructions DNA was extracted. It was ensured that all DNA samples were pure before using them for subsequent analysis. The concentration of each DNA sample was standardized to 100 ng μL^{-1}. All DNA samples obtained were subsequently confirmed as bacterial DNA through PCR assays using the 16S rRNA gene, a universal primer for bacterial DNA[21]. In a single reaction mixture of 50 µL, 1 µL each of 1 pmol of forward and reverse primers for the 16S rRNA (Table 1) was added to a PCR microtube containing Accupower Multiplex PCR premix (Bioneer Corporation, South Korea). In addition, 46 µL of sterile molecular biology grade water (Sigma-Aldrich, Inc. USA) and 2 µL of 100 ng μL^{-1} template DNA were added. The PCR amplification was carried out using a 96 well plate of a Techne TC-512 thermal cycler (Bibby Scientific, UK). The amplification process involved an initial denaturation at 94°C for 3 min; 30 cycles of 94°C for 30 sec, annealing at 55°C for 30 sec followed by extension at 72°C for 1 min and a final extension at 72°C for 10 min.

Detection of ESBL Genes (*bla*$_{SHV}$ and *bla*$_{TEM}$) in clinical and environmental isolates of *E. coli*: Two separate PCR assays were used for the detection of *bla*$_{SHV}$ and *bla*$_{TEM}$ genes, primarily, because the primers used in their detection produced very similar amplified products. A list of primers used in this study is presented on Table 1. Consequently, in a reaction mixture of 50 µL, 1 µL each of 1 pmol of forward and reverse primers for either *bla*$_{SHV}$ or *bla*$_{TEM}$ genes were added to a PCR microtube containing Accupower Multiplex PCR premix (Bioneer Corporation, South Korea) together with 46 µL of sterile molecular biology grade water and 2 µL of 100 ng μL^{-1} template DNA. The amplification process involved an initial denaturation at 95°C for 15 min; 30 cycles of 94°C for 30 sec, annealing at 56°C for *bla*$_{TEM}$ or 60°C for *bla*$_{SHV}$ primers, respectively, for 30 sec followed by extension at 72°C for 30 sec and a final extension at 72°C for 7 min[22] (Table 1).

For each PCR product, 10 µL were loaded in each of the wells of 1.5 % (w/v) agarose gel containing 0.001% (w/v) ethidium bromide (Sigma-Aldrich, Inc. USA) and a 100 bp DNA ladder (Sigma-Aldrich, Inc. USA) was used as a molecular weight marker. The PCR amplicons were separated by electrophoresis at 100 V for an hour. Each gel was visualised under an ultraviolet trans-illuminator (UVP products, UK) and photographic records were made using a Canon digital camera S110 12.1 Mega Pixels.

Statistical analysis: Chi-square test (SPSS version 16) was performed to analyze all data obtained at a 5% level of significance.

RESULTS

Distribution of ESBL producers in *E. coli* isolates from clinical and environmental sources: The phenotypic confirmatory test for ESBL producers using a bacterial lawn of the isolates showed clear zones of varying diameters around each 2nd or 3rd generation cephalosporin (Fig. 1).

The distribution of ESBL producers in all *E. coli* isolates from clinical and environmental sources is presented in Table 2. There was a significantly ($p<0.001$) lower percentage of ESBL producers in both clinical and environmental sources

Table 1: Primers used for confirmation of bacterial DNA and detection of ESBL genes

Name	Sequence (5' to 3')	AT (°C)	Product size (bp)	References
27F	GAGTTTGATCCTGGCTCAG	59	1500	Lane[21]
1492R	GGTTACCTTGTTACGACTT			
TEM F	GAGTATTCAACATTTCCGTGTCGC	56	865	Zhang *et al.*[22]
TEM R	TACCAATGCTTAATCAGTGAGGC			
SHV F	ATGCGTTATATTCGCCTGTG	60	860	Zhang *et al.*[22]
SHV R	TTAGCGTTGCCAGTGCTTGATC			

AT: Annealing temperature, ESBL: Extended spectrum β-lactamase, F: Forward primer, R: Reverse primer

