Molecular Characterization of Extended-Spectrum Beta-Lactamase Producing Urinary *Escherichia coli* Isolated in Brong-Ahafo Regional Hospital, Ghana

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Abstract

Introduction. Antimicrobial resistance is a growing international problem resulting from the enzyme β-lactamase production by bacteria, to degrade antibiotics, especially β-lactam antibiotics. In Brong Ahafo Regional Hospital, Sunyani, these antibiotics are heavily depended upon for the treatment of serious infections, but unfortunately high proportions of bacterial isolates in the hospital, have been found to be resistant to the commonly prescribed antibiotics. This study aims to determine the prevalence of ESBL-producing E. coli so as to determine if ESBL are responsible for the high antimicrobial resistance seen at the Brong-Ahafo Regional Hospital, Sunyani.

Methods. The study was a cross sectional study, involving 51 E. coli isolates from urine samples of both in- and out-patients between January and December, 2014. Antimicrobial susceptibility profiles of the isolates were determined by the Kirby Bauer disc diffusion method against 12 antibiotics. The isolates were screened for ESBL production, and then confirmed by the combined disc method. The isolates were tested for the presence of ESBL $bla_{CTX\text{-}M}$ and bla_{TEM} by conventional PCR.

Results. Non-repeat 1,302 midstream urine samples were cultured from which 200 different pathogens were isolated. Of the 200 isolates 51 were E. coli. Isolates obtained from Community isolates were 16(37.2) and isolates from in-patients were 27(62.8). Resistant strains were detected to all the 12 antimicrobials tested. Low proportions were sensitive to cephalosporins (cefotaxime and ceftazidime), both recording 8/51(15.7%), and quinolones (nalidixic acid and ciprofloxacin), 7/51(13.7%) and 8/51(15.7%) respectively. The isolates had varied susceptibility to aminoglycosides, with low sensitivity of 1/32(2.0%) to gentamicin, but a high proportion of 47/51(92.2%) was sensitive to amikacin. High proportion of the isolates [43/51(84.3%)] were ESBL phenotype, and was found to be significantly associated ($p<0.001$) with antimicrobial resistance among the isolates. The most prevalent ESBL genotype was Bla$_{TEM}$ with 26 (66.7%) and bla$_{CTX\text{-}M}$ 28(71.8%), but 9(23.1%) ESBL phenotypes tested negative for both bla$_{TEM}$ and bla$_{CTX\text{-}M}$ genes. 22 (56.4%) of the isolates had both bla$_{TEM}$ and bla$_{CTX\text{-}M}$ genes.

Conclusion. High proportions of E. coli isolates from urine in Sunyani produce ESBLs. Both bla$_{TEM}$ and bla$_{CTX\text{-}M}$ were prevalent and linked to the high levels of drug resistance found in the locality. Increased antibiotic stewardship and stringent infection control measures along with the testing for ESBL must be instituted in the hospital.

Keywords: Extended-Spectrum Beta-lactamase, UTI, antibiotic resistance, CTX-M and TEM.

Introduction

Overuse of antibiotics is regarded as one of the many reasons for the rising trend of resistance observed among different pathogens, (Gonçalves *et al.*, 2016) all over the world and grave concern has been expressed about the increase in the numbers of organisms that are multi-drug resistant and the difficulty encountered in their treatment (Hima-Lerible *et al.*, 2003; Howladar and Gandhi, 2016). In the choice of antimicrobial for treatment, β-lactam antimicrobials are preferred because they are highly effective against several bacterial pathogens, with minimal side effects and reduced toxicity, (Chiriac *et al.*, 2016) as a result, β-lactam antibiotics are frequently prescribed and oftentimes indiscriminately (Newman *et al.*, 2006; Bassetti *et al.*, 2016). As a result, resistance to β-lactam antibiotics is substantially on the ascendancy becoming a frequent problem encountered in medical practice (Fisher *et al.*, 2005). As the introduction of third generation cephalosporins into any community comes along with it the emergence of ESBLs, (Pitout *et al.*, 2004; Paterson and Bonomo, 2005; Perez *et al.*, 2007). β-lactamase production effectively terminates β-lactam activity and serve as the predominant resistance mechanism among Gram-negative bacteria including *E coli* (Bonnet, 2004; Hertz *et al.*, 2016). ESBLs have extended activity against alternative antibiotic groups like the aminoglycosides and the fluoroquinolones, so ESBL-producing organisms become resistance to them ((Ramazanzadeh, 2010).

