Int J Clin Exp Med 2016;9(6):11051-11057
www.ijcem.com /ISSN:1940-5901/IJCEM0021294

Original Article

Dissemination of CTX-M extended-spectrum β-lactamases (ESBLs) among *Escherichia coli* and *Klebsiella pneumoniae* in Al-Madenah Al-Monawwarah region, Saudi Arabia

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Received December 2, 2015; Accepted April 1, 2016; Epub June 15, 2016; Published June 30, 2016

Abstract: Reports on extended-spectrum β-lactamases (ESBL) producers and the genes responsible for ESBL phenomenon are few in Saudi Arabia. Hence, we determined the prevalence of ESBL in *K pneumonia* and *E. coli* from Al-Madenah Al-Monawwarah and characterized the predominant ESBL gene in these isolates, taking into consideration the emergence of CTX-M gene in the community. Three hundred and fifty nine (n = 359) gram negative isolates were collected from Prince Sultan Military Hospitals in Al-Madenah Al-Monawwarah, KSA. Identification of the isolates was done by using conventional biochemical methods and BD phoenix 100 system. ESBLs were screened according to CLSI guidelines. ESBLs positive strains were tested for the presence of ESBL encoding genes using Multiplex PCR with specific primers for the detection of CTX-M, SHV and TEM genes. Out of the total 359 enterobacterial isolates, *E. coli* was isolated from 189 (52.6%) and *K. pneumonia* from 87 (24.2%). ESBL was demonstrated in 85 (23.7%) of the total 359 isolates. Urine samples were the most frequent in this study (61.7%) followed by blood (15.1%), pus cells (8.2%), wound (4.5%), sputum (4.1%), eye swap (2.7%) and high vaginal swap (2.7%). CTX-M was found to be the most dominant gene (74.1%) followed by TEM (31.8%) and SHV (14.1%). High resistance of ESBL producers was observed among antibiotics belonging to different families including Aztereonam (95.3%), Cephalothin (95.3%), Ampicillin (95.3%), Ciprofoxacin (72.9%), Trimethoprime-Sulfamethaxazole (71.8%), Norfloxacin (68.2%), Levofloxacin (60.0%), Amikacin (33.9%) and Gentamicin (24.7%). The study concluded that all ESBL genes were carried by *K. pneumoniae* and *Escherichia coli* with the proved prevalence of CTX-M in Al-Madenah community, which reflects the continuous strategies followed by *Enteriobactertiacea* to evade antimicrobial action. This represent challenge to the clinicians in this important part of Saudi Arabia as ESBLs, being a cause of outbreaks, is a public health concern.

Keywords: CTX-M gene, extended spectrum beta lactamases, *E. coli*, *K. pneumoniae*, multiplex PCR, Saudi Arabia

Introduction

Resistance to β-lactam antibiotics is older than the discovery of the first β-lactam, penicillin [1]. It is well known that there are naturally occurring, chromosomally mediated β-lactamases in many species of gram-negative bacteria. These enzymes show some sequence homology to penicillin-binding proteins, thus, they are supposed to have been evolved from them [2]. TEM-1 was described in the early 1960s as the first plasmid-mediated β-lactamase in gram-negatives. Within a few years after its first isolation from a single strain of *E. coli,* the TEM-1 β-lactamase spread all over the world and is now present in several *Enterobacteriaceae* including *Pseudomonas aeruginosa, Haemophilus influenzae, and Neisseria gonorrhoeae* [3]. *SHV-1* is another common plasmid mediated β-lactamase capable of hydrolysing extended-spectrum cephalosporins. It was documented in *Klebsiella pneumoniae* and *E. coli* in 1983, based on genetic and functional characteristics [4]. These enzymes were known as

Table 1. Target genes, sequences of primers and ampilicons size

Genes	Primer	Sequences	Ampilicon size	
bla_{SHV}	SHV-F	5'AGGATTGACTGCCTTTTTG3'	392	*K. Colm et al., 2003*
	SHV-R	5'ATTTGCTGATTTCGCTCG3'		
bla_{TEM}	TEM-C	5'ATCAGCAATAAACCAGC3'	*516*	*K. Colm et al., 2003*
	TEM-H	5'CCCCGAAGAACGTTTTC3'		
bla_{CTXM}	CTX-MF	5'TTTGCGATGTGCAGTACCAGTAA3'	*544*	*M. Edelstein et al., 2003*
	CTX-MR	5'CGATATCGTTGGTGGTGCCATA3'		

