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Resistance prevalence profile of *Klebsiella pneumoniae* in the Intensive Care Units of Al-Shatby Pediatric Hospital, Alexandria, Egypt

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Abstract

Klebsiella pneumoniae has been identified as an opportunistic pathogen associated with both community-acquired and nosocomial infections mainly among patients admitted to the Intensive care units (ICUs). Some resistant genes were transferred vertically or horizontally within many microbial communities, directly between bacteria through plasmids or integrin. Different techniques including; phenotypic and genetic ones were used to evaluate the presence of β-lactamases among the isolated strains of *K. pneumoniae*. This bacterial species is the most commonly isolated pathogen (95 isolates) from all the examined samples (51.35%). Current results revealed that 30 and 14 strains of *K. pneumoniae* are positive to Extended spectrum β-lactamase (ESBL) production, and AmpC β-lactamase producers, respectively. On the other hand, modified carbapenem inactivation and modified Hodge test (MHT) were used to assess the carbapenem resistant strains. It is observed that all the β-lactamase producers' strains are also carbapenemase producers, whereas only nine strains (30%) are MHT positive. The Polymerase Chain Reaction (PCR) technique revealed that TEM, BETA, NDM and IPM genes are found on the bacterial plasmid (100%). However the presence of the β-lactamase genes on the bacterial DNA varied among the different strains. The presence of the resistance genes on the bacterial plasmid may signify the resistance acquired upon the previous exposure of this bacterium to the different antibiotics. The aims of the current work were to isolate *K. pneumonia* from Al-Shatby hospital ICUs, to determine the incidence of its β-lactamases, and to decide the frequency of acquisition of 12 different genes among ESBL *K. pneumoniae* isolates.

Keywords: *Klebsiella pneumoniae*; Multi-drug resistance; β-lactamases; Gene

1. Introduction

During the past decades, the emerging problem of ESBL producing bacteria has received a great attention. Doi *et al*., (2013) reported that the most commonly important ways by which the Gram-negative bacteria can resist β-lactam antibiotics are through the production of enzymes (β-lactamase enzymes) capable of hydrolyzing the β-lactam ring of the antibiotics.

According to Falagas *et al*., (2014), *K. pneumoniae* carbapenemase (KPC) enzyme is the most commonly encountered enzyme among these isolates. Lutgring and Limbago, (2016) study revealed that the mechanisms of carbapenem resistance in the family Enterobacteriaceae are multifarious. They consist of β-lactamases carbapenem hydrolyzing enzymes construction, and resistance in line for the combination of other factors including ESBLs or AmpC β-lactamases hyper-production. The Carbapenemase Producing Carbapenem-Resistant Enterobacteriaceae (CP-CRE) can spread rapidly, and their detection may warrant implementation of more-intensive infection control interventions than would be employed for none-CP-CRE (CDC. 2013). In addition, Tamma *et al*., (2016) reported that CP-CRE has more hazardous influence than none-CP-CRE. The Gram-negative β-lactamases are regulated by several genes such as; blaCTX-M, blaTEM and blaSHV (Monstein *et al*., 2007).

The Ambler's molecular classification is based on the nucleotide and amino acid sequences of the β-lactamases enzymes. Four classes (A, B, C and D) are documented as conferring to Ambler's classification. The classes (A, C and D) have serine deposit, while the class B has cysteine or histidine residue singly or together (Ambler, 1980; Papp-Wallace *et al*., 2018). This study aimed to evaluate the incidences of the resistant MDR, ESBL, and CRKP genes of the *K. pneumoniae* isolates recovered from the ICUs of Al-Shatby Pediatric Hospital, Alexandria, Egypt.

2. Materials and methods

2.1. Sampling and collection of the bacterial isolates

This prospective cohort study was conducted over 7 months from the 1st of January to the 31th of July, 2017, at the Surgical sites ICU, Neonates ICU "NICU", and Pediatric ICU "PICU", of Al-Shatby Pediatric Hospital. The different clinical samples were collected from the mid-stream urine, blood, and from the broncho-alveolar lavage (BAL).