In Ghana these antibiotics are the mainstay for the treatment of many infections including UTI, but routine screening of isolates for ESBLs is not done, because of the additional cost it constitutes to the patient, lack of testing facilities to the technicians, and the general lack of interest among clinicians (Newman *et al.*, 2006). Heavy dependence of β-lactam antibiotics leads to the development of β- lactamases and resistance as evident in high resistance proportions reported from teaching Hospitals in Ghana mainly Korle Bu Teaching Hospital(KBTH) (Opintan and Newman, 2007; Obeng-Nkrumah *et al.*, 2013) and Komfo Anokye Teaching Hospital (KATH) (Feglo *et al.*, 2013). Studies on ESBLs have not been conducted from secondary referral hospitals such as the Brong Ahafo Regional Hospital, in Sunyani. This study therefore is to determine the prevalence of ESBL phenotype and genotypes among *E. coli* strains obtained from UTI patients at Sunyani and to determine their antimicrobial susceptibility patterns.

METHODS

Study design and setting

This was a cross-sectional study conducted at the Brong Ahafo Regional Hospital, which is a 300-bed and secondary referral hospital in the Brong Ahafo Region, with population of about two million inhabitants. The Region has 19 districts with Sunyani as the Regional capital.

Urine samples for the study were obtained from patients referred to the Microbiology Laboratory of the hospital for UTI investigations. Patients who gave their informed consent had their samples included in the study. Ethical clearance was obtained from the Institutional Ethics Committee of the Brong- Ahafo Regional Hospital, Sunyani.

Bacterial isolation

Early morning, mid-stream urine samples were received from patients at the Microbiology laboratory and plated on cysteine lactose electrolyte deficient (CLED) agar using a 1/400μm calibrated loop and pure colonies of ≥25 growing on CLED agar after overnight incubation at 37°C were considered significant for diagnosis of urinary tract infection. This is equivalent to growth of 10^5 colony forming units/ml of one organism type and was considered significant bacteriuria. Where there were mixed growth of different organisms it was considered contamination and then rejected.

The growths were identified by their colonial growth morphology on CLED medium. Lactose fermenting colonies, suspected to be *E. coli* were tested further using Gram stain (Gram negative rods), positive in the motility test and then indole and methyl red positivity, citrate negative and Voges-Proskauer negative tests.

Antibiotic susceptibility testing

Antibiotic sensitivity of the isolates was determined by the modified Kirby-Bauer method according the CLSI recommended guidelines (20th Inf.

Suppl. M100-S20). Each set of tests was controlled using susceptible *E. coli* control strain (ATCC 25922). Antimicrobials and their concentrations tested included: Ampicillin/Sulbactam 20ug, amikacin 30ug, cefotaxime 30ug, ceftazidime 30ug, chloramphenicol 30ug, ciprofloxacin 5ug, gentamicin 10ug, ofloxacin 5ug, nalidixic acid 10ug, tetracycline 30ug, Levofloxacin 5ug, and ceftizoxime 30ug.

ESBL Screening and confirmation

In the routine susceptibility testing strains with reduced zone diameters of <27mm and <22mm respectively to cefotaxime and or ceftazidime were suspected of producing ESBLs according to CLSI guidelines (CLSI, 2011). All suspect isolates in the screening test were confirmed using the cefpodoxime discs alone and combined discs of cefpodoxime and clavulanic acid (30µg+10µg) (Oxoid GmbH, Wesel, Germany). Isolates that produced increased zones of ≥5mm between cefpodoxime disc alone and the combined disc is considered an ESBL. *Escherichia coli* ATCC 25922 was used as ESBL negative control and *Klebsiella pneumoniae* ATCC 700603 was used as ESBL positive control.

Molecular Characterization of ESBLs

All confirmed ESBL phenotypes were tested for *Bla*$_{\mathrm{TEM}}$ and *bla*$_{\mathrm{CTX\text{-}M}}$ by PCR according previously described techniques (Monstein *et al.*, 2007) and (Mulvey *et al.*, 2003) Mulvey *et al.*, 2004).