Table 2. Frequency of different isolates among study subjects

Bacterial Isolates	No. (%)
Escherichia coli	189 (52.6)
Klebsiella pneumoniae	87 (24.2)
Proteus spp.	23 (6.4)
Acinitobacter spp.	19 (5.3)
Providencia spp.	12 (3.4)
Enterobacter cloacae	12 (3.4)
Serretia marrcescenc	4 (1.1)
Citrobacter spp.	3 (0.9)
Sallmonella spp	3 (0.8)
Others	7 (1.9)
Total	359 (100)

extended spectrum β-lactamases (ESBLs) because of their spectrum of activity against oxyiminocephalosporins [5, 6]. Over 150 different ESBLs have been described nowadays in *P. aeruginosa* and in different genera of *Enterobacteriaceae* worldwide.

The prevalence of ESBL phenotype expression in bacterial isolates is geographically variable. While high prevalence rates were found in studies from Turkey (58%), Latin American countries (30-60%), New York (44%), Italy (37%) and Portugal (34%), much lower rates of 3-8% were reported in Sweden, Japan and Singapore [7].

In the Arabian Peninsula, ESBL detection rates reported in data from the Kingdom of Saudi Arabia ranged from 8.5% to 38.5% [8, 9]. Moreover, (31.7%) of ESBLs were reported in Kuwait [10]; but the highest level (41%) was from the United Arab Emirates [11]. The present study aimed to estimate the prevalence of ESBLs in Al-Madenah Al-Monawwarah and to determine the frequency of different ESBL genes with special concentration on CTX-M.

Materials and methods

Study type, population and duration

This is a cross-sectional laboratory based study. Different clinical specimens (n = 359) were collected from patients with signs and symptoms of bacterial infection, who attended to microbiology lab at Prince Sultan Military Hospital, Al-Madenah Al-Monawwarah during the period from January 2014 to August 2015. Patients were included in this study after being given their informed consents. Basic data were collected by using a standard data questionnaire.

Clinical specimens, bacterial isolates, and their identification

Various clinical samples were collected; urine, sputum, blood, wound, high vaginal and eye swabs were obtained from patients with symptoms of bacterial infection. Identification of the organisms was carried out by the BD phoenix 100 (USA).

Phenoptypic detection of ESBLs

Phoenix gram-negative panel which were included in the Phoenix ESBL confirmatory test were used to screen the presence of ESBLs among the study isolates. Then the BD phoenix 100 ESBL positive isolates were confirmed using E-test method as recommended by CLSI. The following antibiotics were used: amikacin (30 μg gentamicin (10 μg), imipenem (10 μg), cephalothin (10 μg), aztreonam (30 μg), ampicillin (30 μg), trimethoprim -sulfamethoxazole (30 μg), ciprofloxacin (5 μg) and levofloxacin (10 μg). *E. coli* ATCC 25922 and *K. pneumonia* ATCC 700603 were used as positive control (ESBL producers) in this project.

