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Dominance Of Chromosome-Borne CTX-M Extended Spectrum Beta-Lactamases (ESBL) in Gram-Negative Clinical Isolates

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Abstract

Background: Antibiotic resistance caused by Extended Spectrum beta-lactamase enzymes (ESBL) often lead to poor clinical outcomes in the treatment of bacterial infections of different aetiologies. The ESBLs have continued to emerge in different types with continued expansion in substrate specificity. In this study, conducted at the Federal Medical Centre, Owerri, Imo State Nigeria, from 2017 to 2019, we aimed to detect the presence of ESBL in multidrug resistant clinical isolates and to identify the types and location of the ESBL genes**.**

Materials and Methods: In this comprehensive study, we isolated and characterized seventy-seven multidrug-resistant isolates, including 41 isolates of Escherichia coli, 26 isolates of Pseudomonas aeruginosa, and 10 isolates of Klebsiella pneumoniae, all from urine and wound swab specimens. Phenotypic ESBL detection was carried out using a double-disc synergy test (DDST). Plasmid DNA was extracted using the alkaline lysis method and the TENS method. The product was subjected to gel electrophoresis on 0.8% agarose. Genomic DNA was extracted by the Norgens Genomic DNA extraction method. The extract was amplified on Multiplex Polymerase Chain Reaction (PCR) using a pair of each of three primers, namely: TEM (Temoniera), SHV (Sulfhydryl variable), and CTX-M (Cefotaximase–Munich) genes. The amplified product was subjected to gel electrophoresis on 1.5% agarose. The bands were visualized in an ultraviolet transilluminator in a photo documentation system.

Results: Up to 40.3% of the isolates were positive for ESBL phenotype. However, 66.7% of those positive for ESBL phenotype showed bands for ESBL genotype. One hundred percent of all ESBL positive genotypes possess CTX-M genes, while 31.3% showed co-expression of CTX-M and SHV genes. None of the isolates showed any band in the region of TEM gene. The predominance of Chromosomal borne CTX-M ESBL with 31.3% co-expression of SHV ESBL in multidrug-resistant isolates is hereby reported, with a total absence of both TEM ESBL and plasmids.

Conclusion: Massive infection control measures are needed to curb the rapid spread of drug-resistant genes. Prudent antibiotic stewardship and rational antibiotic policy are also needed to prevent the selection/induction of drug resistance under antibiotic pressure.

Keywords: Extended Spectrum Beta-lactamase, CTX-M, SHV, TEM, Genes, Multidrug resistance.

INTRODUCTION

Antibiotic resistance caused by Extended Spectrum beta-lactamase enzymes (ESBL) often lead to poor clinical outcomes in treating bacterial infections of different aetiologies (1, 2). The ESBLs have continued to emerge in different types with widening substrate specificity (3). The most common types include TEM

(named after Temoniera, the name of the index patient), SHV (named from sulfhydryl variable, a unique feature of the enzyme), and the latest, CTX-M (named after the enzyme's first substrate, cefotaxime (a third generation cephalosporin) and Munich, a city in Germany where it was first detected) (4). These enzymes were usually carried on plasmids (5) and have a high capacity to inactivate the third generation cephalosporins (also called oxyimino cephalosporins), including cefotaxime, ceftazidime and ceftriaxone, and Monobactams (Aztreonam) but inactive on a subgroup of the second generation cephalosporins called cephamycins (cefoxitin and cefotetan) and carbapenems (example imipenem) (6). Characteristically, the enzymes are inactivated by beta-lactamase inhibitors, including clavulanate, sulbactam, and tazobactam. The genes spread rapidly both vertically and horizontally (3). The ESBL genes are usually co-expressed with genes coding for resistance to other classes of antibiotics (7), thus compounding the multidrug resistance scenario. Several reasons have been adduced for the continuous emergence of multiple antibiotic resistance isolates. Such reasons include mutations that, among other things, alter the penicillin-binding proteins of the bacteria (8), thereby preventing attachment; altering the porin channels (9), thereby reducing permeability; altering efflux pumps (10), thereby increasing ejection of the antibiotics, and enzymatic inactivation (11). The efficiency of these mechanisms of drug resistance is fueled by selection pressure occasioned by inappropriate antibiotic use (12, 13), such as self-medication, under-dosing, over-usage, unnecessary usage, etc. This study was conducted to detect the presence of ESBL in multidrug-resistant clinical isolates and to identify the types and locations of the ESBL genes. The test organisms were isolated from clinical specimens collected from the Federal Medical Centre, Owerri, Imo State, Nigeria, from 2017 to 2019. Ethical approval was obtained from the hospital's Ethical Committee. Isolation of the organisms, antimicrobial susceptibility testing, and ESBL phenotyping were done at Salvation Hospital Owerri, Nigeria. Molecular analysis was done at the Molecular Laboratory Services section of Teddy and Thaddeus Nigeria Company, Lagos.

