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Molecular Epidemiology of Extended-Spectrum β-Lactamase-Producing *K. pneumoniae* in Northern Jordan

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Abstract: The incidence of the Extended-Spectrum β-Lactamase (ESBL producing strains among clinical Klebsiella isolates has been steadily increasing over past years. The resulting limitations on the therapeutic options demand new measures for the management of *K. pneumoniae* hospital infections. A total of 112 *K. pneumoniae* isolates were isolated from different wards of the three major hospitals in Northern Jordan. The isolates were identified using bacteriological methods, biochemical testing and disc diffusion technique. ESBL producing isolates were detected using the double disk diffusion method and Polymerase Chain Reaction (PCR). ESBL producing strains constituted 31.25% of all recovered isolates. The percentage of aztronam resistance isolates among all samples was 78% (87/112). 35 (100%) isolates were resistant to cefuroxime followed by 34 (97.1%) of isolates were resistant to cefpodoxime and aztronam. The 97% of ESBL producing strains were susceptible to imipenem and 89% were susceptible to ciprofloxacin which suggesting that imipenem is the drug of choice as a therapeutic option for *K. pneumoniae* infections. Further more, out of 35 isolates; 33 (94.3%) and 28 (80%) were positive for TEM and SHV genes, respectively. *K. pneumoniae* has been added to the growing list of pathogens which are susceptible to a very limited number of antibiotic therapies.

Key words: *K. pneumonia*, antibiotic, extended-spectrum β-lactamase, PCR, therapeutic, ciprofloxacin

INTRODUCTION

Klebsiella pneumoniae is most frequently recovered from clinical specimens and can cause many infections including pneumonia. *K. pneumoniae* can also, cause a variety of extrapulmonary infections including enteritis and meningitis, urinary tract infections and septicemia (Koneman *et al*., 1997; Greenwood *et al*., 2002; Lin *et al*., 2014).

The use of β-Lactam antibiotics has been impeded by the appearance of highly resistant strains of *K. pneumoniae* called Extended Spectrum β-Lactamases (ESBL) producers that produce extended spectrum β-lactamases (Martins-Loureiro *et al*., 2001; Pessoa-Silva *et al*., 2003; Somily *et al*., 2015).

The vast majority of ESBL are derivatives of TEM-1 and TEM-2 (common plasmid-mediated β-lactamases of *Escherichia coli*) or SHV-1 (the chromosomally encoded enzyme of *K. pneumoniae*). These enzymes are capable of hydrolyzing a wide range of β-lactamases including the most recently developed cephalosporins but not active against carbapenemes (Arlet *et al*., 1995; Bradford, 2001). The incidence of the ESBL producing strains among clinical Klebsiella isolates has been steadily increasing over past years. The resulting limitations on the therapeutic options demand new measures for the management of *K. pneumoniae* hospital infections (Rasheed *et al*., 2000).

In this project, we aimed to investigate the molecular epidemiology of ESBL positive strains among *K. pneumoniae* recovered from clinical specimens and to evaluate the susceptibilities of ESBL positive isolates to antibiotic useful in overcoming resistance traits that may be associated with ESBL production.

MATERIALS AND METHODS

Bacterial isolates: A total of 112 *Klebsiella pneumoniae* isolates were isolated from the intensive care unit, internal medicine, surgery, pediatric and neonatal intensive care unit wards. These isolates were received by the microbiology laboratories of the three major hospitals in

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Northern Jordan: King Abdullah University Hospital, Princess Basma Teaching Hospital and Princess Rahma Teaching Hospital.

The isolates were identified using traditional bacteriological methods and biochemical testing with the API 20E (BioMerieux, Marcy L'Etoile, France) according to the manufacturer's recommendations. The isolates were stored at -80°C in 15% glycerol (v/v) in tryptic soy broth until use.

Antimicrobial susceptibility testing: The isolates were tested for their antimicrobial susceptibilities by disc diffusion technique according to the CLSI guidelines (CLSI, 2012a). Using discs from Oxoid (Oxoid, UK). The Minimum Inhibitory Concentration (MIC) of each antibiotic was determined using the dilution and diffusion method on Mueller-Hinton agar with E-test strips (BioMerieux, Marcy L'Etoile, France). The results were interpreted according to the current CLSI guidelines (CLSI, 2012b).