Table 1. Primers for amplification of *bla*$_{\mathrm{TEM}}$ and *bla*$_{\mathrm{CTX\text{-}M}}$

Gene		**Primer sequence (5"-3")**	**size**	**Source**
Bla$_{\mathrm{TEM}}$	TEM-F	ATGTGGCAGYACCAGTAARGTKTGGC	593	Monstein & LE, (2007)
	TEM-R	TGGGTRAARTARGSACCAGAAYCAGCG		
Bla $_{\mathrm{CTX\text{-}M}}$	CTX-M-U1	ATGTGCAGYACCAGTAARGTKATGGC	593	Mulvey et al. (2003)
	CTX-M-U2	TGGGTRAARTARGTSACCAGAAYCAGCGG		

Source: Bla CTX-M (Mulvey et al.,2003), and Bla TEM(Monstein & LE, 2007) Key for standard Mixed base symbols: R-A,G; Y-C,T; M-A,C; K-G,T; S-C,G; W-A,T; H-A,C,T; B-C,G,T; V-A,C,G; D-A,G,T(Integrated DNA Technologies, Inc , USA)

Data Processing and Analysis

Continuous data were expressed as mean ±SD and categorical data expressed as proportion. Resistance proportions were compared using chi-square tests. In all cases a p-value <0.05 was considered significant. The data were analyzed using Stata/IC 10.0 for windows (StataCorp LP, USA, http://www.stata.com).

RESULTS

Antibiotic susceptibility pattern of *E. coli*

Non-repeat midstream urine samples numbering 1,302 were cultured. Significant bacteriuria was detected and 200 different pathogens were isolated. Of the 200 isolates 51 were *E. coli*. *E. coli* isolates obtained from community isolates were 16(37.2) and 27(62.8) were obtained from in-patients. All the 51 *E. coli* isolates were tested against 12 different antibiotics namely Ampicillin/Sulbactam, nalidixic acid, cefotaxime, ceftazidime, chloramphinicol ciprofloxacin, ceftizoxime, tetracycline, ofloxacin, gentamicin, amikacin and levofloxacin, using the Disk-diffusion method.

High proportions of the *E. coli* isolates were resistant to the various antimicrobial agents tested. The highest resistant proportions were found against tetracycline and ampicillin where they recorded (50/51 (98.0%) and (38/51 (74.5%) respectively. Varied resistant proportions were detected to the quinolones with 43/51(84.3%) to nalidixic acid and ofloxacin recorded 36/51(70.6%), levofloxacin 27/51(52.9%) and ciprofloxacin 42/51(82.4%). Resistant proportions were detected against aminoglycosides and cephalosporins tested and the details are presented in table 2.

Table 2. Antibiotic susceptibility pattern of *E. coli* isolated from urine at Sunyani.

E. coli (n=51)	AS	NX	CTX	CAZ	CH	CP
Sensitive	5(9.8%)	7(13.7%)	8(15.7%)	8(15.7%)	16(31.4%)	8(15.7%)
Resistant	38(74.5%)	43(84.3%)	42(82.4%)	42(82.4%)	34(66.7%)	42(82.4%)
Intermediate	8 (15.7%)	1 (2.0%)	1 (2.0%)	1 (2.0%)	1 (2.0%)	1 (2.0%)
Total	**51**	**51**	**51**	**51**	**51**	**51**

E. coli (n=51)	CL	TE	OF	GM	AK	LE
Sensitive	31(60.8%)	-	11(21.6%)	1(2.0%)	47(92.2%)	11(21.6%)
Resistant	12(23.5%)	50(98.0%)	36(70.6%)	30(58.8%)	2 (3.92%)	27(52.9%)
Intermediate	8 (15.7%)	1 (2.0%)	4 (7.8%)	1 (2.0%)	2 (3.9%)	13(25.5%)
Total	**51**	**51**	**51**	**32**	**51**	**51**

AS: *Ampicillin/Sulbactam*; NX: *Nalidixic acid*; CTX: *Cefotaxime*; CAZ*: Ceftazidime; CL: Ceftriaxone; CH: Chloramphenicol; CP: Ciprofloxacin; TE: Tetracycline; OF: Ofloxacin; GM: Gentamicin; AK: Amikacin; LE: Levofloxacin.*

Prevalence of ESBL in urinary *E. coli* isolated from Sunyani

Aminoglycoside resistance was also significantly associated with ESBL expression ($p<0.0006$). Twenty-seven strains (90.0%) were resistant to at least 1 of the 2 tested aminoglycosides with most frequently observed resistance against gentamicin 27(90.0%), while about 15(35%) were resistant to quinolones. Of the 51 *E. coli* isolates, 43 tested positive and 8 tested negative for ESBL production. There was no significant association between ESBL production, gender and age (p<0.42) and (p<0.18) respectively (Table 3).

