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A simple multiplex PCR for assessing prevalence of extended-spectrum β-lactamases producing *Klebsiella pneumoniae* in Intensive Care Units of a referral hospital in Shiraz, Iran

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ABSTRACT

Objective: To identify three common genes (bla_{TEM}, bla_{SHV} and bla_{CTX-M}) responsible for ESBL production in *Klebsiella pneumoniae (K. pneumoniae)* isolated from Intensive Care Units of Namazi Hospital, Shiraz, Iran. **Methods:** A total of 60 non-repetitive nosocomial isolates from 60 patients were selected during 2009-2010. The phenotypic identification of ESBL production was confirmed by Double Disk Synergy Test (DDST) according to CLSI guidelines. The ESBL's genotype was then analyzed by multiplex PCR of bla_{TEM}, bla_{SHV} and bla_{CTX-M} genes and DNA sequencing. **Results:** The primary susceptibility tests of *K. pneumoniae* showed that among 10 examined antibiotics, the most resistant and susceptible antibiotics identified in this study were ampicillin and imipenem, respectively. The phenotypic determination of ESBL by DDST showed that 60% (n=36) of isolates produced ESBL. Multiplex PCR of genes among *K. pneumoniae* isolates showed that 39% (n=18) of them have TEM, 39% (n=18) of them have both CTX-M and TEM and 13% (n=8) of them have TEM, SHV, CTX-M. **Conclusions:** Our findings reveal the high prevalence (60%) of ESBL producing *K. pneumoniae* from ICU patients along with a new pattern of bla_{TEM} distribution differ from other countries.

1. Introduction

Extended-spectrum- β-lactamases (ESBLs) are enzymes produced by Gram negative bacteria which were first described in the 1980s. They have been detected in *Klebsiella* species, and sufbsequently in *Escherichia coli (E. coli)* , *Pseudomonas aeruginosa (P. aeruginosa)* , *Serratia marcescens (S. marcescens)* and other Gram-negative bacilli which have emerged as a significant mechanism of resistance to oxyimino-cephalosporin antibiotics during the last 2 decades[1]. The resistance to broad-spectrum cephalosporins and aztreonam is mostly mediated by β-lactamases by hydrolyzing the β-lactam ring[2]. The ESBL's genes are usually found on plasmids, along with genes which are responsible for resistance to aminoglycosides and trimethoprim-sulfamethoxazole. Furthermore, *Klebsiella pneumoniae* (*K. pneumoniae*) isolates harboring ESBLs are notably more frequently resistant to quinolones than non-ESBL-producing strains and are an important cause of nosocomial infections, which the main population at risk is infants and immunocompromised hosts[3, 4].

ESBLs occur mainly in *Klebsiella* species and *Escherichia coli (E. coli)* but may also exist in other members of Enterobacteriaceae[5]. Extended-spectrum β-lactamases (ESBLs) are the rapidly evolving group of β-lactamase enzymes, which are able to hydrolyse all cephalosporins and aztreonam but are inhibited by clavrulanic acid[6-10]. In general, most ESBLs are mutants of classical plasmid mediated SHV and TEM genes. ESBLs were first described in *K. pneumoniae* from Western Europe but are now widely

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disseminated all over the world[9]. There are frequent reports for nosocomial outbreaks of infections caused by ESBL producing bacteria, but the prevalence of ESBL-mediated resistance is not established well enough for most hospitals[5].

ESBLs have a high epidemiological and clinical importance which are responsible for therapeutic failure and increase hospital costs[11]. The multidrug-resistant infections caused by Gram negative bacilli that produce ESBL enzymes have been reported with increasing prevalence of antimicrobial resistance in intensive-care units and are associated with considerable mortality and morbidity[12].

Factors such as the transmissibility of resistance determinants mediated by transposons, plasmids, and gene cassettes in integrons along with the broad utilizations of antibiotics, contribute to the rise in antibiotic resistance in bacterial pathogens[13]. The current therapy for ESBL-producing *K. pneumonia* infection is Imipenem as an example of broad-spectrum agent. However, there are several reports of therapeutic failures of this drug due to multiple β-lactamases producer strains. Owing to the limited therapeutic options for some of these organisms, the extended-spectrum β-lactamases producers will challenge clinical microbiologists and clinicians[14]. In view of the limited information about ICU-associated infections in referral hospital in Shiraz, the largest metropolis in south of Iran, it is necessary to understand the prevalence rates and pattern of such nosocomial infection to obtain satisfactory results in managing these infections. Therefore, in this study, we set up a cross-sectional study in order to determine the prevalence rate and phenotypic and genotypic characteristics of *K. pneumonia* infections. The molecular characterization of ESBL genes (TEM, SHV and CTX-M) from clinical isolates of *K. pneumonia* collected from ICU was carried out by Multiplex PCR. By using this method, the TEM, SHV and CTX-M genes could be identified simultaneously and characterized at molecular level[15].

