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Molecular Characterization of Extended-Spectrum Beta-Lactamases in *Escherichia coli* and *Klebsiella pneumoniae* in Accra, Ghana

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Abstract
Extended-spectrum beta-lactamases (ESBLs) are plasmid-mediated beta lactamases that are capable of hydrolysing beta-lactams except carbapenems and cephamycins. The ESBL types include SHV, TEM and CTX-M, OXA, PER and VEB-1. The most common ones isolated from clinical specimen are the CTX-M, SHV and TEM. The specific ESBL-producing organisms have different genetic characteristics which mark their identification at the molecular level. This work sought to determine the genetic characterization of ESBL-producing *K. pneumoniae* and *E. coli* in Accra. The molecular investigations of the ESBL-coding genes included extraction of 100 DNA templates of phenotypic ESBL-producing isolates by boiling method, preparation of the PCR reaction mixture using appropriate primers, standard PCR reaction in a thermocycler, agarose gel electrophoresis, bands visualization by ultraviolet trans-illumination and bands photography using a Kodak EDAS 290 gel documentation system. The results significantly ($p<0.05$) indicated that of the 100 ESBL producers, 90(90%) possess CTX-M genes and 25(25%) had TEM genes. None of the ESBL producers possesses SHV genes. Seventy (70%) of the ESBL producers possess only CTX-M genes and 5(5%) had only TEM genes. Twenty (20%) of the isolates had both CTX-M and TEM genes. Of the 100 ESBL phenotypes, 78(78%) and 2(2%) were positive for CTX-M-1group and CTX-M-9group ESBL genes respectively. Organisms producing CTX-M-type ESBL are more prevalent in Accra than other ESBL types. CTX-M-1group producing isolates dominated the ESBL phenotypes with CTX-M-15 likely to be the dominate CTX-M-type ESBL. There is the need for further studies into the characteristic transmission, pathogenesis, antibiotic resistance expression, and infection control of CTX-M-type ESBL and TEM-type ESBL in Accra.
Keywords: Extended spectrum beta-lactamase, CTX-M genes, TEM genes, SHV genes, Molecular

1.0 Introduction

Extended-spectrum beta-lactamases (ESBLs) are plasmid-mediated beta lactamases that are capable of hydrolysing beta-lactams except carbapenems and cephamycins. They are inhibited by beta-lactamase inhibitors such as clavulanic acid, sulbactam and tazobactam. They have been found in the *Enterobacteriaceae* and other Gram-negative bacilli. ESBL producing isolates are predominantly *Klebsiella pneumoniae* and *Escherichia coli* (Paterson and Bonomo, 2005). The ESBL types include SHV, TEM and CTX-M, OXA, PER and VEB-1. The most common ESBL genes isolated from clinical specimen are the CTX-M, SHV and TEM (Paterson and Bonomo, 2005). It has been observed that the same organism may harbour two or more ESBL genes, which may change the antibiotic resistance phenotype (Yamasaki *et al*., 2003).

The CTX-M enzymes are replacing SHV and TEM enzymes as the prevalent type of ESBLs in urinary tract infections, bloodstream and intra-abdominal infections (Falagas and Karageorgopoulos, 2009). The phylogenic study reveals five major groups of acquired CTX-M enzymes (Bonnet, 2004). The CTX-M-1group (Group I) includes CTX-M-$_1$, CTX-M-$_3$, CTX-M-$_{10}$, CTX-M-$_{12}$, CTX-M-$_{15}$, CTX-M-$_{22}$, CTX-M-$_{23}$, CTX-M-$_{28}$, CTX-M-$_{29}$ CTX-M-$_{30}$ and CTX-M-$_{68}$. The CTX-M-2group (Group II) includes CTX-M-$_2$, CTX-M-$_4$, CTX-M-$_5$, CTX-M-$_6$, CTX-M-$_7$ and CTX-M-$_{20}$. The CTX-M-8group (Group III) includes one plasmid-mediated member, CTX-M-$_8$. The CTX-M-9group (Group IV) includes nine plasmid-mediated enzymes (CTX-M-$_9$, CTX-M-$_{13}$, CTX-M-$_{14}$, CTX-M-$_{16}$, CTX-M-$_{17}$, CTX-M-$_{19}$, CTX-M-$_{21}$, CTX-M-$_{24}$ and CTX-M-$_{27}$). The CTX-M-25group (Group V) includes the CTX-M-$_{25}$ and CTX-M-$_{26}$ enzymes (Bonnet, 2004). The CTX-M-$_{15}$ and CTX-M-$_{14}$ seems to be the most widespread globally, while many of the other CTX-M ESBLs tend to be more limited in their distribution (Heffernan *et al*., 2007).