All the bacterial isolates used throughout this work were kindly provided and phenotypically identified by the Surveillance Microbiology Department Strain Bank, at Al-Shatby Pediatric Hospital, Alexandria, Egypt. The strains of *Klebsiella* ATCC 13883, *Klebsiella* 1705 (ESBL +ve), *Klebsiella* 1706 (ESBL -ve), and *Escherichia coli* ATCC 25922, were kindly provided by the Naval Medical Research Unit (NAMRU) no.3, Cairo, Egypt.

2.2. Identification of the bacterial isolates

The morphological features of each bacterial isolate was studied (Noor *et al*., 2014), and then identified biochemically (Barrow and Feltham, 1993). Additional identification for the selected *Klebsiella* isolates was carried out using Thermo Fisher Scientific RapIDTM ONE System (REF: R8311006); which was an identification system based on enzyme technology, and Vitek 2 automated system (bioMerieux, Marcy l'Etoile, France), at the Medical Research Center (MRC), Faculty of Medicine, Alexandria University, Alexandria, Egypt.

2.3. Evaluation of the prevalence of resistance among the *K. pneumoniae* strains

The disc-diffusion assay (Bauer *et al*., 1959) was evaluated using different groups of common antibiotics including; aminopenicillins, 1st generation cephalosporins, aminoglycosides (tobramycin), ureidopenicillins, trimethoprim-sulfamethoxazole, and monobactams, and then incubated at 35± 2˚C for 24 h.

2.4. Detection of the Extended-spectrum β-lactamase in *K. pneumoniae*

The Double-disc synergy test (DDST) (Jaspal *et al*., 2013) and the CLSI confirmatory test [Recommends a phenotypic confirmatory combined-disk test for ESBL production in Enterobacteriaceae. It consists of measuring the growth-inhibitory zones around both cefotaxime (CTX) and ceftazidime (CAZ) disks with or without clavulanate (CA)] (Aggeliki *et al*., 2014), were used for detection of the ESBL. The DDST was carried out using Cephalosporins discs which were placed in the petri plates next to a centrally placed disc of clavulanic acid (amoxicillin/ clavulanic acid 20/ 10 μg), according to CLSI. (2015). AmpC β-lactamases were clinically important cephalosporinases and Cefoxitin (Jacoby, 2009).

2.5. Phenotypic carbapenamase assembly recognition of *K. pneumoniae*

The Modified Hodge test (MHT) (Saito *et al*., 2015), and the Reformed Carbapenem Inactivation Method (CLSI, 2017; Bayraktar *et al*., 2018) were carried out in the selected bacterial isolates.

2.6. Determination of the *K. pneumoniae* resistance genes using molecular techniques

The DNA of the selected *K. pneumoniae* isolates was extracted using Thermo Scientific GeneJET Genomic DNA Purification Kit, while the plasmids of these isolates were extracted using GEBRI kit. To detect the presence of β-lactamases genes, normalization of the DNA and the plasmid concentrations were performed to avoid the dissimilarities between both concentrations before use. About twelve primers were designed (Table 1) and the genes used to mark the β-lactamase class A were; TEM, CTX, KPC, Beta and SHV. The β-lactamase Class B (carbapenemase encoding) genes were; VIM, IMP and NDM, whereas the β-lactamase class C encoding gene was FOX. Finally, the β-lactamase class D encoding genes were OXA-10, OXA-24 and OXA-58 (Pérez-Pérez and Hanson, 2002; Mariana *et al*., 2004).

