Original Article

The Coexistence of Extended-Spectrum β-lactamase and Metallo-β-Lactamase Genes in Gram-Negative Bacteria

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Abstract

The coexistence of different classes of beta-lactamases in multiple drug-resistant (MDR) bacteria has widely increased, thus rising diagnostic and treatment challenges. The study aimed at evaluating the prevalence of extended-spectrum beta-lactamase (ESBLs) and Metallo-β-lactamase (MBL) genes in confirmed ESBL producing Gram-negative (G-) bacteria. A stock of 220 G- isolates were studied for producing ESBL and MBL using phenotypic and PCR methods. The results showed that ESBLs were identified in 35.4% of the isolates, out of which 32.1% were MBLs producers, the most prevalent isolates were *K. pneumoniae* (15.4%), *E. coli* (7.7%), and *Ps. aeruginosa* (6.4%), respectively. PCR test confirmed the presence of ≥1 ESBL and MBL genes in 78.2% and 73.1% of the isolates, respectively. CTX-M was the most prevalent gene (70.5%) followed by IMP (61.5%), TEM (27%), VIM (21.8%), and SHV (21.8%). Also, 39.7% of the isolates carried two genes, while 10.8% carried three genes, and 20.5% carried 4 genes. The combination (CTX-M+ IMP) was the most frequent (33.4%) among the isolates. The high prevalence of ESBLs and MBLs in combination was noted, especially in *E. coli* and *K. pneumoniae*. Continuous monitoring of β-lactamases coexistence in G- bacteria will help to stop their dissemination and control their spread.

Keywords: Coexistence, ESBLs, MBLs, Gram-negative, Bacteria, Carbapenemase

Introduction

Worldwide, multidrug-resistant (MDR) bacteria have become a global problem due to the treatment challenges and a great issue to public health [1, 2]. They are estimated to cause more than 700,000 deaths worldwide every year and may exceed 10 million deaths by 2050 and lead to a high economic loss [3]. Infections by MDR bacteria can result in failure of treatment, in addition to the high cost of medical treatment and hospitalization stay [4]. Generally, antimicrobial resistance mechanisms are categorized into four types: (1) limitation of drug uptake; (2) modification of a drug target; (3) inactivation of a drug; (4) activation of drug efflux. Worldwide, the production of extended-spectrum β-lactamases (ESBLs) by Enterobacteria is a real threat to public health [5, 6]. There are three major families of ESBLs, namely; CTX-M, SHV, and TEM, besides a large variety of other types of enzymes. CTX-M enzymes are the most prevalent ESBLs worldwide [7]. ESBL-producing G- bacteria are resistant to cephalosporins, penicillins, monobactams, and even carbapenems [8]. These organisms are sometimes resistant to fluoroquinolones and aminoglycosides. Metallo-β-lactamases (MBLs) or (carbapenemase producers) are known as class B, they have a broad spectrum and can resist all beta-lactam antibiotics except monobactams. They are neither inhibited by tazobactam, sulbactam, clavulanate, nor metal chelators (EDTA) [9, 10]. The spread of the VIM- and IMP-type MBL in Gram-negative bacteria, has been noticed since 1990 [11, 12]. The spread of MBL genes induces the emergence of multidrug-resistant bacteria. Aminoglycosides and cephalosporins (third generation) are generally used in the treatment of infections caused by G- pathogens. These antibiotics become less effective due to the synthesis of β-lactamase by these bacteria. Carbapenems are the current choice to treat infections caused by ESBL producers; however, resistance to carbapenem by the production of carbapenemases in G- bacteria is fast running out and may leave treatment options with potentially toxic drugs such as polymyxin and colistin. Now the MBL poses a therapeutic challenge worldwide [13] because these enzymes are capable of hydrolyzing penicillins in addition to the 3rd- and 4th-generation cephalosporins (carbapenems) [14]. The

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coexistence of different classes of β-lactamases in gram-negative bacteria has been reported widely, thus rising diagnostic and treatment challenges [15]. It is an indication of an increase in the resistance mechanisms, thus rendering the antibacterial drug ineffective. The study aimed to evaluate the coexistence of ESBLs and carbapenemase-producing genes in confirmed ESBLs-producing Gram-negative bacteria.