2. Materials and methods

2.1. Bacterial isolates

A total of 60 consecutive non-repetitive clinical isolates of *K. pneumoniae* were collected from Intensive Care Units (ICUs) Namazi Hospital in Shiraz, Iran and were selected for the molecular study from the clinical samples including blood (n=2), urine (n=31), sputum (n=12), C.S.F (n=6), eye (n=3) and wound (n=6) over a 6 month period from Dec 2009 to Jun 2010. The isolates were biochemically identified based on the colony morphology on Eosin Methylene Blue, Blood agar, Mac Conkey agar and it was speciated by standard biochemical tests[16]. The isolates were stored in trypticase soy broth with 30% glycerol at −70 ℃ for further analysis.

2.2. Antibiotic susceptibility testing

The antibiotic susceptibility of bacteria was primarily carried out by the disk diffusion method using Mueller Hinton agar plates according to the NCCLS guidelines[18]. The antibiotics tested were ceftazidim, (30 μg) , cefotaxime (30 μg), cefepime (30 μg), aztreonam (30 μg), ampicillin (10 μg) , ciprofloxacin (5 μg), gentamicin (10 μg), amikacin (30 μg), imipenem (10 μg), co-trimoxazole (25 μg) (Hi-media, India).

Minimum inhibitory concentration (MIC) was determined by the E-test (AB-Biodisk, Sweden) for cefotaxime antibiotic based on CLSI standard procedure[17].

2.3. Screening for ESBL producing strains

The *K. pneumoniae* strian was considered to have an ESBL phenotype if it demonstrated a reduced inhibition zone diameter for ceftazidime (≤22 mm) or cefotaxime (≤ 27 mm) or ceftriaxaone (≤ 25 mm) or aztreonam (≤ 27 mm)[9].

The double disk synergy test (DDST) was used to screen for ESBL producing strains which showed resistance to one or more third generation cephalosporins. Cefotaxime (30 μg), ceftazidime (30 μg) and aztreonam (30 μg) were placed on Mueller Hinton agar plates adjacent to an amoxicillin-clavulanic acid disk (20 μg of amoxicillin and 10 μg of clavulanic acid). The ESBL production is inferred when the cephalosporin inhibition zone is expanded by the clavulanate (≥5 mm). *K. pneumoniae* ATCC 700603 and *E. coli* ATCC 25922 were used as positive and negative controls for ESBL production, respectively[18].

2.4. Multiplex PCR amplification

All isolates were screened for the resistance genes SHV, TEM, and CTX-M by Multiplex PCR assay using universal primers (Cinnagen Co, Tehran, Iran) and the detailed information of these primers are listed in Table 1. DNA extraction was performed using a heat shock method[15]. Initially, PCR annealing temperature gradient was performed and all subsequent Multiplex PCR reactions were carried out in a final 25 μL volume containing 2.5 μL of 10X PCR reaction buffer, 1 μL DNA solution, 0.5 μL $MgCl_2$ (50 mM), 0.5 μL of each gene-specific primer (10 pmoL), 0.5 μL (3 U/mL) Hot Star *Taq* Mastermix DNA polymerase (Qiagen) and 0.5 μL deoxynucleoside triphosphates mix (dNTPs, 10 mM). PCR amplifications were performed on a MJ MINI DNA thermal cycler (BioRad, Australia).

PCR amplification conditions are as follows: initial denaturation step at 95 ℃ for 15 min: 30 cycles of denaturation at 94 ℃ for 30 s, annealing at 60 ℃ for 30 s and

Table 1
Primer used for PCR and DNA sequencing.