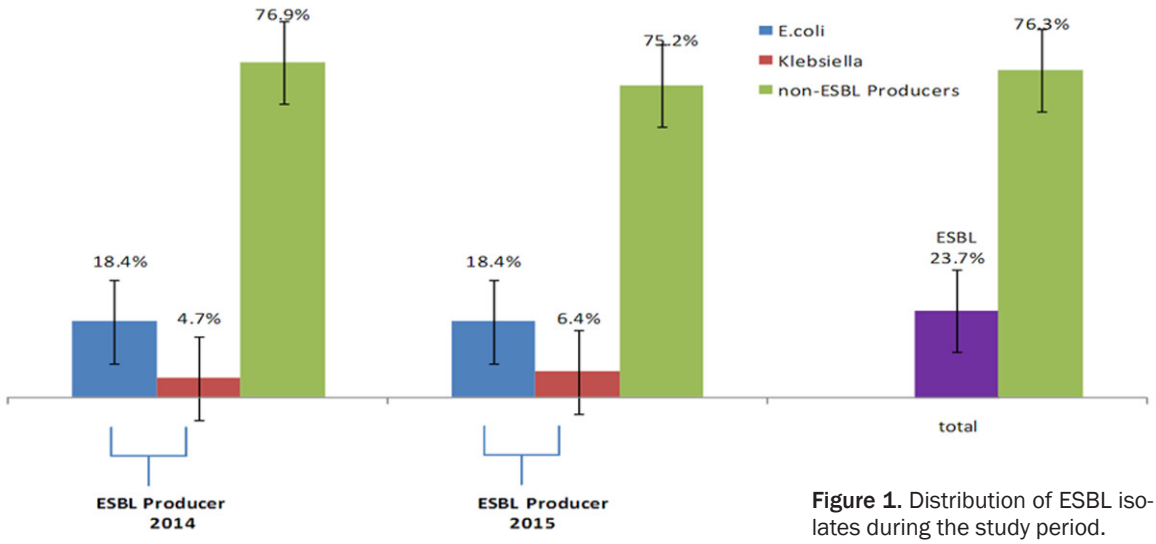

Figure 1. Distribution of ESBL isolates during the study period.

Table 3. Antimicrobial Resistance Pattern of ESBL and non-ESBL Producers

No.	Antibiotic	ESBL Producers no. (%)	Non-ESBL Producers no. (%)	*P* value
1.	Ciprofoxacin	62 (72.9)	55 (20.1)	< 0.05
2.	Norfloxacin	58 (68.2)	51 (18.6)	< 0.05
3.	Amikacin	28 (32.9)	23 (8.4)	< 0.05
4.	Gentamicin	21 (24.7)	50 (18.3)	< 0.001
5.	Levofloxacin	51 (60.0)	52 (19.0)	< 0.05
6.	Cephalothin	81 (95.3)	94 (34.3)	< 0.05
7.	Ampicillin	81 (95.3)	143 (52.2)	< 0.05
8.	Aztereonam	81 (95.3)	25 (9.2)	< 0.05
9.	Trimethoprime-Sulfamethaxazole	61 (71.8)	82 (30.0)	< 0.05

Molecular detection of ESBLs

Extraction of DNA: Bacterial DNA was extracted from all pure ESBLs producer isolates using ready kits from Thermo Scientific GeneJET Genomic, Leithuania. Purity of the isolated DNA was monitored by NanoDropper 2000 (Thermo SCIENTIFIC, USA).

Multiplex PCR assay: PCR assay, searching for bla_{TEM}, bla_{SHV}, and bla_{CTX-M} genes among the 85 ESBLs positive isolates, was adopted as described by Karmele et al., 2003 with minor modification [9]. Briefly, 25 µL reaction mixtures was prepared which contained 12.5 µL of 2× GoTaq Green Master Mix PCR buffer (Promega, USA), 0.2 µL of each primer and 9.3 µL of nuclease free water. Then, 2 µL of template DNA was added separately to each reaction tube with a final volume of 25 µL/Reaction. The PCR primers which were used in this study were listed in (**Table 1**).

The PCR protocol was adopted as follows: one single cycle of denaturation step for 10 minutes at 98°C followed by 32 repeating cycles, each consisting of a denaturation (30 seconds at 94°C), annealing (30 seconds at 54°C) and extension (1 min at 72°C), followed by a final extension at 72°C for 10 minutes.

PCR ampilicons were visualized on 2% agarose gel using 1X TBE buffer which contains 0.5 µg/mL of ethedium promide and finally the PCR products were photographed under UV lamp (SYNGENE, UK) and bands sizes were compared to a 100 bp DNA ladder.

Analysis

Data were analyzed using the SPSS statistical software package, Version 16. Chi-square test was used to recognize differences within the data. All *p*-values < 0.05 were considered as statistically significant.