The specific ESBL-producing organisms have different genetic characteristics which mark their identification at the molecular level. This genetic diversity in the various ESBL-producing organisms may reflect characteristic differences in relation to pathogenesis, antibiotic resistance expression, response to therapy, transmission and infection control. Genetic characterization of ESBL-producing organisms is also essential for epidemiological use. This work seeks to determine the genetic characterization of ESBL-producing *K. pneumoniae* and *E. coli* in Accra.

2.0 Materials and Methods

2.1 Materials

Glycerol broth, blood agar and MacConkey agar were prepared according to manufacturers' guidelines. Vitek 2 Compact System (bioMérieux, Marcy I'Etoile, France) was used to identify the isolates and determine ESBL phenotypes. Water bath was used to heat the colony suspension and centrifuge was used to spin the suspension to extract the bacteria DNA. BIOR GenePro thermocycler was used to perform the polymerase chain reaction (PCR) under controlled reaction conditions with specific primers. PCR products were used to perform agarose gel electrophoresis with 1X TAE buffer, 2% agarose gel and 0.5µg/ml ethidium bromide at 120V for 45minutes. The bands on the gels were visualized by ultraviolet trans-illumination and photographed using a Kodak EDAS 290 gel documentation system.

2.2 Study Sites

Lactose fermenting bacterial isolates were collected from the Central Laboratory of the Korle Bu Teaching Hospital (KBTH) and Advent Clinical Laboratories; both in the Accra Metropolis, Ghana. The PCR was performed at the molecular biology laboratory of the School of Allied Health Sciences, University of Ghana and the bands on the gels were visualized and photographed at the molecular biology laboratory of the Microbiology Department of University of Ghana Medical School.

2.3 Sample Size

A sample size of 100 DNA templates extracted from ESBL- producing *K. pneumoniae* and *E. coli* isolates.

2.4 Inclusion Criteria

Non-duplicate pure cultures of ESBL-producing *K. pneumoniae* and *E. coli* isolates were used in the work.

2.5 Exclusion Criteria

All isolates not confirmed as ESBL-producing *K. pneumoniae* and *E. coli* were excluded.

2.6 Determination of ESBL Phenotypes

The isolates were identified as *K. pneumoniae* and *E. coli* based on their gram stain reaction and biochemical reaction characteristics in the ID test cards wells using Vitek 2 system (bioMérieux, Marcy I'Etoile, France). The Vitek 2 system was used to concurrently determine the ESBL phenotypes based on the simultaneous assessment of the inhibitory effects of cefotaxime, ceftazidime and cefepime, alone and in the presence of clavulanic acid in accordance with the CLSI.

2.7 Molecular Analysis of ESBL-coding Genes

The molecular investigations of the ESBL-coding genes included extraction of DNA templates of phenotypic ESBL-producing isolates by boiling method, preparation of the PCR reaction mixture using appropriate primers, standard PCR reaction in a thermocycler, agarose gel electrophoresis, bands visualization by ultraviolet trans-illumination and bands photography using a Kodak EDAS 290 gel documentation system.