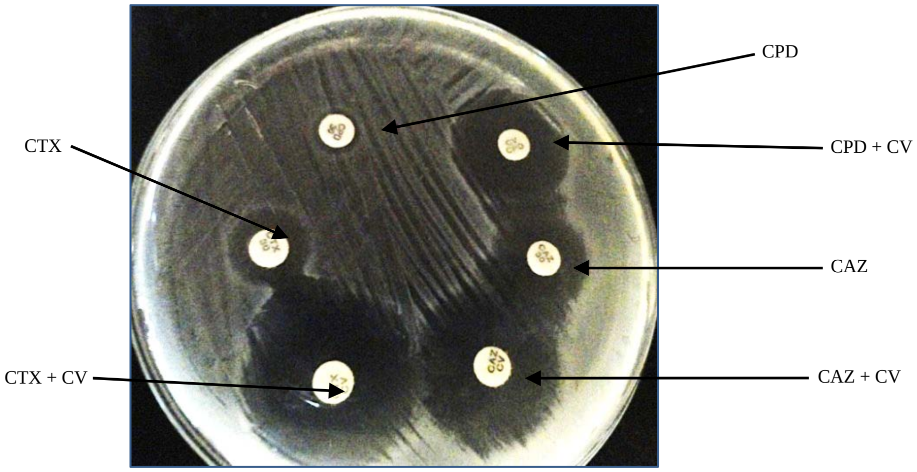

Fig. 1: Phenotypic confirmation of ESBL production by *E. coli* isolates using three sets of antibiotics with and without clavulanic acid

CAZ: Ceftazidime (30 µg), CTX: Cefotaxime (30 µg), CPD: Cefpodoxime (30 µg), CV: Clavulanic acid (10 µg)

Table 2: Distribution of ESBL-producing and non-ESBL-producing *E. coli* strains isolated from clinical and environmental samples

Sample type	ESBL producers	Non-ESBL producers	Total
Clinical	78 (29.8)^{ac}	183 (70.1)^{d}	261 (100.0)
Environmental	16 (12.5)^{b}	112 (87.5)	128 (100.0)
Total	94 (26.5)	295 (73.5)	389 (100.0)

χ^2 = 14.2 (p<0.0001), a: p<0.0001-ESBL producers significantly lower than non-ESBL producers for clinical samples, b: p<0.0001- ESBL producers significantly lower than non-ESBL producers for environmental samples, c: p<0.0001-ESBL producers in clinical samples significantly higher than in environmental samples, d: p<0.0001- Non-ESBL producers in environmental samples significantly higher than in clinical samples

Table 3: Prevalence of bla_{SHV} and bla_{TEM} genes among ESBL producing and non-ESBL producing-*E. coli* isolates

	Environmental isolates (n = 128)		Clinical isolate (n = 261)		
Gene	ESBL +ve	ESBL -ve	ESBL +ve	ESBL -ve	Total
Neither bla_{TEM} nor bla_{SHV}	2 (12.5)	94 (83.8)	4 (5.1)	138 (75.4)	238
bla_{TEM}	4 (25.0)	6 (5.4)	20 (25.7)	9 (4.9)	39
bla_{SHV}	8 (50.0)	12 (10.8)	37 (47.4)	34 (18.6)	91
bla_{TEM}+bla_{SHV}	2 (12.5)	0 (0.0)	17 (21.8)	2 (1.1)	21
Total	16 (100.0)	112 (100.0)	78 (100.0)	183(100.0)	389(100.0)

ESBL: Extended spectrum β-Lactamase, +ve: Positive;, -ve: Negative

as compared to non-ESBL producers. The number of ESBL producers in clinical samples was also significantly (p<0.0001) higher than in the environmental samples. A similar trend was recorded for non-ESBL producers (p<0.0001).

Furthermore, 29.9% of *E. coli* isolates of clinical origin were found to be ESBL producers. A lower proportion of 12.5% of environmental *E. coli* isolates were found to be ESBL producers.

Characterization of ESBL producing genes in *E. coli* isolates from clinical and environmental sources: The DNA extraction resulted in 389 DNA samples with 261 and 128 from clinical and environmental *E. coli* isolates, respectively (Fig. 2a). An agarose gel picture resulting from a confirmation of DNA extract as bacterial DNA is shown on Fig. 2b.

The results obtained from the characterization of ESBL genes in all 389 *E. coli* isolates are summarized in Table 3. The single detection of the bla_{TEM} gene among phenotypically confirmed non-ESBL producers was recorded in 5.4 and 4.9% of environmental and clinical *E. coli* isolates respectively (Table 3). However, 10.8 and 18.6% of non-ESBL environmental and clinical *E. coli* isolates, respectively, were found to carry only bla_{SHV}. There was no detection of either gene for 12.5 and 5.1%, respectively, of the phenotypically confirmed ESBL-producing isolates obtained from environmental and clinical samples. Among the *E. coli* isolates phenotypically confirmed as ESBL-producing, 21.8% from clinical samples possessed both bla_{TEM} and bla_{SHV} genes while only 1.1% of the phenotypically confirmed non-ESBL producers from clinical