Phenotypic detection of ESBL production: ESBL producing isolates were detected using the double disk diffusion method with ceftazidime (30 μg) ceftriaxone (30 μg) efpodoxime (10 μg) and cefepime (30 μg) (Oxoid, UK) according to CLSI recommended guidelines (CLSI, 2012b). Discs were placed around a central disk of Amoxicillin-Clavulanic Acid (20-10, 30 μg, Oxoid, UK) 30 mm center to center on a Mueller Hinton agar plate seeded with the test organism being tested for ESBL production. Plates were then incubated aerobically at 37°C for 18-24 h and the diameter of the inhibition zones (if any) around the antimicrobial disks were measured in (mm) and recorded on a special data sheet according to the manufacturer's instructions. *K. pneumoniae* ATCC 700603 was used as a positive control ESBL strain. Any augmentation (increase in zone of inhibition = 5 mm) of the inhibition zone between the central amoxicillin-clavulanic acid disk and any of the 4 antibiotic disks showing resistance or intermediate susceptibility was recorded and the organism was thus considered as an ESBL producer.

Pcr amplification of bla_{tem} and bla_{shv} genes: The amplification of β-lactamase genes was carried out by Polymerase Chain Reaction (PCR) using the procedures described by Chang *et al.* (2001). The DNA extraction protocol was done according to Promega technical genomic DNA purification manual (Anonymous, 2004). The PCR reactions were performed in a PCR thermal cycler using the primers listed in Table 1.

Table 1: The primes for PCR amplification of bla_{TEM} and bla_{SHV} genes

Primer	Sequence
bla_{TEM}	5'-ATAAAATTCTTGAAGACGAAA-3'
	5'-GACAGTTACCAATGCTTAATCA-3'
bla_{SHV}	5'-GGGTTATTCTTATTTGTCGC-3'
	5'-TTAGCGTTGCCAGTGCTC-3'

The 1 mL of cell suspension centrifuged at 13,000-16,000 xg for 2 min to pellet the cells. The supernatant was removed and suspended in 600 μL of nuclei lysis solution, then incubated at 80°C for 5 min to lyse the cells. Then 3 μL of RNase solution was added to each cell lysate and the tube was inverted 2-5 times then incubated at 37°C for 15-60 min. The next step was adding of 200 μL of protein precipitation solution and vortexed vigorously at high speed for 20 sec. Samples were incubated on ice for 5 min and then centrifuged at 13,000-16,000×g for 3 min. The supernatant containing the DNA was transferred to a clean 1.5 mL microcentrifuge tube containing 600 μL is opropanol and gently mixed by inversion then centrifuge at 13,000-16,000×g for 2 min. The supernatant was carefully poured off and 70% ethanol was added and gently inverted several times to wash the DNA pellet. Consequently the tubes were centrifuged at 13,000-16,000×g for 2 min, the ethanol was aspirated carefully and finally, 100 μL of rehydration solution was added to the tubes and incubated at 65°C for 1 h . DNA samples were stored at 2-8°C until use. The genomic DNA extracted has been photographed under UV light by running the DNA on ethidium bromide stained TBE agarose gel with genomic DNA marker (lambda Hind III). Positive and negative controls for TEM and SHV genes were included in the PCR assay.

Statistical analysis: Results of antimicrobial susceptibility testing and double disk synergy test were analyzed for their significance ($p<0.05$) using SPSS 21 Software (SPSS Version 21 for Windows, Chicago, IL, USA) to measure the mean value, standard deviation and confidence interval of the difference.

Ethical approval: The research has been carried out after obtaining the approval from the Ethical Committee, College of Medicine, Jordan University of Science and Technology, Irbid, Jordan.

RESULTS AND DISCUSSION

One hundred and twelve *K. pneumoniae* isolates were isolated from clinical specimens received at the clinical laboratories from 3 hospitals in the North of Jordan. Not all the collected samples were *K. pneumoniae*, 18% of samples were ignored in this study and only patient samples that were positive for *K. pneumoniae* were included in the study.