Materials and Methods

Identification of Samples

220 G- bacteria stocks were previously collected from 5 tertiary hospitals during pilgrimage seasons in Makkah, KSA between 2018 and 2020, and kept at -70°C. The isolates were identified with a VITEK-2 Compact System using AST-GNI cards (Biomerieux) and biochemical tests including motility, urease, citrate utilization, Indole, Kligler iron agar (KIA), and oxidase tests.

Susceptibility Tests

The susceptibility of bacteria to antibiotics was done using the VITEK 2 system (AST no. 12 card) and the disc-diffusion method following the standards of the Clinical and Laboratory Standards Institute [16]. The antibiotics included; cefepime (30µg), ceftriaxone, cefotaxime, ceftazidime, amikacin, gentamicin, amoxicillin/clavulanic acid, ciprofloxacin, imipenem, ertapenem, and meropenem (Oxoid, UK).

Screening for ESBLs -Producing Strains

ESBL production was confirmed by Double-Disc Synergy Test. An inoculum Standard of (0.5 McFarland) of each isolate was prepared and then inoculated on MHA plates. ceftriaxone (30µg) and Ceftazidime (30µg) discs were then applied with a distance of 1.5cm away from amoxicillin-clavulanic acid (20µg/10µg) disc and then incubated at 37 °C for 18h. Positive results were confirmed when the zone of inhibition was extended towards amoxicillin/clavulanic acid disc >5mm.

EDTA-Disc Synergy Test

MBL production by bacterial isolates was tested using EDTA-Disc Synergy Test. The plates were swabbed with 0.5-McFarland isolates at turbidity standards. After brief drying, two 10µg meropenem discs were placed 20mm apart, and 10µl EDTA (0.5M) was added to one disc. Zone enhancement in the area between 2 discs indicated a positive result.

PCR Testing

DNA of each isolate was extracted using the boiling method with modification and used for PCR [17]. Multiplex PCR was carried out to detect β-lactamase genes blaCTX-M, blaTEM, blaSHV, blaIMP, and blaVIM-1. **Table 1** shows the primers used for PCR. Each reaction had a volume of 25µL, including 10µL master mix (Promega, USA), 1.0µL of each primer, 4µL DNA template, and 10µL purified distilled water. The multiplex PCR reaction was initiated with a pre-denaturation step at 94°C for 5min, followed by 35 amplification cycles (denature at 94 °C (30s), annealing at 55°C (30s), and extension at 72 °C (1min)), then finalized with an extension at 72°C for 10min. The PCR products were detected by 1% agarose gel electrophoresis.

Ethics Statement

The study was approved by the institutional review board of the faculty of medicine at Umm Alquraa University. Consent was obtained from each participant.

Statistical Analysis

Statistical analysis of data was performed using SPSS software (v.25, IBM, US). Different variables were compared using the Chi-Square test, and a p-value <0.05 was considered statistically significant.

Table 1. The primers used for PCR

No.	Primer	Sequence	Size	A.T.	Gene	Ref.
1	TEMF	ATGAGTATTCAACATTTCCGTG	840	55	bla_{TEM}	[18]
2	TEMR	TTACCAATGCTTAATCAGTGAG				
3	SHV S1	ATTTGTCGCTTCTTTACTCGC	1051	55	bla_{SHV}	[18]
4	SHV S2	TTTATGGCGTTACCTTTGACC				
5	CTX-M/F	TTTGCGATGTGCAGTACCAGTAA	544	56	bla_{CTX-M}	[18]
6	CTX-M/R	CGATATCGTTGGTGGTGCCATA				
7	Bla VIM-F	TTTGGTCGCATATCGCAACG	500	66	bla_{VIM}	[19]
8	Bla VIM-R	CCATTCAGCCAGATCGGCAT				
9	Bla IMP-F	GTTTATGTTCATACWTCG	432	45	bla_{IMP}	[19]
10	Bla IMP-R	GGTTTAAYAAAACAACCAC				