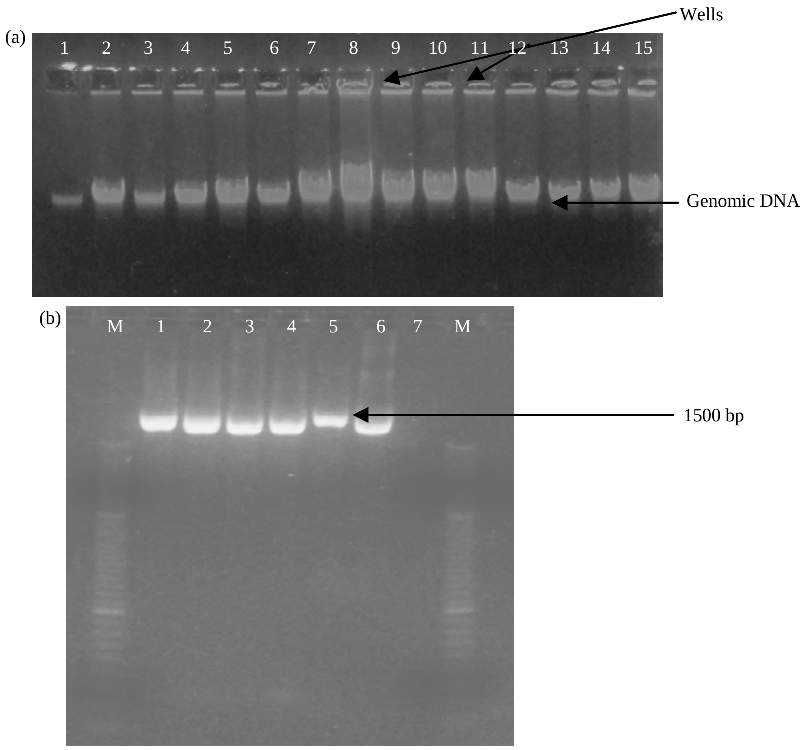

Fig. 2(a-b): (a) Ethidium bromide stained 1% agarose gel showing 15 representative genomic DNA samples. Lane 1-15, genomic DNA extracted from *E. coli* isolates and (b) Ethidium bromide stained 2.5% agarose gel showing PCR products obtained after confirmation of DNA samples as bacterial DNA. Lane 1, 2, 3, 4, 5 and 6, presence of 16 S rRNA gene; Lane 7, absence of 16 S rRNA gene; Lane M, 20 bp molecular marker

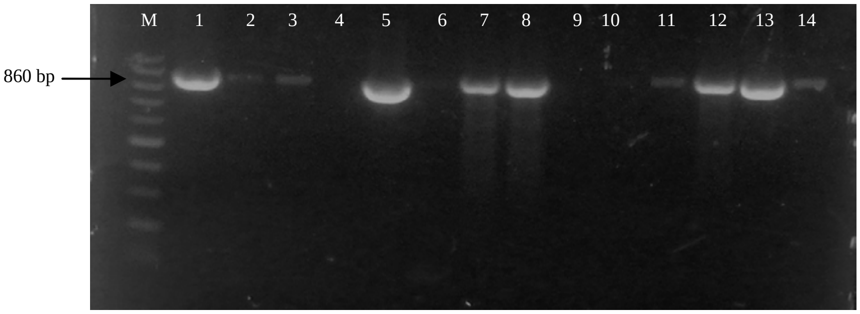

Fig. 3: Ethidium bromide-stained PCR amplification products on a 1.5% agarose gel showing the presence or absence of the *bla*$_{SHV}$ gene among *E. coli* isolates

Presence of the *bla*$_{SHV}$ gene in lanes 1, 2, 3, 5, 7, 8, 11, 12, 13 and 14 and absence in lanes 4, 6, 9 and 10, Lane M: 100 bp ladder

samples had both ESBL genes and none for environmental isolates (Table 3). A representative gel photograph obtained during the screening for the presence of the *bla*$_{SHV}$ gene in *E. coli* isolates is shown on Fig. 3. Another representative gel photograph obtained during the screening for the presence of the *bla*$_{TEM}$ gene in *E. coli* isolates is also shown on Fig. 4.

DISCUSSION

In this study, among the phenotypically confirmed ESBL producing *E. coli* isolates, 21.8% of the isolates from clinical samples possessed both *bla*$_{TEM}$ and *bla*$_{SHV}$ genes. Phenotypically confirmed non-ESBL producers, however, comprised 1.1% of the clinical isolates whilst none of the

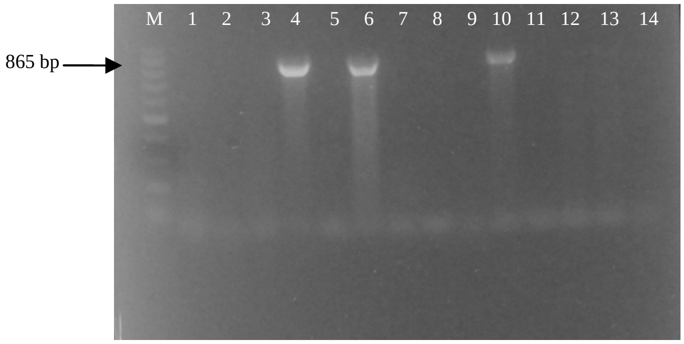

Fig. 4: Ethidium bromide-stained[2] PCR amplification products on a 1.5% agarose gel showing the presence or absence of the *bla*$_{TEM}$ gene among *E. coli* isolates

