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Antibiotic Resistance Profile of CTX-M-type 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 most common ESBL types include CTX-M, TEM and SHV. 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. This work sought to determine the characteristic antibiotic minimum inhibition concentrations (MICs) and antimicrobial sensitivity profile of CTX-M-type ESBLs in Accra. Hundred (100) DNA templates were extracted from ESBL-producing *K. pneumoniae* and *E. coli* isolates. The specific ESBL types were determined by polymerase chain reaction with specific primers and reaction conditions. The MICs of the antibiotics were determined using Vitek 2 Compact System (bioMérieux, Marcy I'Etoile, France). The results showed that CTX-M-type ESBL have cefotaxime MIC in the resistant range of >64 µg/ml. The CTX-M-type ß-lactamases showed co-resistances to gentamicin (88.6%), tetracycline (71.4%), trimethoprim-sulphamethoxazole (98.6%).The resistance of CTX-M-type ESBL producing organisms to fluoroquinolones have been well established in this work with resistances in ciprofloxacin (71.4%) and norfloxacin (71.4%) with MIC_{90} being >4 µg/ml and >16 µg/ml respectively. The beta-lactam-beta-lactamase inhibitor combination of piperacillin-tazobactam was more susceptible to CTX-M-type ESBL than amoxicillin-clavulanate. Imipenem and amikacin has been established as the *in vitro* drug of choice for the management of organisms producing CTX-M-type ESBL in this present work. Efforts should be made to control the increasing prevalence of CTX-M-type producing organisms in the communities and hospital settings in Accra with their adverse multiple-drug resistance.

Keywords: Extended spectrum beta-lactamase, CTX-M-type ESBL, Resistance, Antibiotics

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*. The most common ESBL isolated from clinical specimen are the CTX-M, SHV and TEM (Paterson and Bonomo, 2005).

Because ESBL enzymes are plasmid mediated, the genes encoding these enzymes are easily transferable among different bacteria. Most of these plasmids not only contain DNA encoding ESBL but also carry genes conferring resistance to several non-beta-lactam antibiotics. Consequently, most ESBL isolates are not only resistant to penicillins, cephalosporins and monobactams but also to other classes of antibiotics including aminoglycosides, fluoroquinolones, tetracyclines, nitrofurantoin and sulfamethoxazole-trimethoprim. Treatment of these multiple drug-resistant organisms has proven to be a therapeutic challenge (Todar, 2008).

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. Unfortunately, there is no published work on the antimicrobial resistance

characteristics of the ESBL types in Accra. This work seeks to determine the characteristic antibiotic minimum inhibition concentrations (MICs) and antimicrobial sensitivity profile of CTX-M-type ESBLs 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, determine minimum inhibition concentration (MIC) of selected antibiotics and interpret the MICs according to CSLI breakpoints. 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 70 CTX-M-type- 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 Molecular Determination of CTX-M ESBL-coding Genes

The molecular investigations of the CTX-M ESBL-coding genes included extraction of DNA template from the 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

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

DNA template was extracted by a simple boiling method. 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.

2.6.2 PCR Detection of CTX-M ESBL-encoding Gene

Polymerase chain reaction of CTX-M 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 and their corresponding PCR conditions (table 2). 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)
CTX-M-F	TTTGCGATGTGCAGTACCAGTAA	CTX-M	590
CTX-M-R	CGATATCGTTGGTGGTGCCATA		

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

Target Gene	PCR Condition
CTX-M	Initial denaturation for 5min at 94°C; 30 cycles of 94°C for 30s, 60°C for 30s, 72°C for 60s; Final extension at 72°C for 7min.

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**	

2.6.3 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.

2.7 Antibiotic Sensitivity Testing

The Vitek 2 system (bioMérieux, Marcy I'Etoile, France) was used to determine the MIC of the selected antibiotics and their therapeutic significance. The 17 antibiotics used were ampicillin, amoxicillin/clavulanic

acid, piperacillin, piperacillin/tazobactam, cefazolin, cefoxitin, cefotaxime, ceftazidime, cefepime, imipenem, amikacin, gentamicin, ciprofloxacin, norfloxacin, tetracycline, nitrofurantoin and trimethoprim/ sulfamethoxazole.

3.0 Results

3.1 Antibiotic Susceptibility among Isolates with CTX-M ESBL Genes

Of the 70 CTX-M ESBL-producing organisms, the number and percentage of isolates in the susceptible MIC breakpoint and the MIC at which 50% of the CTX-M ESBL producers were susceptible to a particular antimicrobial agent (MIC_{50}) and MIC at which 90% of the CTX-M ESBL producers were susceptible to a particular antimicrobial agent (MIC_{90}) were recorded in table 4 below:

Table 4 Antibiotic Susceptible among Isolates with CTX-M ESBL genes (n=70)

Antimicrobial Agent	No.(%) of Susceptible Isolates	MIC(µg/ml)	
		MIC_{50}	MIC_{90}
Ampicillin	0(0.0)		
Amoxicillin/Clavulanic acid	12(17.1)	8	8
Piperacillin	0(0.0)	-	-
Piperacillin/Tazobactam	24(34.3)	≤4	16
Cefazolin	0(0.0)	-	-
Cefoxitin	50(71.4)	≤4	8
Cefotaxime	0(0.0)	-	-
Ceftazidime	13(18.6)	4	4
Cefepime	52(74.3)	2	8
Imipenem	70(100.0)	≤1	≤1
Amikacin	67(95.7)	≤2	16
Gentamicin	8(11.4)	≤1	≤1
Ciprofloxacin	19(27.1)	0.5	1
Norfloxacin	20(28.6)	2	2
Tetracycline	17(24.3)	≤1	4
Nitrofurantoin	23(32.9)	≤ 16	32
Trimethoprim/Sulfamethoxazole	1(1.4)	≤20	≤20