3. Results

3.1. Sample collection and identification of the bacterial isolates

A total of 1470 samples were collected from 949 ICU hospitalized patients that were distributed as follows: Surgical, PICU and NICU (11.59, 15.91 and 72.49%, respectively) of the patients admitted. A total of 1217 out of 1470 samples are considered sterile, whereas 68 samples are considered as contaminated samples. The most commonly isolated pathogen from all the examined samples is *Klebsiella* sp. representing 51.35% (95 out of the total 185 isolated pathogens), and is distributed as 5.27, 25.26 and 69.47% in urine, BAL and blood samples, respectively. The Vitek 2 and RapID™ one systems were used for identification of *Klebsiella* sp., and they confirmed their identification as *K. pneumoniae* with > 99.9% confidence level. Results in Table (2) revealed that the incidence of Gram-negative bacterial isolates detected in the ICUs' is 69.18%, while it is 18.91% for Gram-positive bacteria. The incidence of the isolated bacterial spp. revealed that *Klebsiella* sp. is the most commonly isolated pathogen followed by *Candida albicans*, Coagulase negative Staphylococcus (CONS), and *Pseudomonas aeruginosa* with; 51.35, 11.89, 7.57 and 6.49%, respectively.

3.2. Evaluation of prevalence of resistance among the *K. pneumoniae* strains

The prevalence of antibiotics resistance among the isolated strains of *K. pneumoniaea* revealed that they are resistant to aminopenicillins, 1st generation cephalosporins, aminoglycosides (tobramycin), ureidopenicillins, trimethoprim-sulfamethoxazole, and monobactams. The *K. pneumonia* strains exhibited high resistance pattern to colistin and meropenem antibiotics (81 and 55.7%, respectively), as clear in Table (3), Fig. (1). These results may represent a high risk and provide very limited options for treatment of bacterial infections. However, it may raise an alert for a surveillance program to monitor the bacterial trends and antibiotics resistance patterns in Egypt.

Table 1. Primers sequences used for screening of genes encoding β-lactamases classes A-D

Class	Primer	Sequence	Product size (bP)
Class A	CTX	F: ATGTGCAGYACCAGTAARGTKATGGC R: GGTRAARTARGTSACCAGAAYCAGCGG	550
	Beta	F:ATAAAGGCGTGCTGCTGTTT R:TGTTCGCGGTTTTGTTCATA	686
	KPC	F: GCTTTCT(T/G)GCTG(C/G)CGC(T/C)GTGCT R: AGCCAATCAAC(A/C)A(A/G)CTGCTG(C/A)CGC	412
	SHV	F: TGTATTATCTC(C/T)CTGTTAGCC(A/G)CCCTG R: GCTCTGCTTTGTTATTCGGGCCAAGC	868
	TEM	F: TCGCCGCATACACTATTCTCAGAATGAC R: CAGCAATAAACCAGCCAGCCGGAAG	1100
Class B	VIM	F: GATGGTGTTTGGTCGCATATCGCAAC R: CGAATGCGCAGCACCAGGATAGAA	500
	IPM	F: AC(G/A)GG(C/G/T)GGAATAGAGTGGCTTAA(T/C)TCTC R: TTCAGG(C/T)A(A/G)CCAAACYACTASGTTATCT	432
	NDM	F: CGAAAGTCAGGCTGTGTTGCGC R: GACCGCCCAGATCCTCAACTG	475
Class C	FOX	F: AACATGGGGTATCAGGGAGATG R: CAAAGCGCGTAACCGGATTGG	190
Class D	OXA-10	F: ATGGTGTCTTCGTGCTTT R: TCTTACTTCGCCAACTTCT	564
	OXA-24	F: GTACTAATCAAAGTTGTGAA R: TTCCCCTAACATGAATTTGT	246
	OXA-58	F: CCCCTCTGCGCTCTACATACAACATC R: AAGTATTGGGGCTTGTGCTGAGCATAG	599

Table 2. Distribution of the bacterial isolates recovered from the different clinical samples