Results

Bacterial isolates

Identification of the isolates was carried out by using BD phoenix 100. Results reflected high burden of *E. coli* (189, 52.6%) followed by *K. pnumoniae* (87, 24.2%). The frequency of the

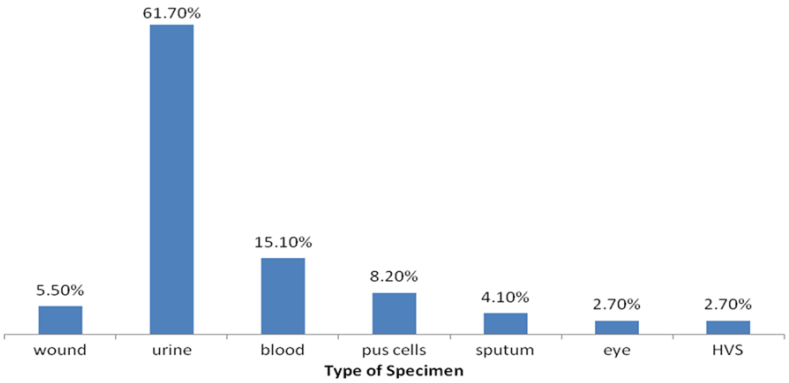

Figure 2. Frequency of ESBLs isolates among different clinical samples.

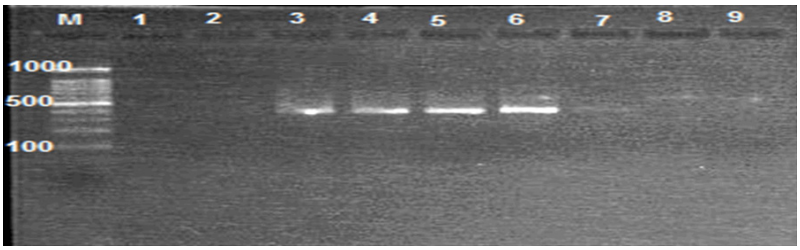

Figure 3. 2% Agarose gel electrophoresis of PCR products. Lane M: 100 bp molecular weight marker, Lane 1: negative control, Lane 3: *bla* SHV positive control *K. pneumoniae* KPN05 (392 bp), Lanes: 2, 7, 8, 9 are tested isolates showing negative results for *bla* SHV gene, Lanes 3, 4, 5, 6 are tested isolates with positively amplified *bla* SHV gene.

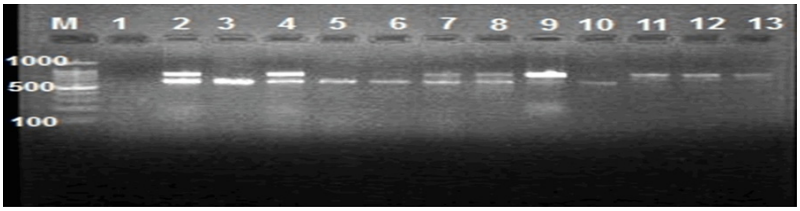

Figure 4. 2% Agarose gel electrophoresis of PCR products. Lane M: 100 bp molecular weight marker, Lane 1: negative control, Lane 2: TEM positive control (512 bp) and CTX-M positive control (619 bp), Lanes 3, 5, 6 and 10 tested isolates showing positive results for TEM gene, Lanes 9, 11, 12 and 13 are tested isolates with positively amplified *CTX-M gene*, Lane 4, 7 and 8 tested isolates showing positive results for both CTX-M and TEM genes.

different Enterbacteriacae isolated from study subjects is shown in **Table 2**.

Antimicrobial susceptibility testing

Of the total 359 bacterial isolated, 85 (23.7%) strains were defined as ESBLs and 274 (76.3%) were defined as non-ESBLs. Among the total 85 ESBL, *E. coli* was found to be the most frequent (66/77.6%), while *K. pneumonia* was (19/22.4%), (**Figure 1**). Furthermore, all ESBL and non-ESBL producers were tested against different empirical antibiotics, the results reflected significantly high rate of resistance among ESBL isolates compared with non-ESBL producers ($P < 0.05$) as shown in **Table 3**.

Distribution of ESBL among different clinical samples

Extended spectrum β-lactamases producers (ESBLs) were more frequent in urine specimens (61.7%), followed by blood (15.1%), pus (8.2%), wound (5.5%), sputum (4.1%), eye (2.7%) and high vaginal swap (2.7%) as illustrated in **Figure 2**.

Multiplex PCR in the detection of ESBL genes

All of the 85 isolates that were phenotypically flagged as ESBL producers were genotyped. The results were as follows: 63 isolates (74.1%) possessed the CTXM gene, 27 (31.8%) of the isolates possessed TEM gene, and only 12 (14.1%) possessed the SHV gene (**Figures 3**, **4** and **Table 4**). Furthermore, CTXM and TEM genes together were presented in 15.3% of the ESBL isolates while CTXM together with SHV genes were presented in 8.2% and SHV combined with TEM were found in 3.5% of the total ESBLs.