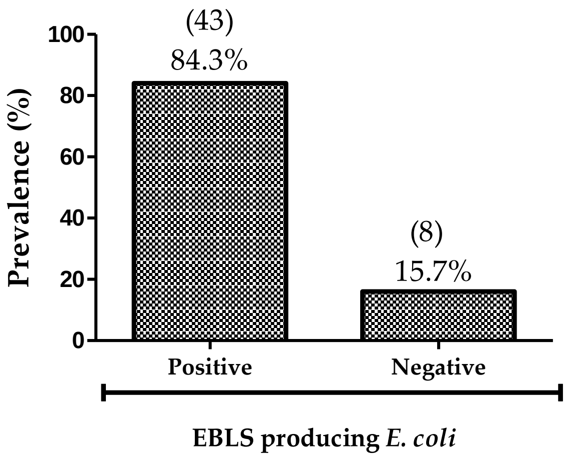


Fig 1 Prevalence of EBL producing *E. coli* isolated from Sunyani

Table 3. Association of Gender and Age with ESBL production in *E. coli*

	Total n=51	ESBL Positive n=43	ESBL Negative n=8	p-value
Gender				
Male	18	14 (32.6%)	4 (50.0%)	0.4297
Female	33	29 (67.4%)	4 (50.0%)	-
Age group (years)				
Under five years	9	8(18.6%)	1 (12.5%)	0.1828
Adults	42	35(81.4%)	7 (75.0%)	-

Values are presented as frequency (percentage). Difference between proportions was tested using Chi-square. p<0.05 was considered statistically significant.

There was significant association between ESBL production and multi-drug resistance. Antimicrobial resistance was significantly associated with ESBL production (p<0.0001) (Table 4).

Table 4 Comparison between antimicrobial drug resistance and ESBL production in *E. coli*

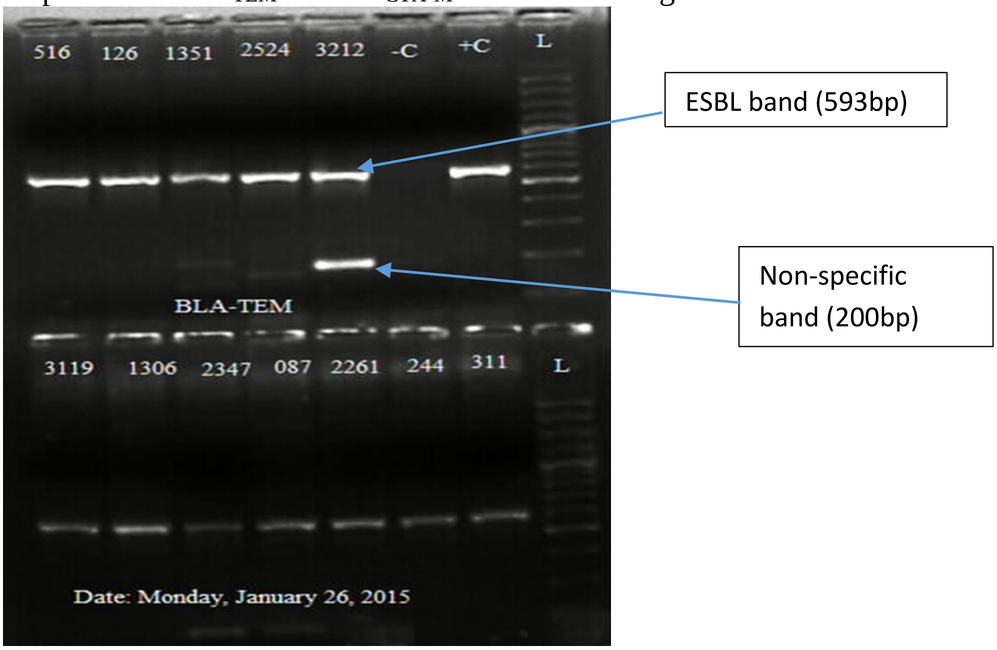
	ESBL isolates N=43	**Non-ESBL isolates N=8**	**Total (N=51)**
	% Resistance	% Resistance	**P-values**
Cefotaxime	42/42(100.0)	0/42(0.0)	**< 0.01**
Ceftazidime	42/42(100.0)	0/42(0.0)	**< 0.01**