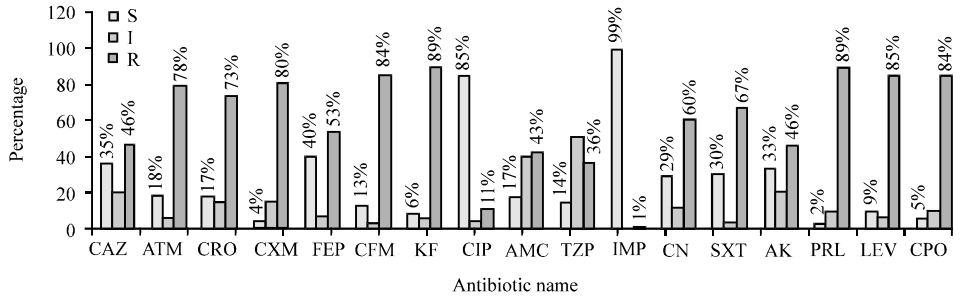

Fig. 1: Antimicrobial sensitivity among all *K. pneumoniae* isolates: CAZ; Ceftazidime, ATM; Aztronam, CRO; Ceftriaxone, CXM; Cefuroxime, FEP; Cefpime, CFM; Cefixime, KF; Cephalothin, CIP; Ciprofloxacin, AMC; Amoxicillin-Clavulanic Acid, TZP; Piperacillin/Tazobactam, IPM; Imipenem, CN; Gentamicin, SXT; Sulphamethoxazole/trimethoprim, AK; Amikacin, PRL; Piperacillin, LEV; Levofloxacin, CPD; Cefpodoxime, S; Sensitive, I; Intermediate, R; Resistant, A; Augmentation

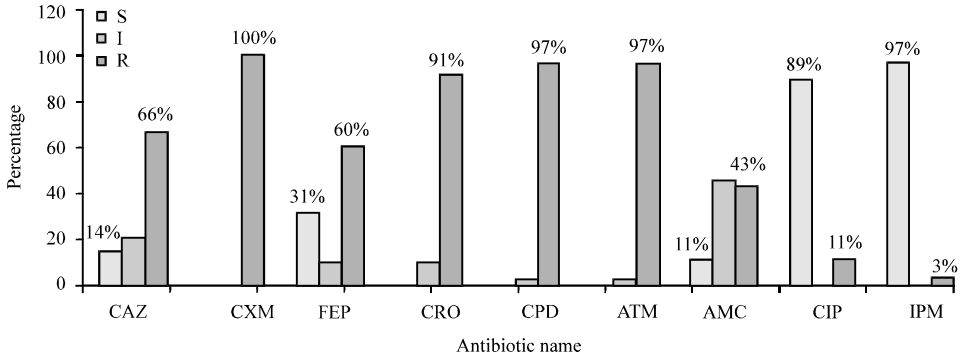

Fig. 2: Antimicrobial susceptibility among ESBL producers of *K. pneumoniae* isolates: CAZ; Ceftazidime, CXM; Cefuroxime, FEP; Cefpime, CRO; Ceftriaxone, CPD; Cefpodoxime, ATM; Aztronam, AMC; Amoxicillin-Clavulanic Acid, CIP; Ciprofloxacin, IPM; Imipenem, S; Sensitive, I; Intermediate, R; Resistant, A; Augmentation

Disk diffusion method: The disk diffusion method was carried out for all *K. pneumoniae* isolates (112 samples) to screen for their susceptibility to different antibiotics as shown in Fig. 1. The percentage of Aztronam resistance isolates among all samples was 78% (87/112) and the susceptibility for other antibiotics showed different resistance percentages including PRL, KF, CPD, CFM, CXM, CRO, SXT, FEP, CAZ, AK, CN, AMC, TZP, CIP and LEV and their resistance percentages were 89, 89, 84, 84, 80, 73, 67, 53, 46, 46, 29, 17, 14, 11 and 9%, respectively. The most intermediate resistance pattern among isolates was found in TZP 50%, followed by AMC 40%. 97% of ESBL producing strains were found susceptible to IPM followed by 89% were found susceptible to CIP.

Detection of esbl production: Out of the 112 *K. pneumoniae* isolates included in this study, 35 isolates (31.25%) were found to be ESBL producers using the double disk diffusion method. The ESBL producers isolates (31.25%, p = 0.001) showed various susceptibility pattern for different antimicrobial drugs. 35 (100%) isolates were resistant to CXM, 34 isolates (97.1%) were resistant to CPD and ATM and 32 (91.4%) isolates were resistant to CRO. In the other hand, 97% of ESBL producers were susceptible to IMP and 89% were susceptible to CIP as shown in Fig. 2.

PCR method and gel electrophoresis: The PCR method was done to detect the presence of TEM and SHV genes for the 35 ESBL produce isolates. After the genomic DNA was extracted, the agarose gel electrophoresis was run to ensure that the extraction process was carried out probably as shown in Fig. 3.