MIC_{50}: MIC at which 50% of the CTX-M ESBL were susceptible to a particular antimicrobial agent

MIC_{90}: MIC at which 90% of the CTX-M ESBL were susceptible to a particular antimicrobial agent

3.3 Antibiotic Resistance among Isolates with CTX-M ESBL Genes

Of the 70 CTX-M ESBL-producing organisms, the number and percentage of isolates in the resistant MIC breakpoint and the MIC at which 50% of the CTX-M ESBL producers were resistant to a particular antimicrobial agent (MIC_{50}) and MIC at which 90% of the CTX-M ESBL producers were resistant to a particular antimicrobial agent (MIC_{90}) were recorded in table 5 below:

Table 5 Antibiotic Resistance among Isolates with CTX-M ESBL genes (n=70)

Antimicrobial Agent	No. (%) in Resistant Ranges	MIC (µg/ml)	
		MIC_{50}	MIC_{90}
Ampicillin	70(100.0)	≥32	≥32
Amoxicillin/Clavulanic acid	20(28.6)	≥32	≥32
Piperacillin	70(100.0)	≥128	≥128
Piperacillin/Tazobactam	43(61.4)	≥128	≥128
Cefazolin	70(100.0)	≥64	≥64
Cefoxitin	9(12.9)	32	≥64
Cefotaxime	70(100.0)	≥64	≥64
Ceftazidime	30(42.9)	≥64	≥64
Cefepime	6(8.6)	≥64	≥64
Imipenem	0(0.0)	-	-
Amikacin	0(0.0)	-	-
Gentamicin	62(88.6)	≥16	≥16
Ciprofloxacin	50(71.4)	≥4	≥4
Norfloxacin	50(71.4)	≥16	≥16
Tetracycline	50(71.4)	≥16	≥16
Nitrofurantoin	47(67.1)	≥512	≥512
Trimethoprim/Sulfamethoxazole	69(98.6)	≥320	≥320

MIC_{50}: MIC at which 50% of the CTX-M ESBL were resistant to a particular antimicrobial agent

MIC_{90}: MIC at which 90% of the CTX-M ESBL were resistant to a particular antimicrobial agent

4.0 Discussion

The genetic diversity in the various ESBL-producing organisms may reflect characteristic differences in relation to antibiotic resistance expression. This study sought to establish the minimum inhibition concentrations (MICs) of beta-lactams, beta-lactam/beta-lactamase inhibitor combinations and non-beta-lactams among CTX-M-type ESBL producers. It has been suggested that organisms producing CTX-M-type beta-lactamases typically have cefotaxime MICs in the resistant range (>64 µg/ml) (Paterson and Bonomo, 2005) as has been confirmed in this present study. In this study the CTX-M-type ESBL hydrolysed ceftazidime with 42.9% of its MIC in the resistant range of >64 µg/ml confirming the report of Sturenburg and colleagues (2004) that CTX-M-type ESBLs may actually hydrolyse ceftazidime and confer resistance to it. This was also corroborated by Poirel and colleagues (2001) as well as Ramdani-Bouguessa and colleagues (2006). On the other hand only 8.6% of the cefepime MIC were in the resistant range at variance with Tzouvelekis and colleagues who reported that CTX-M-type beta-lactamases hydrolyse cefepime with high efficiency (Tzouvelekis *et al*., 2000).

In this study, CTX-M-type ESBL showed co-resistances to gentamicin, tetracycline and trimethoprim/sulphamethoxazole and is confirmed in the findings of Ramdani-Bouguessa and others (2006) who established co-resistance of CTX-M-type ESBL in gentamicin and trimethoprim/sulphamethoxazole in Algeria. Ben Ami and colleagues (2006) also corroborated this study where CTX-M-producing *E. coli* were co-resistant to trimethoprim/sulphamethoxazole and gentamicin. According to Bonnet (2004), CTX-M-type ESBL co-resistance to non-beta-lactams may be attributed to genetic structures such as sul1-type integrons.

As in the studies by Pitout and Laupland (2008), Ben Ami (2006), this study confirms that strains producing CTX-M enzymes were substantially resistant to ciprofloxacin. These reports contradict the findings of Ramdani-Bouguessa and colleagues (2006) who recorded low resistance rate to ciprofloxacin in Algeria. In this study CTX-M ESBL producing isolates were resistant to piperacillin/tazobactam than amoxicillin/clavulanic acid. This contradicts the study of Bush and others (1993) that tazobactam exhibits greater inhibitory activity to CTX-M-type ESBL than clavulanic acid.

This study has established that none of the CTX-M-type ESBL showed resistance to imipenem and amikacin which confirm them as the drug of choice for treating CTX-M-type ESBL-producing infections. This is consistent with the findings of Oteo and others (2010) and Sana and colleagues (2011) which concluded that imipenem and amikacin were ideal for the treatment of severe ESBL-producing organisms.

5.0 Conclusion

The outcome of this work affirmed elevated resistances to gentamicin, ciprofloxacin, norfloxacin, nitrofurantoin, tetracycline, trimethoprim-sulphamethoxazole and the beta-lactam-beta-lactamase inhibitor combination of piperacillin-tazobactam and amoxicillin-clavulanate by *K. pneumoniae* and *E. coli* producing CTX-M-type ESBL. Imipenem and amikacin have been established as the *in vitro* drug of choice for the management of organisms producing CTX-M-type ESBL in this present work. Conscientious efforts should be made to arrest the public health menace of CTX-M-type ESBL producing organisms in the communities and hospital settings in Accra.

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