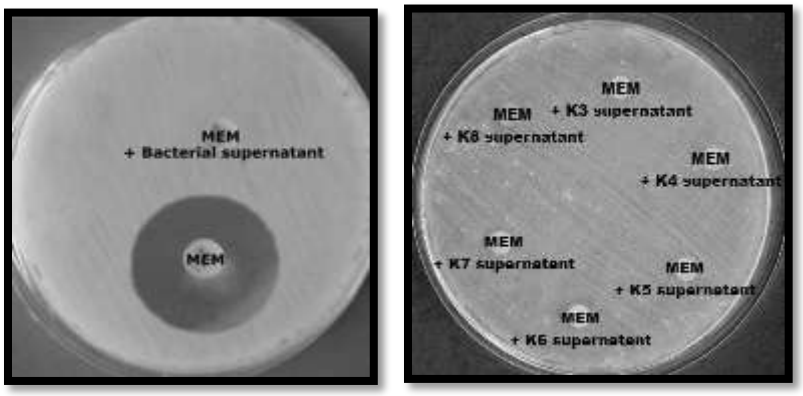
Name of the bacterial isolates		Number of isolates (%)	Number and percentage (%) of the isolation sources		
			Blood	Urine	BAL
	C. albicans	22.0 (11.89)	7.0 (3.78)	10.0 (5.40)	5.0 (2.70)
Gram-negative	***Acinetobacter* sp.**	9.0 (4.86)	4.0 (2.16)	0.0 (0)	5.0 (2.70)
	***Citrobacter* sp.**	1.0 (0.54)	1.0 (0.54)	0.0 (0)	0.0 (0)
	E. coli	10.0 (5.40)	7.0 (3.78)	2.0 (1.08)	1.0 (0.54)
	K. pneumonia	95.0 (51.35)	66.0 (35.68)	5.0 (2.3)	24.0 (12.97)
	P. aeruginosa	12.0 (6.49)	0.0 (0)	3.0 (1.4)	9.0 (4.68)
	***Stenotrophomonas* sp.**	1.0 (0.54)	1.0 (0.54)	0.0 (0)	0.0 (0)
	Total Gram-negative	128.0 (69.18)	79.0 (42.70)	10.0 (5.40)	39.0 (21.08)
Gram-positive	**CONS**	14.0 (7.57)	10.0 (5.40)	0.0 (0)	4.0 (2.16)
	E. faecalis	10.0 (5.40)	6.0 (3.24)	4.0 (2.16)	0.0 (0)
	MRSA	7.0 (3.78)	6.0 (3.24)	0.0 (0)	1.0 (0.54)
	S. aureus	1.0 (0.54)	1.0 (0.54)	0.0 (0)	0.0 (0)
	S. pneumoniae	1.0 (0.54)	0.0 (0)	0.0 (0)	1.0 (0.54)
	S. viridans	2.0 (1.08)	1.0 (0.54)	0.0 (0)	1.0 (0.54)
	Total Gram-positive	35.0 (18.91)	24.0 (12.97)	4.0 (2.16)	7.0 (3.78)
	Total	185.0	110.0 (59.46)	24.0 (12.97)	51.0 (27.57)

-Results are averages of three replicates. Where; CONS: Coagulase negative *Staphylococcus*, MRSA: Multi drug resistant *Staphylococcus aureus*, BAL: Broncho-alveolar lavage