The resulted amplified sequence of the TEM gene was 900 bp Fig. 4. Results were reported as negative when there was no appearance of an amplified sequence. The results shown in Table 1 indicated that 33 (94.3%) isolates out of 35 were positive for the presence of TEM gene and the remaining 2 isolates (5.7%) of ESBL producer isolates were negative for TEM gene.

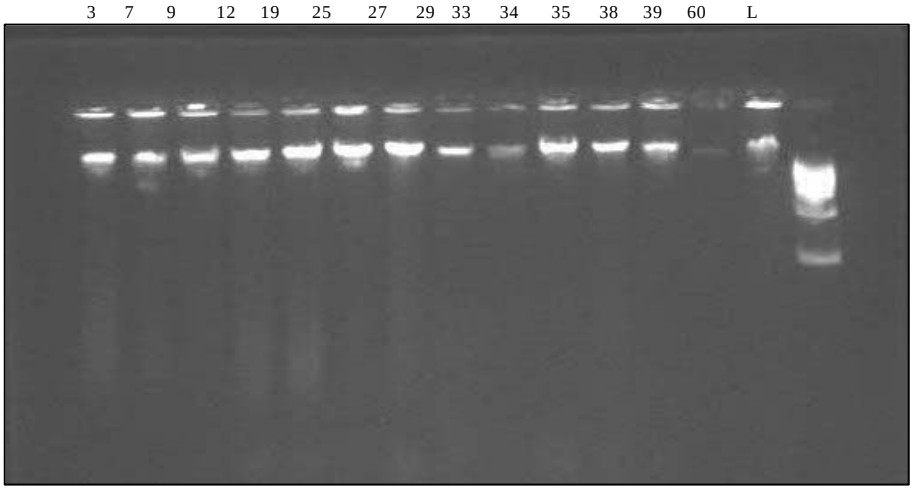

Fig. 3: Agarose gel electrophoresis of genomic DNA; L: 100 bp DNA ladder, isolates numbers: 3, 7, 9, 12, 19, 25, 27, 29, 33, 34, 35, 38, 39, 60 L

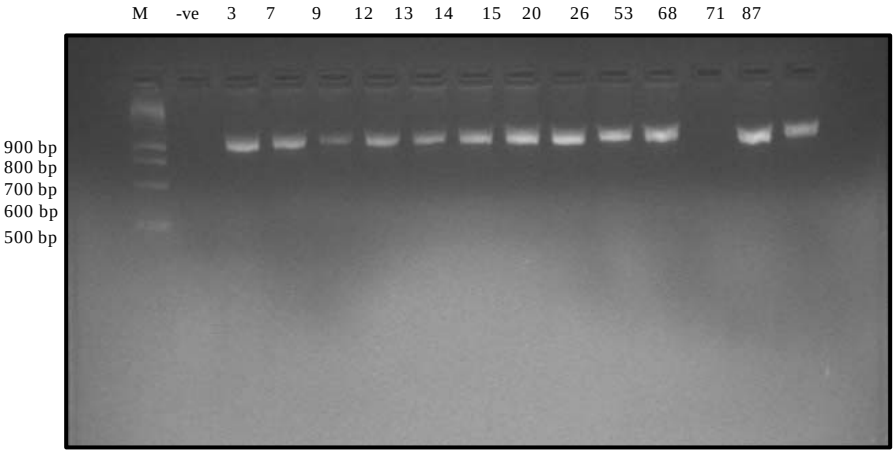

Fig. 4: Agarose gel electrophoresis of PCR for bla TEM gene. M; 1Kbp ladder, -ve; Negative control, 3, 7, 9, 12, 13, 14, 15, 20, 26, 53, 71, 87; TEM genes positive, 68; TEM gene negative

On the other hand, the resulted amplified sequence for SHV gene was 870 bp as shown in Fig. 5. The results shown in Table 2 indicated that 28 isolates (80%) were positive for the presence of SHV gene and the remaining 7 isolates (20%) of ESBL producer isolates were negative for SHV gene.

K. pneumoniae outbreaks most often started in intensive care units, presumably because in these areas patients are exposed to a considerable risk number of potential risk factors for colonization/infection such as misuse or abuse of antimicrobial agents which leads to development of antibiotic resistance (Edmondson *et al*., 1980; Ofek *et al*., 1995; Banerjee *et al*., 2016).