2.7.1 Genomic DNA Extraction of *K. pneumoniae* and *E. coli*

DNA template was extracted by a simple boiling method (Heffernan *et al*., 2007). A loopful of bacterial colony was picked from each isolate and suspended in 100µl of double distilled H_2O in Eppendorf tube. The DNA suspension was incubated at 99°C for 5 minutes and snapped cold on ice for 10 minutes. The cell lysate was then centrifuged briefly at high speed (12.000 rpm for 3 min), and the supernatant containing genomic DNA was transferred into sterile Eppendorf and 5µl of the supernatant was used for PCR reaction. The extracted DNA was stored at -21°C until required for PCR.

2.7.2 PCR Detection of ESBL-encoding Gene

PCR of ESBL-encoding genes was carried out using BIOER GenePro thermocycler. A typical 25µl PCR reaction mixture for a primer set was prepared as shown in table 3. The primers used were already published

primers as shown in table 1 for CTX-M, TEM and SHV genes and their corresponding PCR conditions (table 2). Further PCRs using the primers for CTX-M-1group and CTX-M-9group were performed with isolates that were positive with the universal CTX-M primers (Heffernan *et al*., 2007). Sterile distilled water as negative controls were included in each round of PCR.

Table 1: Primers used for the detection of ESBL genes (Heffernan *et al*., 2007)

Primer Name	Sequence (5'-3')	Target Gene	Size (bp)
SHV-F	GCCGGGTTATTCTTATTTGTCCG	SHV	1007
SHV-R	ATGCCGCCGCCAGTCA		
TEM-F	GTATCCGCTCATGAGACAATA	TEM	966
TEM-R	TCTAAAGTATATATGAGTAAAC		
CTX-M-F	TTTGCGATGTGCAGTACCAGTAA	CTX-M	590
CTX-M-R	CGATATCGTTGGTGGTGCCATA		
CTX-M-1-F	CCCATGGTTAAAAAATCACTG	CTX-M-1 group	891
CTX-M-1-R	CCGTTTCCGCTATTACAAAC		
CTX-M-9-F	GTGACAAAGAGAGTGCAACGG	CTX-M-9 group	857
CTX-M-9-R	ATGATTCTCGCCGCTGAAGCC		

Table 2: PCR conditions used for the detection of ESBL genes (Heffernan *et al*., 2007)

Target Gene	PCR Conditions	
SHV	Initial denaturation for 5min at 94ºC	1 cycle
	94ºC for 30s 68ºC for 30s 72ºC for 60s	35 cycles
	Final extension at 72ºC for 7min	1 cycle
TEM	Initial denaturation for 5min at 94ºC	1 cycle
	94ºC for 60s 55ºC for 30s 72ºC for 60s	35 cycles
	Final extension at 72ºC for 10min	1 cycle
CTX-M	Initial denaturation for 5min at 94ºC	1 cycle
	94ºC for 30s 60ºC for 30s 72ºC for 60s	30 cycles
	Final extension at 72ºC for 7min	1 cycle
CTX-M-1group	Initial denaturation for 10min at 94ºC	1 cycle
	94ºC for 60s 55ºC for 60s 72ºC for 2mins	30 cycles
	Final extension at 72ºC for 5min	1 cycle
CTX-M-9group	Initial denaturation for 10min at 94ºC	1 cycle
	94ºC for 30s 55ºC for 30s 72ºC for 60s	25 cycles
	Final extension at 72ºC for 10min	1 cycle

Table 3: PCR reaction mixture

Reagent	Volume (µl)	Final concentration
Nuclease-free water	16.175	-
10X PCR buffer + $MgCl_2$	2.5	1X
10mM DNTP mix	0.4	200 µM each
10µM forward primer	0.4	0.2µM
10µM reverse primer	0.4	0.2µM
5U/µl Taq polymerase	0.125	0.5U
Template DNA	5	(≤1 µg/reaction)
TOTAL volume	**25**	