Table 3. Antibiogram of all the isolated *K. pneumoniae* strains

Antibiotic group	Antibiotic	Number and resistance percentage (%)				
		Sensitive strains	Intermediately sensitive strains	Resistant strains	R*	S*
Aminopenicillins	**Ampicillin**	0.0 (0)	0.0 (0)	95.0 (100)	<13	>17
1st generation cephalosporins	**Cefazolin**	0.0 (0)	0.0 (0)	95.0 (100)	<19	>23
Aminoglycosides	**Tobramycin**	0.0 (0)	0.0 (0)	95.0 (100)	<12	>15
	Amikacin	21.0 (22.1)	27.0 (28.4)	47.0 (49.4)	<14	>17
Ureidopenicillins	**Pipracillin**	0.0 (0)	0.0 (0)	95.0 (100)	<17	>21
β-lactams-β-lactamase inhibitor	**Amoxacillin/ Clavulanate**	3.0 (3.1)	18.0 (18.9)	74.0 (77.8)	<13	>18
	Ampicillin/ Sulbactam	0.0 (0)	6.0 (6.3)	89.0 (93.6)	>12	>15
	Pipracillin/ Tazobactam	9.0 (9.4)	9.0 (9.4)	77.0 (81)	<17	>21
	Cefoperazone/ Sulbactam	0.0 (0)	15.0 (15.7)	80.0 (84.2)	<15	>21
2nd generation cephalosporins	**Cefoxitin**	39.0 (41)	9.0 (9.4)	47.0 (49.4)	<14	>18
3rd generation cephalosporins	**Cefotaxime**	0.0 (0)	6.0 (6.3)	89.0 (93.6)	<14	>23
	Cefoperazone	0.0 (0)	3.0 (3.1)	92.0 (96.8)	<15	>21
	Ceftriaxone	0.0 (0)	3.0 (3.1)	92.0 (96.8)	<19	>23
	Ceftazidime	0.0 (0)	6.0 (6.3)	89.0 (93.6)	<17	>21
Carbapenems	**Meropenem**	21.0 (22.1)	21.0 (22.1)	53.0 (55.7)	<19	>23
Quinolones	**Ciprofloxacin**	3.0 (3.1)	9.0 (9.4)	83.0 (87.3)	<20	>31
	Trimethoprim-Sulfamethoxazole	0.0 (0)	15.0 (15.7)	80.0 (84.2)	<10	>16
Monobactams	**Aztreonam**	0.0 (0)	0.0 (0)	95.0 (100)	<17	>21
Phenicols	**Cholramphenicol**	51.0 (53.6)	18.0 (18.9)	26.0 (27.3)	<12	>18
Tetracyclines	**Doxycycline**	6.0 (6.3)	9.0 (9.4)	80.0 (84.2)	<10	>14
Polymyxins	**Colistin**	9.0 (9.4)	9.0 (9.4)	77.0 (81)	<11	>14

-Results are averages of three replicates. Where; *According to CLSI. (2015), R: Resistant, S: Sensitive, NRS: Number of Resistant Strains, NIS: Number of Intermediate Strains, NSS: Number of Sensitive Strains, %: Percentage of strain resistance to each listed antibiotic



(a) **(b)**

Fig. 1. Sensitivity of *E. coli* ATCC 25955 (a) and *K. pneumoniae* strains (b) to meropenem antibiotic

3.3. Detection of the Extended-Spectrum β-lactamase in *K. pneumonia* using (DDST) and CLSI confirmatory assays

In a trail to recognize the ESBL producing *K. pneumonia* strains, the double disc synergy (DDST) and CLSI examinations were used. Results in Fig. (2 a, b) demonstrated that 27 and 30 out of the 95 of isolated *K. pneumoniae* strains are ESBL producers using the DDST, while the CLSI confirmatory assays presenting; 28.4 and 31.5%, respectively.

3.4. Screening for the AmpC β-lactamase production

In the current study, 14 out of 30 (46.6%) of ESBL positive *K. pneumoniae* strains are considered as AmpC β-lactamase producing bacteria as shown in Fig. (2c).

3.5. Phenotypic detection of carbapenemases production by *K. pneumoniae*

In a trial to detect carpabenmase production by the bacteria under investigation, a confirmatory test known as Modified Carbapenem Inactivation Method (mCIM) (Virginia *et al*., 2017) was evaluated. Results presented in Fig. (3) revealed that all the 30 strains of the tested bacteria are Extended spectrum β-lactamase (ESBL) positive, and are carpabenemase producers. About 9 out of the 30 ESBL *K. pneumoniae* strains are proved to be carbapenem-resistant *Klebsiella pneumoniae* (CRKP) positive. The MHT is considered positive, because the *E. coli* 25922 strain showed a clover leaf-like indentation along with *K. pneumoniae* growth within the meropenem diffusion inhibition zone.