Jordan is one of the developing, countries where all types of antibiotics are sold over the counter. Third generation cephalosporins, especifically ceftazidime, cefotaxime, ceftraxone used in Jordan and frequently prescribed by practitioners in private clinics, since, the mid-1980 could contributed to the emergence of ESBL producing *K. pneumoniae* (Youssef *et al*., 1999; Rodrigues *et al*., 2014; Desta *et al*., 2016).

In regard to the antibiogram for all isolates, our *K. pneumoniae* isolates were more susceptible to imipenem (100%) ciprofloxacin and levofloxacin (85%) while the most resistant antibiotic among all isolates was for pipercillin and cefazolin with a resistance percentage of 89% and this was higher than a study carried out in Turkey (67.9%) (Ofek *et al*., 1995).

All ESBL producers isolates tested, showed various susceptibility pattern for different antimicrobial drugs, 34 isolates (97.1%) were resistant to CPD and ATM, 100% CXM resistant, 65.7% CAZ resistant, 91.4% CRO resistant

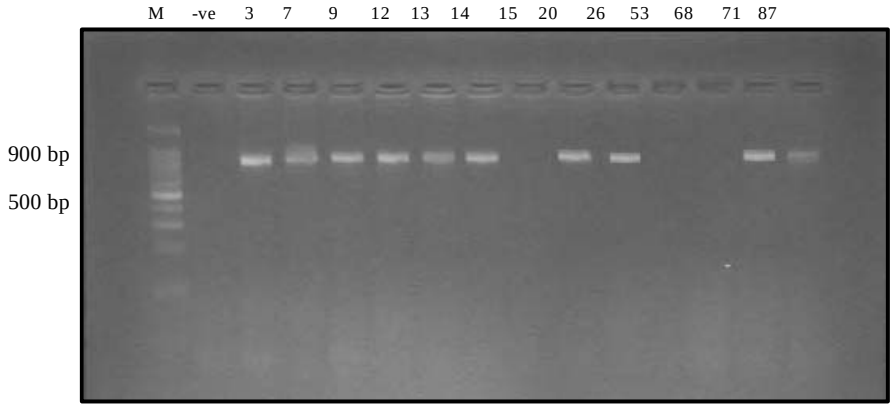

Fig. 5: Agarose gel electrophoresis of PCR for bla SHV gene. M; 1Kbp ladder, -ve; Negative control, 3, 7, 9, 12, 13, 14, 20, 26, 71, 87; SHV gene positive, 15, 53, 68; SHV gene negative

Table 2: Antimicrobial susceptibility and PCR for the 35 ESBL producing K. pneumoniae

Sample No.	PCR detection method (+/-)		Antimicrobial susceptibility pattern							
	bla_{TEM}	bla_{SHV}	CAZ	CXM	FEP	CRO	CPD	ATM	IPM	CIP
3	+	+	R	R	S	I	R	R	S	S
7	+	+	R	R	S	I	R	R	S	S
9	+	+	I	R	R	R	R	R	S	S
10	+	+	R	R	R	R	R	R	S	S
12	+	+	I	R	R	R	R	R	S	S
13	+	+	S	R	R	R	R	R	S	R
14	+	+	R	R	S	I	R	R	S	S
15	+	-	I	R	R	R	I	R	S	S
16	+	+	S	R	R	R	R	R	S	S
18	+	-	I	R	R	R	R	R	S	S
19	+	+	R	R	R	R	R	R	S	S
20	+	+	I	R	R	R	R	R	S	S
25	+	-	R	R	S	R	R	R	S	S
26	+	+	S	R	I	R	R	R	S	S
27	+	+	R	R	R	R	R	R	S	S
28	+	+	S	R	I	R	R	I	S	S
29	+	+	R	R	R	R	R	R	S	S
32	-	+	I	R	R	R	R	R	S	S
33	+	+	R	R	S	R	R	R	S	S
34	+	+	I	R	R	R	R	R	S	S
35	+	+	R	R	R	R	R	R	S	S
38	+	+	R	R	S	R	R	R	S	S
39	+	+	R	R	S	R	R	R	S	S
48	+	+	R	R	R	R	R	R	S	S
50	+	+	R	R	S	R	R	R	S	S
53	+	-	R	R	R	R	R	R	S	R
60	+	-	R	R	R	R	R	R	R	R
62	+	+	R	R	S	R	R	R	S	S
65	+	-	R	R	S	R	R	R	S	S
68	-	-	R	R	R	R	R	R	S	S
71	+	+	R	R	I	R	R	R	S	S
85	+	+	R	R	I	R	R	R	S	S
87	+	+	S	R	R	R	R	R	S	S
91	+	+	R	R	I	R	R	R	S	S
92	+	+	R	R	S	R	R	R	S	R