Ceftizoxime	9/12(75.0)	3/12(25.0)	**< 0.01**
Ciprofloxacin	36/42(85.7)	6/42(14.3)	0.84
Levofloxacin	24/27(88.9)	3/27(11.1)	**< 0.01**
Ofloxacin	32/36(88.9)	4/36(11.1)	**< 0.01**
Nalidixic acid	37/43(86.0)	6/43(14.0)	0.87
Amikacin	2/2(100)	0/2(0.0)	**< 0.01**
Gentamicin	27/30(90.0)	3/30(10.0)	**< 0.01**
Ampicillin-sulbactam	33/38(86.8)	5/38(15.2)	**< 0.01**
Tetracycline	42/50(82.4)	8/50(16.0)	**< 0.01**

Continuous data were presented as mean ± Sd and categorical data presented as proportion. Categorical data were compared to each other using Chi-square analysis. 0.05

Molecular Characterization of ESBLs

The two ESBL genes namely *Bla*$_{TEM}$ and *Bla*$_{CTX\text{-}M}$ tested for were detected among the 43 *E. coli* ESBL phenotypes. *Bla*$_{TEM}$ was detected in 26 (66.7%). *Bla*$_{CTX\text{-}M}$ was detected in 28/43(65.1%) of the isolates. Twenty-two being 51.1% of the isolates were positive for both *Bla* $_{CTX\text{-}M}$ and *Bla*$_{TEM}$ genes. Phenotypically 9(20.9%) of isolates tested positive for ESBL but were negative for both *Bla*$_{CTX\text{-}M}$ and *Bla*$_{TEM}$. Pictures of agarose gel separation of amplicons for *Bla*$_{TEM}$ and *Bla*$_{CTX\text{-}M}$ are shown in Figures 2 and 3.



Isolates 087, 3212 and 2347 showed non- specific bands (presence of two genes) of 593/200 while 876, 947, 2183, 2908, 4173 and 314 are negative for blaTEM +c depicts PCR product of E. coli positive control and –c depicts PCR product of a negative control. L is the molecular weight standard 100bp (Thermo Scientific). Band size 593bps

Fig 2. PCR amplicons on 2% agarose gel for *Bla*TEM genes of E. coli

Isolates 3119**negative for bla CTX-M, +c and –c are positive and negative controls, L is ladder standard 100bp

Fig 3. PCR amplicons on 2% agarose gel for bla $_{\text{CTX-M}}$ genes

DISCUSSIONS

Several studies have demonstrated the presence of β-lactamases in Ghana, but most of these studies were conducted in tertiary referral institutions. Results similar to those obtained in tertiary referral hospitals were expected in Sunyani Hospital, because it is a secondary referral hospital and specialist services exist in this institutions too, just like other regional hospitals in Ghana. Patients are referred from many districts within the region and beyond to seek health care at the regional hospitals. It is cases beyond the competence of the regional hospitals that are referred to the tertiary levels in Kumasi and Accra. This problem of ESBL has not caught the attention of the health authorities and are missing in many medical reports from the health institutions. ESBLs are not just nosocomial infections in big hospitals in Ghana, but are spreading into the communities as well (Feglo *et al.*, 2013). In this present study 43/51(84%) of the urinary *E. coli* isolates produced ESBL and 16(37.2%) were community isolates, an observation, which confirms findings in other studies that ESBL-producing *Enterobacteria* are detectable in both community and hospitalized patients with varying prevalence levels as 63% was reported from Mali (Tande *et al.*, 2009), 43% in Ghana (Feglo *et al.*, 2013), 40% in Niger (Woerther *et al.*, 2011) and 26% in Kenya (Kariuki *et al.*, 2007) and 28.66% in Bangladesh (Islam *et al.*, 2017). It was estimated that ESBL prevalence of 80% in a hospital setting will lead to as much as 80% of treatments failures and 35%

mortality (Perez *et al.,* 2007). The detection of more than 80% ESBL prevalence in Sunyani is of concern as deaths might have arisen from treatment failures as a result of ESBL problem in the Sunyani health facility. There are no ESBL records and ESBL treatment failures are not and cannot be accounted for because the hospital authorities are unaware of it and β-lactam antibiotics are still prescribed. Even if hospital ESBL spread is controlled, the community ESBL auxotrough spread will persist and will replace normal antibiotic susceptible wild type in the community. Alternative antibiotic groups like the aminoglycosides and the fluoroquinolones, have also been affected by the ESBL menace because the enzymes have extended activity to non-beta lactam antibiotics too (Ramazanzadeh, 2010). As demonstrated in the present study, appreciable proportion of *E. coli* resistance was recorded against the quinolones, with resistance proportion to nalidixic acid being 84.3%, ofloxacin (70.6%), Levofloxacin (52.9%) and ciprofloxacin (82.4%).