MATERIALS AND METHODS

The investigation was performed using already identified isolates from clinical specimens of wound swabs and urine that were resistant to at least one antibiotic from three or more different classes of antibiotics (14). Six classes of antibiotics were used according to the BNF (15) classification. They include Penicillins, Cephalosporins, Monobactams, Carbapenems, Aminoglycosides, and Fluoroquinolones. The multidrug resistant isolates include Escherichia coli (n=41), Pseudomonas aeruginosa (n=26) and Klebsiella pneumoniae (n=10).

Double Disc Synergy Test (DDST)

Phenotypic ESBL detection was carried out by double disc synergy (16). Discs of Cefotaxime (30µg), Ceftriaxone (30µg), Ceftazidime (30µg) and Cefpodoxime (30µg) were positioned at a distance of 15mm edge to edge from a disc of amoxicillin-clavulanate containing 20µg amoxicillin + 10µg clavulanate centrally on Mueller-Hinton agar plate seeded with the test organism.

The plates were then incubated for 24 hours at 35°C, and the shape of the zone of inhibition was noted. Isolates exhibiting distinct shapes with potentiation towards amoxicillin + clavulanate disc (known as keyhole effect) were confirmed as producers of ESBL. E. coli NCTC 13353 and E. coli ATCC 25922 were used as positive and negative ESBL controls, respectively. Isolates that showed resistance to the cephalosporins and yet were negative for ESBL using the DDST method were re-tested with Mueller-Hinton agar in which AmpC inhibitor, Cloxacillin (240µg/ml) was supplemented (17).

Plasmid Analysis

Plasmid DNA was extracted by alkaline lysis (18, 19) and TENS (20, 21). The product was subjected to gel electrophoresis on 0.8% agarose.

The DNA was visualized by placing the gel in an ultraviolet transilluminator in a photo documentation system (Clinix Japan, Model 1570).

Molecular Analysis

Genomic DNA extraction was done directly from pure isolates using Norgen Genomic DNA isolation kit (Product # 24700, 2475, Canada) designed to prepare genomic DNA from cultured cells rapidly. The extract was amplified on Multiplex Polymerase Chain Reaction (PCR) using a pair of each of three primers, namely TEM, SHV, and CTX-M genes. The PCR amplifications for the genes were performed using a thermocycler (A & E Laboratories, UK, Model Cyl-005-1).

Amplified products were separated using 1.5 % agarose gel electrophoresis in TAE buffer (Tris-Acetate, EDTA) performed at 70V for 1.5 hours.

A 100-bp DNA ladder digest (Solis Biodyne, Cat No—07-1100050) was employed as a molecular weight marker.

The Gel was stained with 0.5µg/ml of ethidium bromide for 45 minutes and destained in water for 20 minutes. Stained gels were examined under ultra-violet (UV) trans-illuminator in a photo documentation system (Clinix Japan).

The amplicon sizes characteristic of the target genes were band sizes of 534bp corresponding to CTX-M Gene, 754bp corresponding to SHV Gene and 822bp for TEM gene. Therefore, the Gel images were compared with typical result preview of the standard DNA Ladder ran together with the samples as well as the results of the positive and negative control samples. All positive bands corresponding to expected band sizes for each isolate were recorded as positive (+), while the absence of the corresponding band was recorded as negative (-). Faint bands were recorded as undecided amplification or variable result (±).

RESULTS

The isolates were first tested for ESBL phenotype using multiple disc synergy test. All multidrug resistant isolates tested negative initially for ESBL phenotype. However, when Cloxacillin was supplemented in Mueller-Hinton agar (17) to inactivate suspected AMP-C, and the DDST test repeated, the characteristic synergy between clavulanate and the cephalosporins became visible.

Out of 77 multidrug resistant isolates tested, 31 (40.3%) showed the characteristic synergy between the beta-lactamase inhibitor, clavulanate and the third generation cephalosporins and thus were positive for ESBL phenotype (Figure 1).

About 53.8% of the Pseudomonas aeruginosa tested were positive for ESBL phenotype while 29.3% of the E.coli tested were positive (Figure 2).