Primer name	Amplicon size (bp)	Gene name	Nucleotide sequence (5′-3′)
TEM	445	*bla*-TEM-A	TCGCCGCATACACTATTCTCAGAATGA
TEM	445	*bla*-TEM-B	ACGCTCACCGGCTCCAGATTTAT
CTX-M	593	*bla*-CTXM-A	ATGTGCAGYACCAGTAARGTKATGGC
CTX-M	593	*bla*-CTX-M-B	TGGGT RAA RTA RGTSACCAGAA YCAGCGG
SHV	973	*bla*-SHV-A	TCTCCCTGTTAGCCACCCTG
SHV	973	*bla*-SHV-B	CCACTGCAGCAGCTGC(A/C)GTT

*= K is G or T, R is A or G, S is G or C, Y is C or T

extension at 72 ℃ for 2 min followed by a final extension step at 72 ℃ for 10 min[10]. PCR amplicons were separated electrophoretically on a 1% (w/w) agarose gel and stained with ethidium bromide. A molecular marker (Fermentase SMo323 100 bp DNA ladder) was used to assess PCR product size.

2.5. Uniplex PCR amplification of TEM, SHV, CTX-M genes and Sequencing β – lactamase genes

In order to confirm the results obtained by the multiplex PCR amplification, three uniplex PCR amplification assay were carried out targeting the SHV, TEM and CTX-M genes, respectively, using 10 pmol of each gene specific primer as given in Table 1 and a Hot Star *Taq* Mastermix in a final reaction volume of 25 μL. PCR amplification conditions are as follows: initial denaturation step at 95 ℃ for 15 min, 30 cycles of denaturation at 94 ℃ for 30 s, annealing at 56 ℃ for bla_{SHV} 50 ℃ for bla_{TEM}, and 58 ℃ for bla_{CTX-M}, extension at 72 ℃ for 2 min, and a final extension step at 72 ℃ for 10 min. PCR amplicons were separated electrophoretically on 1% (w/w) agarose gel and stained with ethidium bromide at a concentration of 20 μg/100 mL water.

In order to confirm and further identification of the β -lactamase genes detected in PCR assays, DNA sequence analysis of the PCR amplicons was performed.

3. Results

During the study period, a total of 60 isolates of *K. pneumonia* were isolated from a range of clinical specimens of patients hospitalized in intensive care units of Namazi Hospital, Shiraz, Iran. Out of 60 isolates of *K. pneumoniae* that collected and confirmed by biochemical methods a total of 31 (51.67%), 12 (20%), 6 (10%), 6(10%), 3 (5%) and 2 (3.33%) were isolated from urine, sputum, wound, C.S.F, eye culture and blood, respectively.

3.1. Susceptibility test results

The antimicrobial susceptibility test of resistant isolates by means of disk diffusion method showed that the least and most resistance were observed in imipenem (1.66%) and ampicilin (100%), respectively. The resistance rate to other antibiotics is as follow: cefotaxime (56.66%), cefepime (48.34%), ceftazidime (46.67%), co-trimoxazole (43.33%), aztreonam (31.67%), ciprofloxacin (21.66%), gentamicin (13.34%) and amikacine (8.34%) (Table 2). According to phenotypic confirmatory ESBL test; double disk synergy test (DDST), 60% (n=36) of *K. pneumoniae* isolates in our study was ESBL producers. Resistance to co-trimoxazole and ciprofloxacin among ESBL producing *K. pneumoniae* isolates were 27.78% and 16.67%, respectively. *K. pneumoniae* isolates were mainly resistant to multiple antibiotics, but mainly susceptible to imipenem, amikacin and gentamicin. The frequency of resistance among *K. pneumoniae* isolated for cefotaxime as detected by MIC E-test were in intermediate range (24.8 μg/mL).

Table 2
Percentage of susceptibility and resistant antibiotics among 60 clinical isolates *K. pneumoniae* isolated from ICU.

Antibiotics	Resistant	Intermediate	Sensitive
Ceftazidime	28(46.67%)	4(6.66%)	28(46.67%)
Cefotaxime	34(56.66%)	10(16.67%)	16(26.67%)
Cefepime	29(48.34%)	8(13.33%)	23(38.33%)
Aztreonam	19(31.67%)	13(21.66%)	28(46.67%)
Ampicilin	60(100.00%)	0(0.00%)	0(0.00%)
Amikacin	5(8.34%)	3(5%)	52(86.66%)
Gentamicin	8(13.34%)	7(11.66%)	45(57%)
Co-trimoxazole	26(43.33%)	2(3.33%)	32(53.34%)
Ciprofloxacin	13(21.66%)	2(3.33%)	45(75%)
Imipenem	1(1.66%)	0(0%)	59(98.34%)

The MDR phenotypes were detected in 40% (n=24) of *K. pneumoniae* isolates and was defined as resistance to ceftazidime, cephotaxime and cefepime and the most MDR strains (33.34%) were isolated from internal ICU[19].