ESBL carriage was observed to be significantly associated with antimicrobial resistance in *E. coli* isolates tested from Sunyani ($p < 0.01$), as was also observed in India (Jain *et al.,* 2003) Bangladesh (Asna *et al.,* 2017). Studies indicate that patients who have ESBL infection seem to have poorer prognosis than patients infected with non ESBL bacteria (Paterson and Bonomo, 2005), and the reason may be due to treatment failures . This is a serious issue given that immunity at extremes of life is relatively weak, a situation which prescribers must know of and must show optimum care for in the choice of antibiotics meant for patients less than 5years and above 60 years.

There was increased resistance of *E. coli* to most of the antimicrobials tested, with the exception of amikacin to which resistant proportion was low (3.9%). Though 20.26% of community urinary isolates in Morocco were found to be multidrug resistant (Natoubi *et al.,* 2017), elsewhere in China and India, amikacin and nitrofurantoin were reported to be effective drugs for the treatment for UTIs (Shao *et al.,* (2004) and Mandira Mukherjee *et al.,* (2013) respectively. In our study and in Nigeria high proportions of urinary isolates were resistant with all *E. coli* isolates (100%) being resistant to augmentin, gentamycin, streptomycin, tetracyclin and chloramphenicol, and 90.90% resistant to ofloxacin, sparfloxacin and amoxicillin (Elikwu *et al.,* 2017). High resistant proportions seen in this current study is attributable to many factors. Firstly, many hospitals in Ghana do not have the competence and the facility to do bacterial culture, isolation and antimicrobial sensitivity testing, so physicians prescribe without laboratory support. The second factor is the indiscriminate use the drugs, because they are relatively cheap and easily accessible by the patient. The drugs can be purchased off the counter due to lack of enforcement of regulations, and also because some of these antibiotics are taken orally, so they are easy to administer and misuse (Newman *et al.*, 2006). Another link to antibiotic resistance is the increased use of antibiotics in animal husbandry coupled with the production and sale of substandard and fake drugs (Tajick, 2006; Shakoor *et al*., 1977). In the case of substandard drugs physicians prescribe adequate doses, but do not achieve optimal clinical response *in-vivo* leading to the creation of resistant strains.

In this study, *Bla*$_{\mathrm{TEM}}$ was detected in 26 (66.7%) of the ESBL *E. coli* strains and *bla* $_{\mathrm{CTX\text{-}M}}$ tested was detected in 28 (71.8%) of the isolates. These results are similar to other results obtained in studies in tertiary institutions in Ghana where higher *Bla*$_{\mathrm{TEM}}$ were reported than the *Bla*$_{\mathrm{CTX\text{-}M}}$ among *E. coli* strains (Obeng-Nkrumah *et al.*, 2013 ; Labi *et al.*, 2016).

Studies in Europe and America reported more prevalence of SHV and CTX-M-β-lactamase organisms than the TEM (Bonnet, 2004; Naseer and Sundsfjord, 2011), but most studies in Ghana report the TEM ESBL as the most common, just as it is found in the current study in Sunyani. Though tested phenotypically to be ESBLs about 20.9 % of the ESBL-producing isolates in this study were negative for the both *Bla*$_{\mathrm{CTX\text{-}M}}$ and *Bla*$_{\mathrm{TEM}}$ an indication that other ESBLs types could be present.

The increased diversity and complexity of β-lactam resistance in gram-negative organisms, have necessitated the call for stringent infection control and antimicrobial stewardship. Also increased establishment of institutional testing facilities, training of personnel and continuous monitoring of drug resistance Sunyani Hospital is suggested. Testing for other *Bla*$_{\mathrm{GENES}}$ such as for SHV, AmpC, OXA, KPC and IMP and VIM in *E coli* and other members of the *Enterobacteriaceae* is advocated.

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Competing interests: The authors declare that they have no competing interests.

Consent for publication: Not applicable.

Ethics approval: The study was approved by the Joint Committee on Human Research, Publication and Ethics of School of Medical Sciences and Komfo Anokye Teaching Hospital, Ghana.

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