The gel electrophoresis of the plasmids extracted from the isolates with alkaline lysis and TENS methods showed no plasmid bands (Figures 3a, 3b, 3c).

Twenty-four isolates that tested positive for ESBL phenotype were subjected to multiplex PCR test. Fifteen of these (62.5%) actually harbour ESBL genes and one (4.2%) had a faint band (±) for CTX-M gene (Table 1).

All positive isolates (n=16) possess the CTX-M gene, while five of them (31.3%) also possess the SHV gene. None of the isolates harbour the TEM gene (Figure 4).

Table 1: Positive ESBL Phenotype/Genotype

Isolates	Positive ESBL Phenotype	Positive ESBL Genotype	Faint Positive Phenotype (±)	Percentage (%)
Pseudomonas sp	**9**	**6**	**-**	**66.7**
E. coli	**10**	**6**	**1**	**70**
Klebsiella sp	**5**	**3**	**-**	**60**
Total	**24**	**15**	**1**	**66.7**

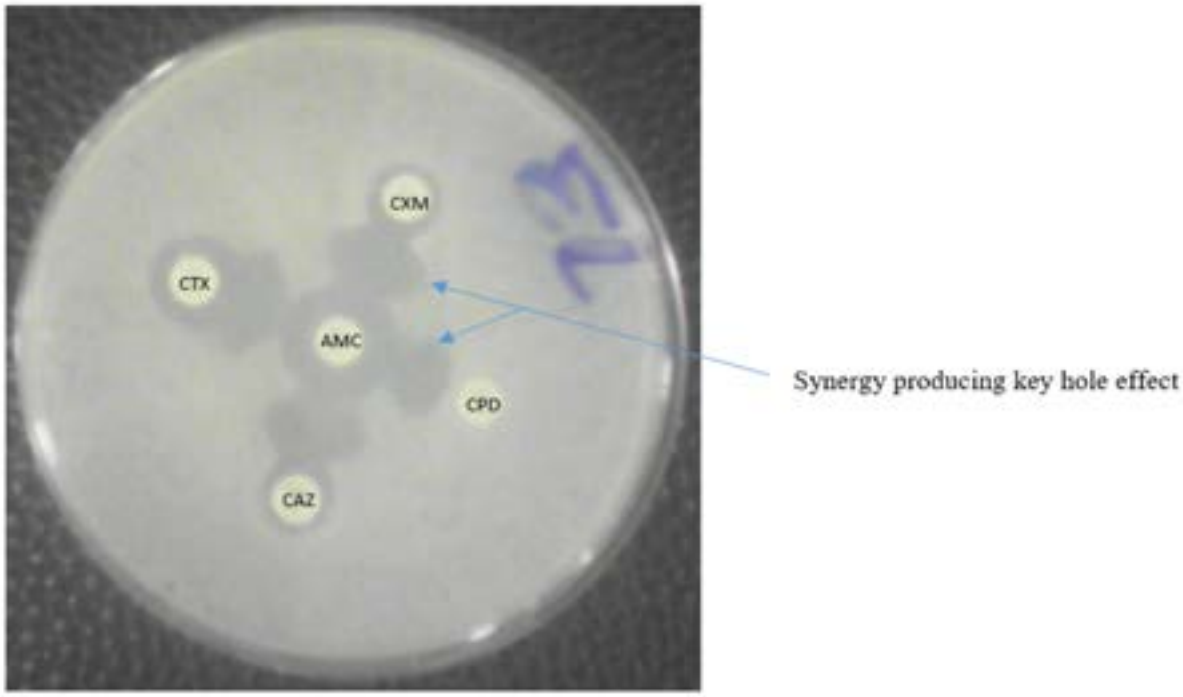

Figure 1: Synergy between beta-lactamase inhibitor and cephalosporins Antibiotic discs