2.7.3 Agarose Gel Electrophoresis

The buffer (1XTAE buffer) was prepared and subsequently used to prepare 2% agarose gel. The suspension was boiled in a microwave for 2 minutes. The molten agarose was allowed to cool to 60°C and stained with 3µl of 0.5µg/ml ethidium bromide (which absorbs invisible UV light and transmits the energy as visible orange light). A comb was inserted into the slots of the casting tray and the molten agarose was poured into the tray. The gel was allowed to solidify for 20 minutes to form the wells. The 1XTAE buffer was poured into the gel tank to barely submerge the gel. Two microliter (2µl) of 10X blue gel loading dye (which gives colour and density to the samples to make it easy to load into the wells and monitor the progress of the gel) was added to 10µl of each PCR product and loaded into the wells. The 100bp DNA ladder was loaded into well 1. The gel was electrophoresed at 120V for 45 minutes using either a midi or a maxi gel system. The bands on the gels were

visualized by ultraviolet trans-illumination and photographed using a Kodak EDAS 290 gel documentation system. The sizes of the PCR products were estimated by comparison with the mobility of a 100bp molecular weight ladder that was ran alongside experimental samples in the gel.

2.8 Statistical Analyses

The data from the work was collated and statistically analysed using using the chi-square test. *P* values < 0.05 were considered significant.

3.0 Results

Of the 100 ESBL producers, 90% possessed CTX-M genes with bands amplicon size of 590bp as in plate 1 and 25% had TEM genes with bands amplicon size of 966bp as shown in plate 2. None of the ESBL producers possessed SHV genes as indicated in table 4. Seventy percent (70%) of the ESBL producers possessed only CTX-M genes and 5% had only TEM genes. Twenty percent (20%) of the isolates had both CTX-M and TEM genes. Of the 100 ESBL phenotypes, 78% and 2% were positive for CTX-M-1group and CTX-M-9group ESBL genes respectively as demonstrated in table 5. CTX-M-1group ESBL genes with bands amplicon size of 891bp and CTX-M-9group with band amplicon size of 857bp are indicated in plate 3 and plate 4 respectively.

Table 4: Occurrence of ESBL-coding genes in *K. pneumoniae* and *E. coli* Isolates by Polymerase Chain Reaction

	Number (%)
ESBL-genes	Isolates (n= 100)
CTX-M	90(90)
TEM	25(25)
SHV	0(0)
CTX-M Only	70(70)
TEM Only	5(5)
CTX-M and TEM	20(20)
Neither CTX-M or TEM	5(5)

Table 5: Occurrence of CTX-M-group ESBL coding genes in *K. pneumoniae* and *E. coli* Isolates by Polymerase Chain Reaction

	Number (%)
ESBL-genes	Isolates (n= 100)
CTX-M-1group	78(78)
CTX-M-9group	2(2)

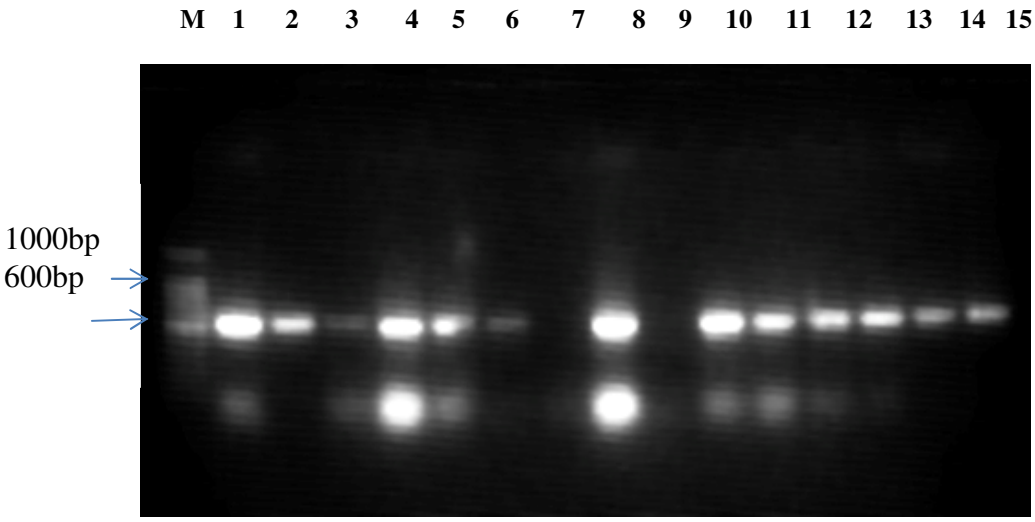

Plate 1 Representative agarose gel electrophoregram of PCR products (band size 590bp) of ESBL gene CTX-M