Lanes 1, 2, 3, 5, 7, 8, 9, 11, 12, 13 and 14, absence of *bla*$_{TEM}$ gene, Lanes 4, 6 and 10, presence of *bla*$_{TEM}$ gene, Lane M: 100 bp ladder

environmental isolates had both ESBL genes (Table 3). The prevalence of ESBL-producing environmental isolates was relatively lower (12.5%) than those of clinical isolates. This indicates clearly that there was some level of multidrug resistance in the environment, suggesting that infections caused by such micro-organisms could be much more difficult to treat. Therefore, the presence of such strains could be a major contributing factor to the rapid emergence of multidrug resistance[23]. The percentage of the phenotypically confirmed ESBL producers among clinical samples collected in this study was lower (29.9%) than the 44.4% reported in a study conducted in Kumasi[9] and 49.3% in Accra[8]. Even though very useful, the phenotypic methods of detection of ESBL production cannot differentiate between the key enzymes that are responsible[5] and this could impede effective health delivery. The expression of extended spectrum β-lactamases (ESBL) as a defense mechanism, by some enteric bacteria, especially *Klebsiella* spp. and *E. coli* continues to be a major source of antimicrobial therapy failure[23,24] and consequently, has serious repercussions for infection control. There are many types of ESBL including *bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{CTX}$ and *bla*$_{OXA}$. However the majority of them are derivatives of *bla*$_{TEM}$ and *bla*$_{SHV}$ enzymes and mostly found in *E. coli* and *K. pneumonia*[25]. Several researchers employ molecular methods for detection of specific genes responsible for ESBL production, which can be used to identify very low levels of resistance that could remain undetected when conventional phenotypic protocols are used[26]. In the current study, the amplification of the two genes *bla*$_{TEM}$ and *bla*$_{SHV}$ using PCR was the method adopted without sequencing of the amplified products. Consequently, 12.5% of environmental and 5.1% of clinical isolates of *E. coli* phenotypically confirmed were negative for either of the two resistance genes, possibly, because they carried some ESBL genes other than *bla*$_{TEM}$ and *bla*$_{SHV}$. It was also found that 16.2% of environmental isolates and 24.6% of clinical ones phenotypically confirmed ESBL-negative *E. coli* possessed either *bla*$_{TEM}$ or *bla*$_{SHV}$. However, among the phenotypically confirmed ESBL-negative *E. coli* isolates, higher proportions of isolates were found to possess *bla*$_{SHV}$ only than *bla*$_{TEM}$ only. This suggested that *bla*$_{SHV}$ was not as good a marker for ESBL detection in *E. coli* as *bla*$_{TEM}$. Similar findings were reported in a study conducted in Pretoria, South Africa, on *K. pneumonia* isolates[27]. It is also possible that the phenotypic method used in this study was not sensitive enough to detect the ESBL producers in those cases thereby leading to false negative results as reported in another study[28,29]. This is a major challenge to phenotypic methods confirming their limitations as diagnostic tools. Thus, phenotypic methods should be backed by the reliable PCR method or other genotypic methods. ESBL genes were identified in environmental *E. coli* isolates at lower frequencies than in clinical samples. It is however worthy to note that if appropriate measures are not taken, the relatively lower levels of resistance observed in the environment are likely to increase steadily in the next few years. The current study pointed to the urgent need for Ghana to effectively implement the policy on antimicrobial use and resistance signed in 2017. This will go a long way to curb the menace in Ghana as other countries including Germany, have taken pragmatic steps to minimize the rate of emergence of multidrug resistance. Also, for effective diagnosis of bacterial and other microbial infections, there is the need to equip health laboratories with some molecular tools to back phenotypic findings. This should allow for correct prescriptions for effective health delivery. Further research will be conducted to characterize the other types of ESBL prevalent in the country including those encoded by the *bla*$_{CTX-M}$ gene.

CONCLUSION

The study has demonstrated that, apart from the hospital, the environment could be a reservoir of *E. coli* carrying bla_{TEM} and bla_{SHV} gene. This calls for the intensification of public education on the risks associated with the misuse of antibiotics.

SIGNIFICANCE STATEMENT

This study reports on the presence of ESBL genes in *E. coli* naturally found in the environment and also from clinical samples. This is a major public health threat as such genes can get integrated into the community thereby contributing to the rise in cases of unsuccessful treatment and mortality in Cape Coast. The findings form very important contributions, as Cape Coast, the famous coastal town of the West African country Ghana, is a major tourist destination in the sub-region.

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