Antibiotic discs

AMC - Amoxicillin/Clavulanate

CAZ - Ceftazidime

CTX - Ceftriaxone

CXM - Cefotaxime

CPD - Cefpodoxime

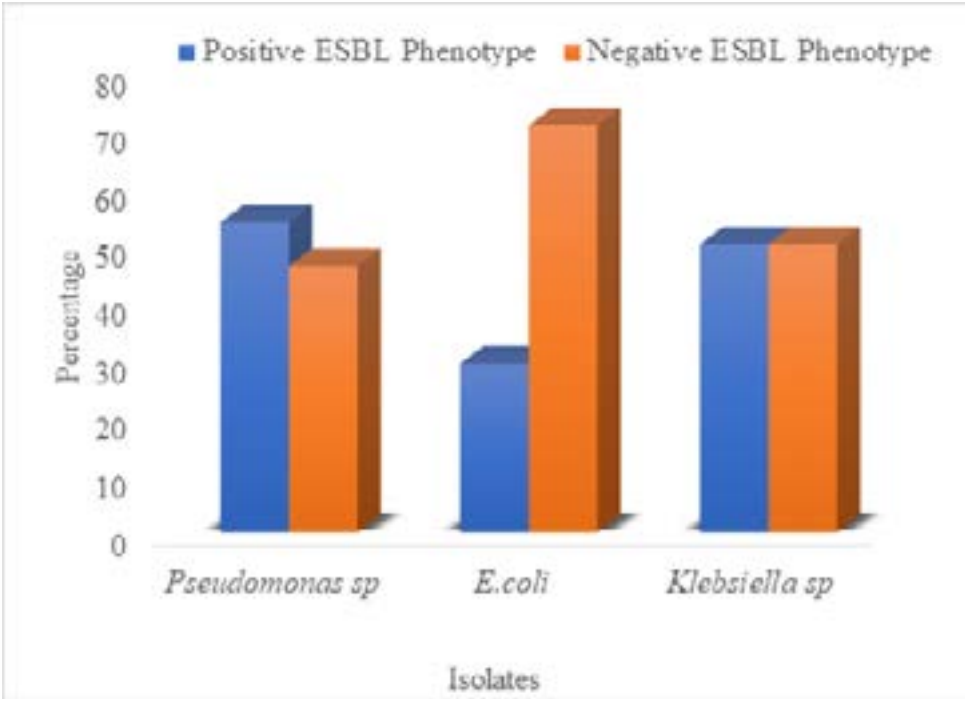

Figure 2: Ratio of positive to negative ESBL phenotypes for each isolate.

Figure 3a

Figure 3b

Figure 3c
Figures 3a, 3b, 3c**:** Image of gel electrophoresis of Plasmid DNA of the isolates
Lane M is HIND III Marker,
Lanes 1-77 Showed no plasmid bands

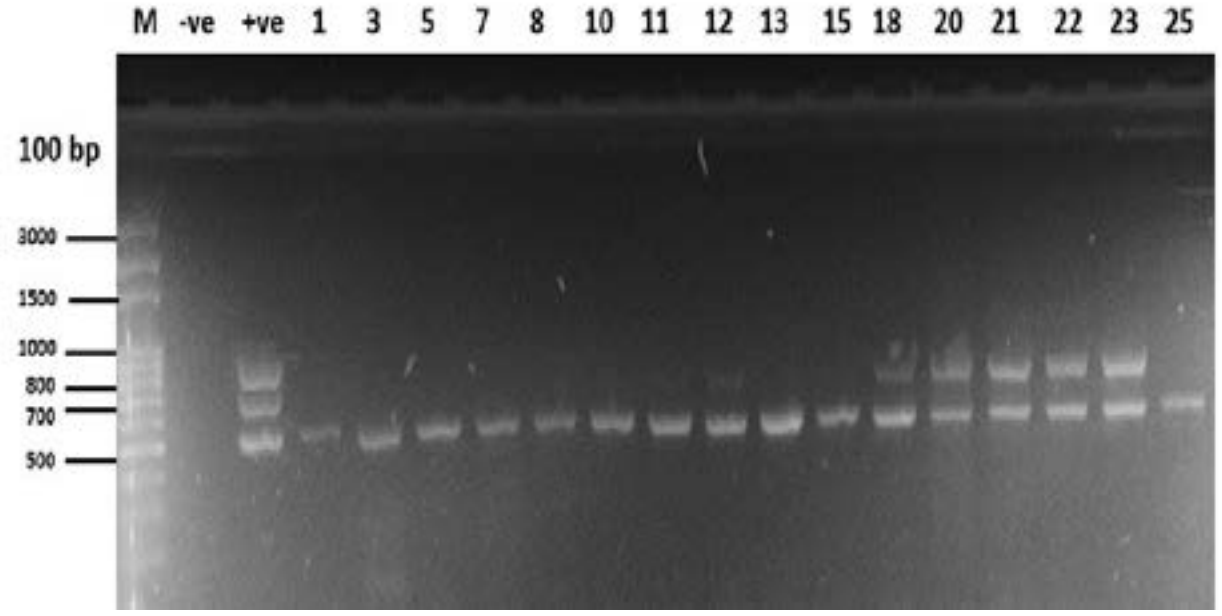

Figure 4: MULTIPLEX-PCR Gel electrophoresis of amplified ESBL positive samples only.