Discussion

Although research regarding ESBL producers drew attention regionally and globally, data in this regard in Al-Madenah Al-Monawwarah area is still limited. Thus, we aimed to shed light on the ambiguities surrounding this important

Table 4. Frequency of ESBL genes among 85 target isolates

Gene	*E. coli* (66) (%)	*K. pneumoniae* (19) (%)	Total (85) (%)
CTX-M	57 (86.4)	6 (31.6)	63 (74.1)
TEM	19 (28.8)	8 (42.1)	27 (31.8)
SHV	3 (4.5)	9 (47.4)	12 (14.1)
CTX-M+SHV	2 (3.0)	5 (26.3)	7 (8.2)
CTX-M+TEM	10 (15.2)	3 (15.8)	13 (15.3)
SHV+TEM	1 (1.5)	3 (15.8)	3 (3.5)

phenomenon in that holy region. Within the Arabian Gulf region, high ESBL prevalence of 31.7% in Kuwait and 41% in the United Arab Emirates [10, 11] has been reported among inpatients. For Saudi Arabia, reported ESBL rates vary from 8.5-38.5% [8, 9]. Thus, in comparison to regional data, the finding of 85/359 (23.7%) ESBL producers in this study are on the middle of the spectrum. This finding is also similar to data reported from surveys in other countries in Europe and Asia [12-14].

Although many species of *Enterobacteriacae* were isolated from different samples, ESBLs were identified only among *E. coli* (66/85; 77.6%), and *K. pneumoniae* (19/85; 22.4%). In this study, the predominance of *E. coli* is similar to, but the frequency is less than what has been reported in data from the Eastern Province of Saudi Arabia (83%) [15]. Other results obtained from different parts of the world demonstrated that *K. pneumoniae* is the most frequent pathogen compared to *E. coli* (52.27%: 46.43%) [16]. Moreover, we reported in this research that ESBL producers are significantly resistant to other drugs that do not belong to beta lactam antibiotics ($P < 0.05$). Previous studies have reported the occurrence of this phenomenon from other parts of the world like India and Sudan [16, 17].

Our data confirmed the existence of the bla_{CTX-M} gene in Al-Madenah Al-Monawwarah with a significant ratio (74.1%). Similar findings were reported from Riyadh, KSA [9, 18] and Makkah [19]. High prevalence of bla_{CTX-M} were also reported from other parts of the world; 71.4% in Sudan, 83.3% in Spain, 41.3% in Mongolia, 59.0% in Bangladesh, 48.5% in India, 50.0% in Taiwan, 84.0% in Thailand and 35.9% in Russia [17, 20-25]. Our findings proved for the first time the high occurrence of CTX-M gene among different ESBL-producing isolates in this special and overcrowded part of Saudi Arabia. Other ESBL genes were also found among the isolates from the enrolled subjects, the results were as follows: SHV gene among all ESBL producers was (3.5% in *E. coli* and 10.6% in *K. pneumonia*) and TEM gene (22.4% in *E. coli*, 9.4% in *K. pneumoniae*), while 15.3% of both *E. coli* and *K. pneumoniae* present as co-producers of CTXM with TEM, 8.2% and 3.5% as co producers of CTXM with SHV and SHV with TEM ESBL genes respectively. Many authors in different part of the world have proved similar results [12, 13].

From this research we can conclude that there is a relatively high proportion of ESBL producers in Al-Madenah Al-Monawwarah (23.7%) compared to other parts of KSA, which will be considered as a major challenge to the health authorities in this region. Putting into account the high emergence of CTX-M gene (74.1%) among the ESBLs of the study subjects, which is known as a serious public health concern worldwide and has been noted to be the cause of outbreaks as reported elsewhere.

Acknowledgements

Authors are so grateful to the staff of the central Laboratory at Prince Sultan Hospital for their help and support, especial thanks to Dr. Miskelyeman A. Elmekki, College of Applied Medical Sciences, Taibah University, for technical assistance. This project was supported by the Deanship of Scientific Research, Taibah University, Al-Madenah Al-Monawwarah, KSA (Grant number 435/6092).

Disclosure of conflict of interest

None.

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