Results and Discussion

In this study, 35.4% (n=78) of Gram-negative bacteria were found to be phenotypic ESBL producers **(Table 2)**, out of which 53.8% (n=42) were *K. pneumonia*, 20.5% (n=16) *E. coli*, 10.3% (n=8) *Ps. aeruginosa*, 9.0% (n=7) *Enterobacter spp.*, 3.8% (n=3) *P. mirabilis*, and 2.6%(n=2) were *A.*

baumannii. Of these 78 isolates, 32.1% (n=25) were found to be MBLs, 15.3% (n=12) belonged to *K. pneumonia*, 7.7% (n=6) belonged to *E. coli*, 6.4% (n=5) belonged to *Ps. aeruginosa* and 2.6% (n=2) belonged to *Enterobacter spp* **(Table 2)**. The study showed that rates of resistance against cephalosporins; cefotaxime, ceftazidime, ceftriaxone, and cefepime were 100%, 98.7%, 96.2%, and 66.7%, respectively. Resistance rates against carbapenems; ertapenem, meropenem, and imipenem, were 38.5%, 32.1%, and 23.1% respectively. Resistance rates against amikacin, gentamicin, ciprofloxacin, and amoxicillin/clavulanic acid antibiotics were 64.1%, 61.5%, 52.5%, and 50.0%, respectively **(Table 3)**. PCR confirmed the presence of ≥1 ESBL genes in 61 isolates (78.2%) as shown in **Table 4** and **Figure 1**. The study found that 57 (73.1%) of the isolates were harboring at least one of the tested MBL genes. About 8 (10.3%) isolates carried only one gene, namely SHV (5.1%), TEM (2.6%), and IMP (2.6%) while 55 (70.5%) of the isolates carried more than one gene **(Table 3)**. Neither CTX-M nor VIM gene was found alone. Among the studied isolates, 31 (39.7%) carried two genes, 8 (10.8%) strains carried three genes, and 16 (20.5%) strains carried 4 genes **(Table 4)**. CTX-M was the highest detected gene (70.5%) followed by IMP (61.5%), TEM (27%), VIM (21.8%), and SHV (21.8%) **(Table 5 and Figure 2)**. The coexistence of two genes (CTX-M+IMP) mostly was seen in 26 (33.4%) isolates. In addition, the coexistence of three genes (TEM+ CTX-M+ IMP) was mostly seen in 6 (7.7%) while the coexistence of four genes (CTX-M+ TEM+ SHV+ IMP) was mostly seen in 6 (7.7%) isolates as shown in **Table 4** and **Figure 1**. The results showed that 17 *K. pneumoniae* isolates carried (CTX-M+ IMP) genes, 4 isolates harbored (TEM+ CTX-M+ IMP), and 4 isolates harbored (CTX-M+ TEM+ SHV+ VIM) genes simultaneously. Moreover, the simultaneous presence of (CTX-M+ IMP) and (CTX-M+ TEM+ SHV+ IMP) genes was observed in 6 and 2 strains of *E. coli*, respectively. Also the presence of (CTX-M+ IMP), (TEM+ CTX-M+ IMP), (IPM+ CTX-M+ VIM+TEM), (IMP+CTX-M+ SHV+ VIM), (CTX-M+ TEM+ SHV+ IMP) genes was observed in 5 separate *Ps. aeruginosa* isolates **(Table 4)**. Significant association was detected between CTX-M and IMP gene carriers and between SHV and VIM gene carriers ($P < 0.05$) **(Table 5)**.

Table 2. The rates of ESBLs and MBLs phenotype among the studied isolates

Species	ESBLs (78)	ESBLs+MBLs (25)
Klebsiella pneumoniae	42 (53.8%)	12 (15.3%)
Escherichia coli	16 (20.5%)	6 (7.7%)
Pseudomonas aeruginosa	8 (10.3)	5 (6.4%)
Enterobacter spp.	7 (9.0%)	2 (2.6%)
Proteus mirabilis	3 (3.8%)	0 (0%)
Acinetobacter baumannii	2 (2.6%)	0 (0%)
Total	78 (100%)	25 (32.1%)

Table 3. Antimicrobial-susceptibility pattern of ESBLs phenotype isolates

Antibiotic	Sensitive	Intermediate	Resistant
Cefotaxime	0 (0%)	1 (1.3%)	78 (100%)
Ceftazidime	1 (1.3%)	0 (0%)	77 (98.7%)
Ceftriaxone	3 (3.8%)	0 (0%)	75(96.2%)
Cefepime	18 (23.0%)	8 (10.3%)	52(66.7%)
Amoxicillin clavulanic acid	32 (41.0%)	7 (9.0%)	39 (50.0%)
Amikacin	28 (35.9%)	0 (0%)	50(64.1%)
Gentamicin	30 (38.5%)	0 (0%)	48 (61.5%)
Ciprofloxacin	23 (29.5%)	14 (18.0%)	41 (52.5%)
Imipenem	59(75.6%)	1 (1.3%)	18 (23.1%)
Meropenem	53 (68%)	0 (0%)	25 (32.1%)
Ertapenem	48 (61.5%)	0 (0%)	30 (38.5%)