Lane M, 100bp DNA ladder,
Lane –ve: Negative control,
Lane +ve: Positive control for all 3 primers; 534bp (CTX-M Gene), 754bp (SHV Gene) and 822bp (TEM gene), anes

1-24, show positive amplification at corresponding band sizes for each amplified primer.

DISCUSSION

The isolates in our study were first tested for ESBL phenotype using multiple disc synergy test. All multidrug resistant isolates tested negative initially for ESBL phenotype. However, when Cloxacillin was supplemented in Mueller-Hinton agar (17), and the DDST test repeated, the characteristic synergy between clavulanate and the cephalosporins became visible in 40.3% of the isolates. This implies that those isolates that became phenotypic ESBL positive also harbour AmpC β-lactamases which masked the phenotypic identification of ESBL initially (22).

Thirty-one isolates (40.3%) showed positive ESBL phenotype. Pseudomonas aeruginosa showed

the highest positive ESBLs (53.8%), trailed by E. coli (29.3%). This contrasts with the lower rate of ESBL (10%) in all E. coli isolates and 21.26% in faecal enteric isolates reported in India (23, 24). The trend depicts increasing ESBL prevalence across the globe. In 2008-2009, the surveillance rate was reported as 6.5% in France, 8.0% in Spain and 11.3% in Germany (25). Also, UK reported 13.4%, Italy reported 14.2%, and 35% in Turkey. A mean 40% was reported across Asia, a little close to 30% around Latin America, a little above 10% around Europe and Africa respectively (26). The same increasing trend had variously been reported across Nigeria. From 16% in Gram negative isolates in Nnewi South East Nigeria (27), to 24.5% in Klebsiella sp isolates in Ilorin Nigeria (28) and 21.6% in Port Harcourt (29). In North-West, Nigeria, 34.3% was reported in Gram-negative isolates (7), while a 37.3% prevalence rate in Klebsiella sp and 36.4% in E. coli isolates were reported (5). All these findings affirm that ESBL-mediated multidrug resistance is widely distributed across the globe, Nigeria included, and calls for concerted efforts to interrupt the dissemination of the menace.

The multiple antibiotic-resistant isolates were subjected to plasmid profile analysis using the Alkaline lysis extraction method. No plasmid band was detected. This warranted the application of a second plasmid extraction method, the TENS method (20) and gel electrophoresis repeated. The result still revealed the absence of plasmid bands. This shows that the isolates harbour no plasmid. This implies that the DNA encoding the multidrug resistance is contained on the chromosome and not on plasmids, as pointed out in a previous report (30). It also means that plasmid transfer cannot exchange the resistance trait between bacteria. This result contrasts the findings in some other studies. Sixty percent of strains of Salmonella were reported to harbour plasmids elsewhere (31), while 9.3% carriage was reported in Turkey (32) and 36.4% in three tertiary health institutions in Nigeria (33). Low molecular weight plasmids were also reported in ESBL isolates in Ebonyi State, Nigeria (34). The absence of plasmids also indicates that these drug resistances arise in these isolates by mutations, as previously suggested (34), rather than by horizontal gene transfer, as postulated by one report (35). However, one previous report pointed out that most ESBL belonging to CTX-M were chromosome-borne (36), and the detection of a few multiple antibiotic-resistant isolates lacking plasmids was reported earlier (30). These findings render inadequate the depiction of ESBL by several authors as plasmid-borne enzymes only (1,4, 37, 38, 39, 40).

Although TEM, the first β-lactamase, was detected in a plasmid (41), several reports have described chromosome-borne β-lactamases, especially the ESBLs (36, 42). The finding of 100% chromosome-mediated ESBL in this study may suggest that the spread of the drug resistance mechanism in the area may not be as rapid as seen in plasmid-medicated enzymes. This, however, does not undermine the risk of dissemination as chromosome genes could be mobilized onto plasmids by gene capture units should any of the isolates acquire plasmids of any form from the environment, even if the plasmids so acquired were not related to ESBL from the onset. Such mobilization had been demonstrated with CTX-M ESBL genes (42). With a similar result, it was postulated that the entire plasmid DNA or part of it could be inserted and integrated into the chromosome (36), possibly mediated by transposons (43). Structures termed 'integrative and conjugative elements' located in bacterial chromosomes have the tendency of being excised from their location in one chromosome and incorporated into another chromosome and, therefore, could be a vehicle for the exchange of resistance genes like ESBLs between bacteria just like plasmids (44). Also, studies have noted the possible action of phage-like elements in facilitating resistance gene mobilization into bacterial genomes (45, 46).