Lane M=100bp marker; Lanes 1-6=PCR positive CTX-M; Lanes 7 and 9 = PCR negative CTX-M; Lanes 8, 10-15 = PCR positive

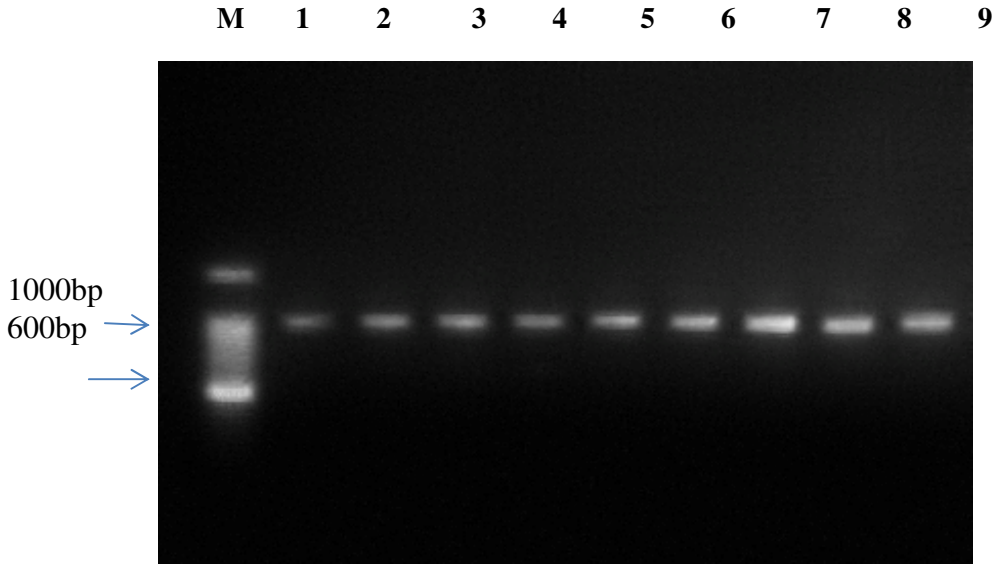

Plate 2 Representative agarose gel electrophoregram of PCR products (band size 966bp) of ESBL gene TEM Lane M=100bp marker; Lanes 1-9 =PCR positive TEM

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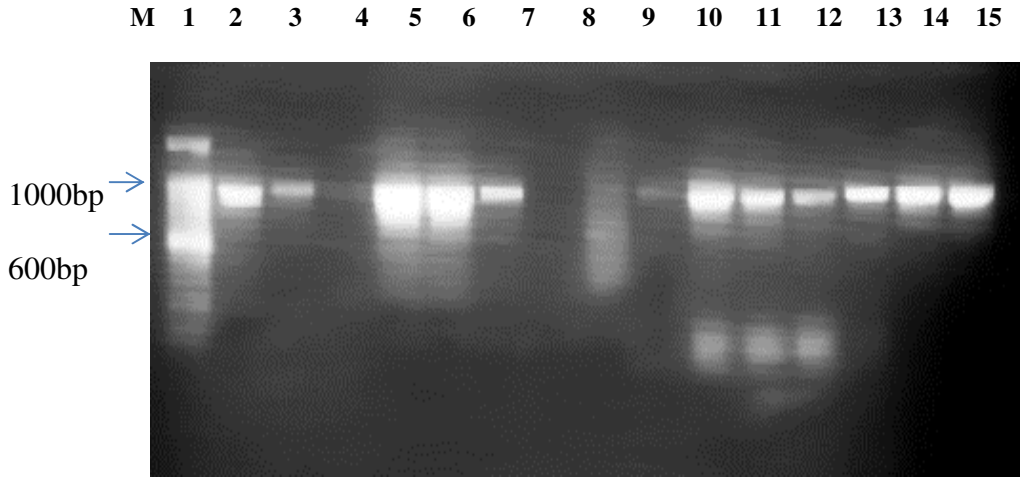