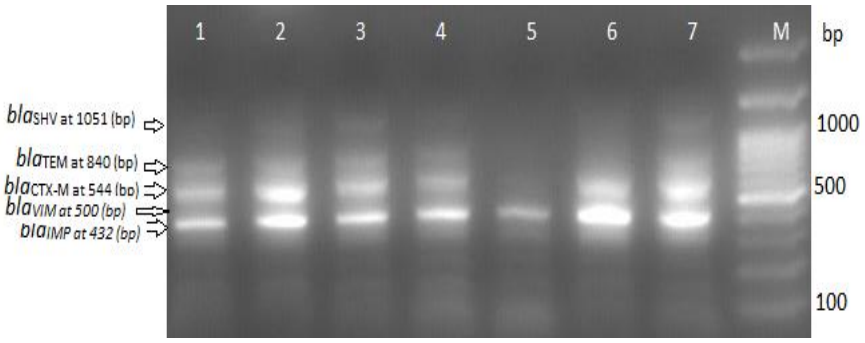

Figure 1. Detection of ESBL and MBL genes on 1.2% agarose gel electrophoresis; lane M: 100bp DNA ladder.

Table 4. Distribution of MBL and ESBL genes among the studied isolates

Gene	*K0 pneumoniae*	*E0 coli*	*Ps0 aeruginosa*	*Ent0 spp*	*P0 mirabilis*	*A0 baumannii*	Total 78 (100%)
TEM	1	1	0	0	0	0	2 (206%)
SHV	3	1	0	0	0	0	4 (501%)
IMP	2	0	0	0	0	0	2 (206%)
CTX-M+ VIM	3	1	1	0	0	0	5 (604%)
CTX-M+ IMP	17	6	1	2	0	0	26 (3304%)
VIM+IMP+CTX-M	1	0	0	0	0	1	2 (206%)
TEM+ CTX-M+ IMP	4	1	1	0	0	0	6 (707%)
CTX-M+ SHV+ TEM+ VIM	4	0	0	0	0	0	4 (501%)
IPM+ CTX-M+ VIM+TEM	1	1	1	0	0	0	3 (308%)
IMP+CTX-M+ VIM+ SHV	2	0	1	0	0	0	3 (308)
CTX-M+ TEM+ SHV+ IMP	2	2	1	1	0	0	6 (707%)
Total	40 (5103%)	13 (1607%)	6(707%)	3(308%)	0 (000%)	1(103%)	63 (8007%)

Table 5. Cross-tabulation between ESBL and MBL genes among the studied isolates

ESBL vs MBL	CTX-M 55 (70.5%)	TEM 21 (27.0%)	SHV 17 (21.8%)	Pearson's chi-square test
IMP 48 (61.5%)	0.000	0.133	0.598	*p*-value
VIM 17 (21.8%)	0.159	0.777	0.002	

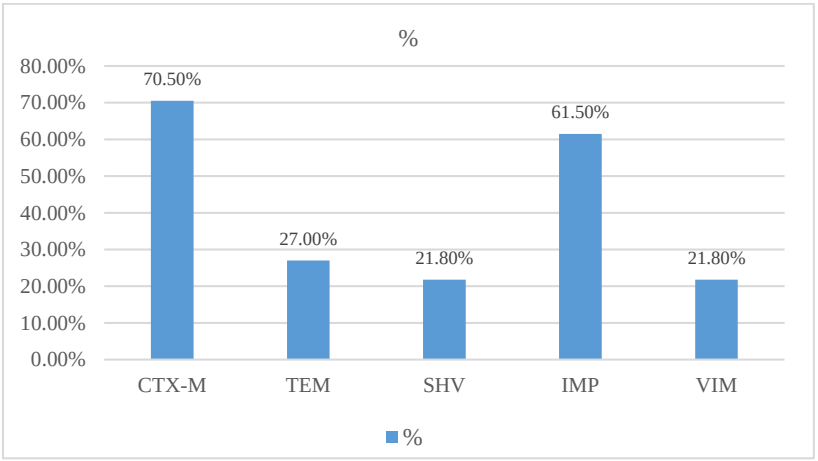

Figure 2. Frequency of MBL and ESBL genes in the studied isolates.