3.2. Annealing temperature gradient of PCR amplification.

A multiplex PCR amplification assay was designed to detect bla_{SHV}, bla_{TEM}, and bla_{CTX-M} genes in ESBL suspected *K.*

pneumoniae strains. Each PCR amplification assay created a unique PCR amplicon (Table 1) discernible from each other when separated on an appropriate agarose gel (Figure 1). PCR amplification of all three genes were done simultaneously, using a primer annealing temperature gradient between 50℃ and 60℃, revealed on optimal annealing temperature between 58℃ and 60℃ (Figure 1).

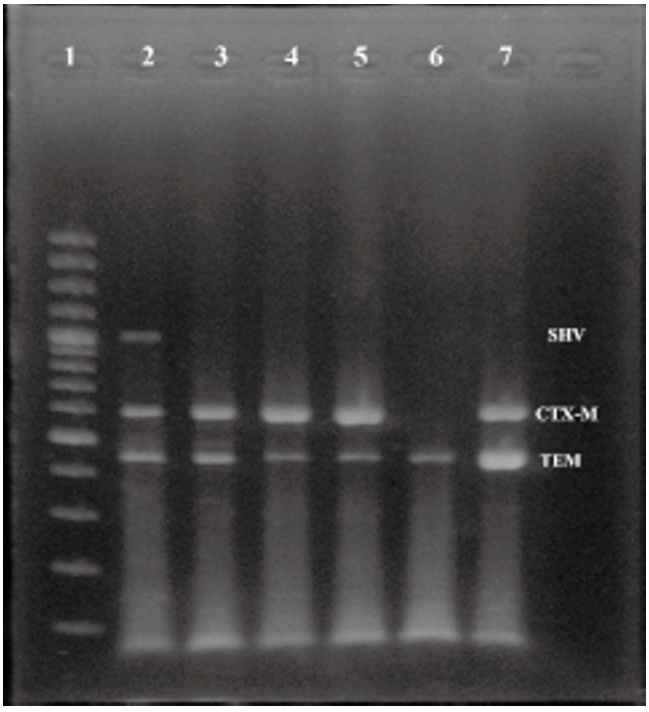

Figure 1. Multiplex PCR for TEM, SHV and CTX-M: 1percent Agarose gel showing products of multiplex PCR amplification from 6 clinical isolates *K. pneumonae* with bla_{TEM} (445 bp), bla_{SHV} (973 bp) and bla_{CTX-M} (593 bp).
Lane 1: The molecular size standard (100 bp) DNA ladder. Lane 2: TEM- CTX-M- SHV, lane 2-3-4-6-7: TEM, CTX-M. Lane 6: TEM.

3.3. Detection of bla_{SHV}, bla_{TEM}, bla_{CTX-M} genes by multiplex PCR amplification

The multiplex PCR assay was tested against 60 *K. pneumoniae* isolates that met and did not meet screening criteria. The ethidium bromide staining of agarose gels revealed a distinct electrophoretic separation of the bla_{SHV}, bla_{TEM}, bla_{CTX-M} PCR amplicons (Figure 1)

Result from agarose gel disclosed that the bla_{TEM} gene alone was present in 38.34% (n=23) of isolates and also the similar rate was observed for [bla_{TEM}, bla_{CTX-M}] genes. The bla_{CTX-M} gene alone was found in 3.33% (n=2) of isolates and the same for both TEM and SHV genes. Finally the three TEM, SHV, CTX-M genes were found in 13.33% (n=8) of isolates (Figure 2).

No PCR products were obtained in 3.33% (n=2) of 60 clinical isolates, demonstrating that the bla_{TEM}, bla_{CTX-M} and bla_{SHV} genes were either not present or that the PCR primer sequences may not produce any PCR products under the conditions used. This may be owing to single nucleotide sequence variations.