Plate 3 Representative agarose gel electrophoregram of PCR products (band size 891bp) of ESBL gene CTX-M-G1

Lane M=100bp marker; Lanes 1-6=PCR positive CTX-M-G1; Lanes 7 and 8 = PCR negative CTX-M-G1; Lanes 9-15 = PCR positive CTX-M-G1

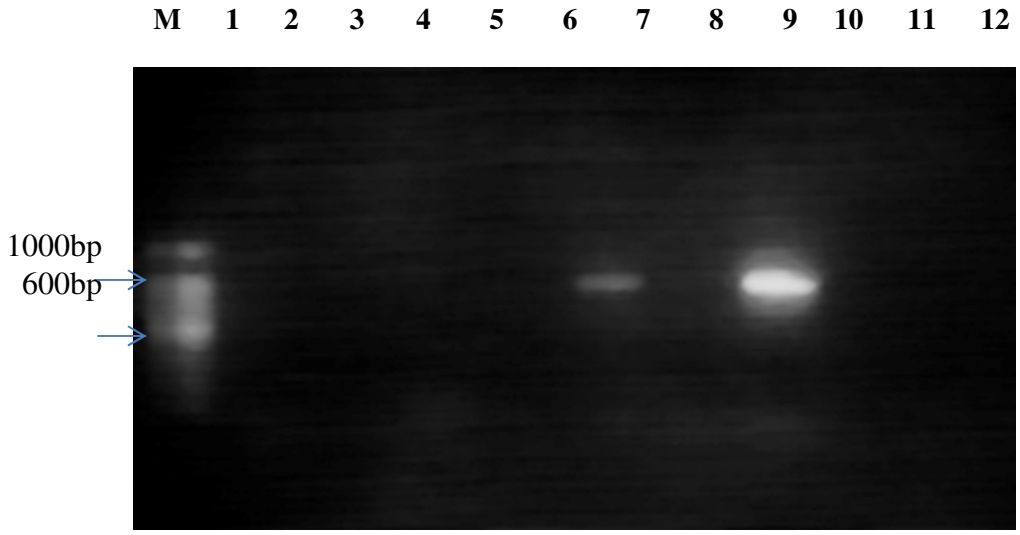

Plate 4 Representative agarose gel electrophoregram of PCR products (band size 857bp) of ESBL gene CTX-M-G9

Lane M=100bp marker; Lanes 7 and 9 =PCR positive CTX-M-G9; Lanes 1-6 and 10-12 = PCR negative CTX-M-G9

4.0 Discussion

CTX-M-type, TEM-type and SHV-type ESBLs are the main types of ESBL produced by bacteria. Some researchers have suggested that CTX-M-type ESBLs are now the most frequent ESBL type worldwide as compared to SHV and TEM-type ESBLs (Paterson and Bonomo, 2005). In this study 90% isolates produced

CTX-M-type ESBL confirming that CTX-M is the dominant ESBL-type in Accra as it is in the rest of world (Falagas and Karageorgopoulos, 2009).