The outbreak of ESBLs-producing Gram-negative bacteria is a major threat in admitted patients. However, over the past few years, the coexistence of ESBL and MBL producers has been increasingly reported worldwide. In this study, the prevalence of MBL and ESBL genes were investigated in clinical isolates of G- bacteria. According to the phenotypic results, the coexistence of ESBL- and MBL-producing *K. pneumonia*, *E. coli*, and *Ps. aeruginosa* was notable. Out of 78 ESBL producers, 32.1% were found to be MBLs (carbapenemase producers). This was higher than that the results of Oberoi *et al.* [20] who reported 8.79% of coexistence, and less than [21] who reported 48.7% of the total *β*-lactamases. In Saudi Arabia, phenotypic studies from Makkah reported positive MBLs production with a range between 38% and 48% [22, 23]. In our study, the phenotypic coexistence of ESBLs and MBLs was common in *K. pneumonia* (15.4%), *E. coli* (7.7%), and *Ps. aeruginosa* (6.4%) isolates, which is near to Oberoi *et al.* [20] who reported the maximum coexistence in *E. coli* (33.34%) and *K. pneumoniae* (16.67%). The study showed that cephalosporins ranged (96.2%-100%) while aminoglycosides ranged (61.5%-64.1%) and in carbapenems ranged (23.1%-38.5%%). Concerning the cephalosporins and aminoglycosides, our finding is near to a study carried in Qatar, which evaluated antimicrobial susceptibility of ESBL-producing Enterobacteriaceae isolated from ICU patients [24]. Concerning the imipenem, our finding disagrees with two studies in Saudi Arabia, which evaluated antimicrobial resistance patterns in ESBL-producing and non-ESBL-producing isolates, found no resistance to imipenem among the studied bacteria [25, 26]. In our study, the strains showed moderate resistance (52.5%) to the fluoroquinolones (ciprofloxacin), which is similar to other findings [27, 28]. The PCR results confirmed that the presence of ESBL and MBL genes results in 78.2% and 73.2% of the isolates respectively. Adam and Elhag, [29] reported the MBL genes prevalence in 45% of the isolates. Our results agree with many studies worldwide [30-32]. Only 5.1% and 2.6% of isolates carried SHV and TEM genes alone, respectively. No isolate carried the CTX-M gene alone. Our result showed that CTX-M was the highest detected ESBL gene (70.5%) which is closed to a study from Pakistan that indicated 72% of isolates carried the CTX-M gene [33]. The CTX-M enzymes have been shown to be the highest ESBL enzyme in different studies [34-36]. In this study, the IMP gene (61.5%) was the highest detected MBL gene followed by VIM (21.8%). Our finding agrees with Anoar *et al.* [37] who reported IMP as the most frequently detected gene (18.6%) while VIM was detected in (10.7%) of the isolates, however, our findings are opposite to others results [29, 38-40]. In Saudi Arabia, Al-Zahrani *et al.* reported only one isolate of VIM [41]. Worldwide, many studies reported various frequencies of MBL genes in different countries [42-44]. In the present study, 17 *K. pneumoniae* isolates harbored (CTX-M+ IMP) genes, 4 isolates harbored (TEM+ CTX-M+ IMP), and 4 isolates harbored (CTX-M+ TEM+ SHV+ VIM) genes. Moreover, 6 *E. coli* isolates carried (CTX-M+ IMP), 2 isolates harbored (CTX-M+ TEM+ SHV+ IMP) genes. Kazemian *et al.* stated that *K. pneumonia* and *E. coli* isolates simultaneously harbored VIM and IMP genes together with ESBLs genes [39]. Worldwide, MBL genes are becoming highly distributed among *K. pneumonia*, *E. coli*, and other Gram-negative bacteria [45-50]. A statistically significant association was detected between CTX-M and IMP gene carriers and between SHV and VIM gene carriers ($P < 0.05$). Hence, whenever such ESBL gene is detected, it should be followed by other beta-lactamase testings.

Conclusion

This study showed a high prevalence of MBL genes in some ESBL producing G- isolates from Makkah Hospitals. The high prevalence of ESBLs and MBLs in combination was noted, especially in *E. coli* and *K. pneumoniae*. Continuous monitoring of β-lactamases synthesis among Gram-negative bacteria will help to stop their dissemination and control their spread.

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