CAZ: Ceftazidime, CXM: Cefuroxime, FEP: Cefpime, CRO: Ceftriaxone, CPD: Cefpodoxime, ATM: Aztronam, IPM: Imipenem, CIP: Ciprofloxacin, S: Sensitive, I: Intermediate, R: Resistant, A: Augmentation

and 54.3% FEP resistant as detected by disk diffusion method. Those ESBL producer isolates were considered multi-drug resistant strains but 97 and 89% of them were susceptible to IMP and CIP, respectively.

In regard to ESBL production; one of the most alarming findings of this study is the finding of ESBL production in 31.25% of *K. pneumoniae* isolates. This percentage is similar to the prevalence's of ESBL

production worldwide were found to be 20% in an Italian study (Spanu *et al*., 2002) and 39.5% in a Chinese study (Parasakthi *et al*., 2000).

The ESBL test and bla_{TEM} gene detection by PCR showed that 34 of 35 (97.1%) were ESBL (TEM β-lactamase) producer except the isolate number 68. In the other hand, PCR detection method for bla_{SHV} gene showed that 28 of 35 (80%) were ESBL (SHV β-lactamase) producer except the isolate numbers 15, 18, 25, 53, 60, 65 and 68 were found negative for SHV gene. Isolate number 68 showed ESBL production by double disk synergy test but in PCR method was found negative for bla_{TEM} while bla_{SHV} gene was presence. This study was aimed to seek for bla_{TEM} and bla_{SHV} genes although there are many types of β-lactamases genes that were not studied like IRT and CTX-M β-lactamases. Therefore, the isolate number 68 might harbor one of these genes. The isolates number 28 that harbored both bla_{SHV} and bla_{TEM} genes considered multidrug resistant isolates because they had many possibilities for degrading different generations of cephalosporins and aztreonam.

In comparable with other studies, we have to confirm the incidence of multi-drug resistant *K. pneumoniae* that varies by location from country to another (Pessoa-Silva *et al*., 2003) but by general Quale *et al*. (2002) and Parasakthi *et al*. (2005) confirmed that patient to patient transmission of ESBL positive *K. pneumoniae* had been correlated with the rectal colonization of patients, contamination of environmental surfaces and the hands of clinical staff and the transfer of patients between hospitals and nursing home (Parasakthi *et al*., 2005). In addition, Martin-Lourero *et al*. (2001) confirmed that *K. pneumoniae* caused serious epidemic and endemic nosocomial infection, person to person spread was the most common mode of transmission and nearly 50% of the out breaks occurred in neonatal care units.

Quale *et al*. (2002) found that out of 824 *K. pneumoniae* isolates, 34% were ESBL producer and the susceptibility to ciprofloxacin, ceftriaxone, amikacin, ceftazidime and imipenem were 83, 86, 72, 72 and 99.6%, respectively. The imipenem sensitivity percentage of 0.4% by Quale *et al*. (2002) study is almost a similar percentage of imipenem sensitivity (3%) in our study.

Chang *et al*. (2001) tested 113 isolates of *K. pneumoniae* from 10 hospitals in northern Taiwan for TEM and SHV β-lactamase production. They found that bla_{SHV} gene was amplified from all isolates and TEM-type resistance was found in 32 of the isolates. Furthermore, they reported that imipenem is still a drug to which isolates were susceptible to. Tasli and Bahar (2005) tested 45 transconjugants isolates in Dokuz Eylul University Hospital in Turkey. They found that 77.7% of isolates were ESBL producers 31 and 93.3% were TEM and SHV PCR positive. Nogueira *et al*. (2015) using the PCR analysis identified the presence of bla_{TEM} gene in 4 strains (3 *K. pneumoniae* and 1 *E. coli*) and bla_{SHV} gene in 6 strains (3 of each *K. pneumonia* and *E. coli*).

CONCLUSION

Imipenem resistance in *K. pneumoniae* is a particularly disturbing development in the world of antibiotic resistance. For many strains expressing multiple ampc types and extended spectrum β-lactamases, imipenem was the only therapeutic option for serious infections. Now *K. pneumoniae* has been added to the growing list of pathogens which are susceptible to a very limited number of antibiotic therapies.

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