The percentage of isolates producing CTX-M-type ESBL in this present study is consistent with the study in Kumasi, Ghana by Feglo (2013) who reported 94.4% CTX-M-type ESBL. However, while Feglo (2013) reported that 64.5% of the ESBL isolates possess two genes and 29.9% possess three genes; only 20% of ESBL isolates in this present study had two genes and none possessed three ESBL genes. In a study in Southwest Nigeria, 30 selected multidrug-resistant *K. pneumoniae* strains isolated from patients with urinary tract infections showed 57% CTX-M enzymes (Olysegun *et al*., 2006) which were slightly lower than the 87.1% of CTX-M ESBLs observed in *K. pneumoniae* strains in this study. Reports from Indonesia confirmed the high prevalence (94.5%) of CTX-M-type ESBL (Severin *et al*., 2010). This was also corroborated by Tham and others (2010) who observed that 90% ESBL-positive genes were of CTX-M ESBL-type. This study was consistent with the finding by Heffernan and colleagues (2007) in New Zealand who observed that 96% *E. coli* and *K. pneumoniae* isolates produced CTX-M ESBL genes which is also corroborated by a study done in Canada (Pitout *et al*., 2007). So in this study, Ghana joins North America, South America, Western Europe, Asia and other African nations with high prevalent CTX-M-ESBL producers (Paterson and Bonomo, 2005).

This study has shown that 78% of the CTX-M-type ESBLs were CTX-M-1group with 2% belonging to CTX-M-9group corroborating the study of Tham and colleagues in 2010 who reported that CTX-M-1group was prevalent followed by the CTX-M-9group as do the conclusions of Heffernan and colleagues who also showed that CTX-M-1group (CTX-M-15) and CTX-M-9group (CTX-M-14) seem to be the most widespread (Heffernan *et al*., 2007). However, this study is at variance with the work of Pitout and colleagues in 2007 who found 48% and 37% CTX-M-1group and CTX-M-9group respectively.

The high prevalence of CTX-M-1group ESBL observed in this study confirms the studies in other parts of Africa. In Tanzania, Blomberg and colleagues (2005) discovered the presence of CTX-M-1group (CTX-M-15) ESBL-producing organism for the first time in Africa. In Tunisia, 43 of 47 isolates showed CTX-M-1group (CTX-M-15) and 2 CTX-M-9group (CTX-M-14) (Elhani *et al*., 2010). This consensus of CTX-M ESBL genes dominating the ESBL types was contradicted by the study of Feglo (2013) in Kumasi, Ghana which reported TEM-type ESBL as the dominating ESBL gene.

In this study 25% of the bacterial isolates produced TEM-type ESBL, a result which is lower as reported in Canada (Pitout *et al*., 2007) and New Zealand (Heffernan *et al*., 2007). Furthermore, Severin and colleagues (2010) in a study of 73 ESBL-positive *E. coli* and 72 *K. pneumoniae* strains, TEM-type ESBLs were not detected in any of the isolates. Interestingly Feglo in 2013, reported a high prevalent of 96.2% of TEM-type ESBL phenotypes in a study in Kumasi in contrast to the findings of this study and other reports.

Regarding SHV-type ESBL, none was seen in 100 strains of ESBL phenotypes in this study confirming a study in Algiers by Ramdani-Bouguessa and colleagues in 2006. Low rates were reported in New Zealand (Heffernan *et al*., 2007) and North Lebanon (Sana *et al*., 2011) which contradicts findings in this study. However, higher rates of 32.5% SHV-type ESBL were reported by Feglo (2013) in Kumasi contradicting this work also in Ghana, 38.7% in Cameroon Gangoué-Piéboji *et al*., 2005) while Jones and others also reported that SHV genes were found in 41% and 28% of the ESBL-positive *K. pneumoniae* and *E. coli* isolates respectively (Jones *et al*., 2009) in contrast to this study.

5.0 Conclusion

The findings of this work suggest that organisms producing CTX-M-type ESBL are more prevalent in Accra than other ESBL types. The present study indicated that the CTX-M-1group producing isolates dominated the ESBL phenotypes and CTX-M-15 may be the dominant CTX-M-type ESBL. There is the need for further studies into the characteristic transmission, pathogenesis, antibiotic resistance expression, response to therapy and infection control of CTX-M-type ESBL and TEM-type ESBL in Accra.

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