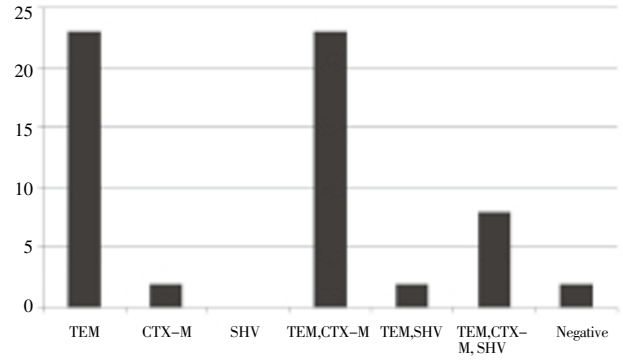

Figure 2. Prevalence extended spectrum beta lactamases genes among bacterial isolates *K. pneumoniae* isolated from ICU.

3.4. Single PCR amplification

After initial annealing temperature optimization of the TEM, CTX-M and SHV primer pairs, isolated DNAs were analyzed for the presence of the three groups of genes of interest. The results achieved were in agreement with the multiplex PCR amplification results (data not shown).

3.5. Partial DNA sequence analysis

DNA sequence analysis of cloned PCR amplicons derived from *K. pneumoniae* bla_{CTX-M} and bla_{TEM} positive strains confirmed the identity of the PCR amplicons as expected using gene - specific bla_{TEM}, bla_{SHV} and bla_{CTX-M} primers.

4. Discussion

The emergence of ESBL-producing Gram-negative bacilli emphasizes on a significant mechanism of resistance to oxyimino-cephalosporin antibiotics. The challenges relating to these increasingly hostile environments have been ascended for their survival, and they have proved to be well adapted to the task. In addition, patient-to-patient transmission is favoured by the extensive use of broad-spectrum antimicrobial agents at ICUs where antibiotic-resistant microorganisms are prevalent[20]. On the other hand, insufficient infection control measures have aggravated this problem, according to numerous reports of worldwide hospital outbreaks of ESBL-producing organisms[21]. Therefore, methods that can precisely identify

the potential expression or presence of these enzymes in clinical isolates on a large scale are thus needed. Yet, most ESBL detection strategies are based on determination of the isoelectric point (pI) and agar diffusion test methodology, which has usually been considered enough to identify the ESBL-producing strains[22]. However, in a large scale these methods are not the preferred options for establishing phenotypic identification[22].

Most researchers in Iran prefer to use phenotypic methods along with the reported prevalence ranging from 55 to 65 per cent[20, 23-25]. Therefore, in the present study along with the phenotypic identification of ESBL-producing *K. pneumoniae* isolates, we set up Multiplex PCR as a rapid method for discrimination of bla_{TEM}, bla_{CTX-M} and bla_{SHV} genes, simultaneously as well as identification of the genotypic pattern of these genes. The phenotype and genotype of 60 clinical *K. pneumoniae* isolates, as causative nosocomial pathogen with high morbidity and mortality, was studied during 2009-2010. In this study, it was revealed that 40% (n=24) of isolates showed MDR phenotype. In comparison to other findings, Imipenem had an excellent antimicrobial activity against almost all MDR isolates (95.84%) followed by amikacin (86.11%), ciprofloxacin (80.56%)[12]. Imipenem and Meropenem were proposed by most investigators as selective drugs for ESBL-positive *Entrobacteriaceae* or in an outbreak setting. However, to maintain the therapeutic value of carbapenems, a fluoroquinolone or aminoglycosides would be preferable choice for this purpose[24]. It should be noted that observation of a single imipenem-resistant isolate in one hospital can alarm the emergence of more resistant isolates. Thus, there is a need for using this and other carbapenems with caution[23].

The present study shows that the bla_{TEM} was found to be predominant followed by bla_{CTX-M} and bla_{SHV}, respectively. These findings are in agreement with those of recent studies in several cities of Iran that had similar pattern of ESBLs distribution[23-25]. However, this pattern is different from that of other countries (e.g. Italy, Egypt and Austria), which CTX-M is the most of β-lactamases[26, 27].

In conclusion, this study highlights the importance of high emergence of ESBLs in Iran with the rate of 60%, which is an alarm to be more aggravated if this trend continued due to lack of rational drug administration policy. Therefore, attempts to keep up current therapeutic options for ESBL producing bacteria by limiting the use of extended spectrum antibacterial agents and 3rd generation cephalosporins along with implementation of infection control measures are crucial. This study also provides the Multiplex PCR could be a simple and fast alternative procedure for an efficient, rapid discrimination of ESBLs in *K. pneumoniae* as well as a rapid tool for epidemiological studies among ESBL isolates.

Conflict of interest statement

We declare that we have no conflict of interest.

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