Sixteen isolates (66.7%) showed amplification of the PCR products at the molecular size of 543bp indicating presence of CTX-M genes. Also, 5 of this 16 CTX-M bearing isolates (31.3%) also showed amplification at the molecular size of 754bp corresponding to SHV gene. The existence of these genes portends impending therapeutic crisis for the wound and urinary tract infections from where the superbugs were isolated. This means poor

prognosis as previously pointed out (47). The rapid development of these genes is blamed on improper antibiotic use (28, 48). E. coli and Pseudomonas aeruginosa have very close prevalence rates of ESBL. Since majority of the genes in this study were CTX-M, it implies that they could not have arisen from mutations in the parent SHV and TEM genes. However, being entirely chromosome-borne in our study leads to difficulty in predicting the origin as CTX-M genes from initial studies were acquired originally from Kluyvera species chromosome via mobile plasmids (49). Several reports implicated international travel and dissemination through meat and other imported raw food products as sources of dissemination of CTX-M (50, 51). It has been reported that most Salmonella enterica isolates harbour the CTX-M enzyme on the chromosome (36). The CTX-M group of ESBLs has been noted as being most prevalent in 7 countries (52, 53) and has been described as having overtaken SHV and TEM in prevalence (54, 55).

Before the discovery of CTX-M, Ceftazidime resistance was applied as an ESBL surrogate marker; however, with the appearance of cefotaximase-bearing isolates susceptible to Ceftazidime, the practice could not be realistic anymore (56).

While reviewing several reports from different countries, it was observed that once CTX-M enters any locality, it dominates the other ESBL variants (TEM and SHV) by displacing or superimposing over them (57), and this is possibly what has happened in this present study. The high rate of CTX-M has been attributed to the co-expression of genes that are resistant to other drugs like aminoglycosides and quinolones, which encourages co-selection pressure (58). The rise in CTX-M prevalence has further been attributed to the occurrence and extraordinary distribution of the CTX-M genes in plasmids/transposons and the sustenance of the DNA in successful strains, implying efficient clonal spread (59). In this study, however, since our CTX-M genes in this study were chromosome-borne, dissemination through clonal spread (vertical transmission) is the feasible route rather than plasmid transfer. Furthermore, the occurrence of CTX-M enzyme with other β-lactamases had been reported earlier and was underscored as a common strategy to promote resistance to multiple antibiotics (57). All the factors that favor the dissemination of CTX-M ESBL are effectively driven by strong and efficient selection pressure mounted by the widespread and overt use of many antibiotics in clinical conditions (1, 60). This was strengthened by the realization that the parent CTX-M enzyme earlier discovered was resistant to Cefotaxime alone and susceptible to Ceftazidime, but now many reports of Ceftazidime-resistant CTX-M abound (61). It has been reported that 60% of CTX-M variants show simultaneous resistance to Cefotaxime and Ceftazidime (57). This means that Ceftazidime has become one of the agents of selection responsible for CTX-M diversification (62).

CONCLUSION

It is concluded here that CTX-M Extended Spectrum Beta-lactamase is the predominant ESBL in this research. There is a 31.3% co-expression of CTX-M and SHV ESBLs in the multidrug-resistant clinical isolates. We further report that the MDR genes were entirely contained in the chromosome of the isolates with a total absence of TEM ESBL and plasmids. The total absence of plasmids in all isolates appears to be unusual compared to previous reports in other areas. Mass infection control measures must be adopted to curb the rapid spread of drug-resistant genes. We further recommend prudent antibiotic stewardship and rational antibiotic policy to prevent the selection/induction of drug resistance under antibiotic pressure.

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work.

CONFLICT OF INTEREST: The authors declare that there are no conflicts of interest

ABBREVIATIONS

ESBL: EXTENDED SPECTRUM BETA-LACTAMASE

CTX-M: CEFOTAXIMASE–MUNICH (Enzyme that inactivates Cefotaxime first discovered in Munich, Germany)

DDST: DOUBLE DISC SYNERGY TEST

TEM: Temoniera, a type of ESBL, name of a patient from whom the first ESBL was detected

SHV: Sulfhydryl variable, a type of ESBL named from the characteristics of the ESBL

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