Fig. 2. Detection of ESBL *Klebsiella pneumoniae* using DDST (a), CLSI confirmatory test (b), and AmpC β-lactamase (c)

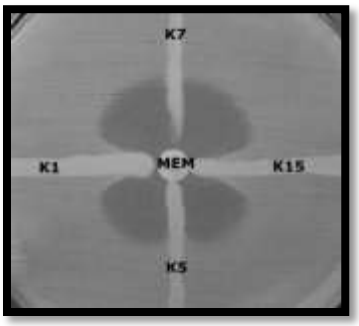


Fig. 3. Detection of carbapenemase production by *K. pneumoniae* strains (K1, K5, K7 and K15) using the Modified Hodge test (MHT)

3.6. Screening for the β-lactamase genes in the *K. pneumoniae* strains using PCR

The PCR technique was used to amplify the genes of the 9 selected *K. pneumoniae* strains, where strain 1- 9 proved to be Multi drug resistant (MDR), ESBL and CRKP, and were compared to the reference strains (ESBL +ve 1705, ESBL -ve 1706, and bacteria with ATCC 13883) (Fig. 4-7). It is interesting to note that the TEM and BETA genes are detected on the plasmid in all the 9 tested strains of *K. pneumoniae*. However the percentage of their presence on the DNA varied among the *K. pneumoniae* strains (TEM and BETA, 44.44 and 33.33%, respectively). Results revealed that NDM and IPM genes are present (100%) on the plasmid in all the tested *K. pneumoniae* strains. However their percentages are variable i.e. VIM and NDM are present in 11.11 and 66.66% on the DNA, respectively. The presence of the FOX gene on the DNA and the plasmid of all tested strains are variable (11.11 and 55.55%, respectively). The OXA-10 and OXA-24 genes are variably detected on the plasmid (22.22 and 11.11%, respectively). The current results demonstrated that the ICU's patients harbored most of the carbapenem-resistant strains, with the highest percentage of carbapenemases resistance genes are detected in *K. pneumoniae*.

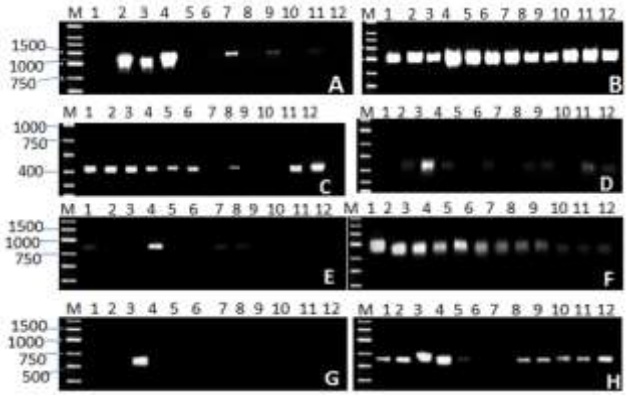


Fig. 4. Agarose gel electrophoresis showing: PCR detection of TEM on DNA (A) and plasmid (B), KPC on DNA (C) and plasmid (D), SHV on DNA (E) and plasmid (F), BETA on DNA (G) and plasmid (H)

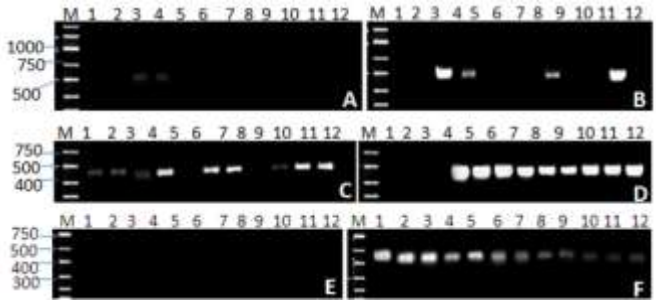


Fig. 5. Agarose gel electrophoresis showing: PCR detection for VIM on DNA (A) and plasmid (B), NDM on DNA (C) and plasmid (D), IPM on DNA (E) and plasmid (F)

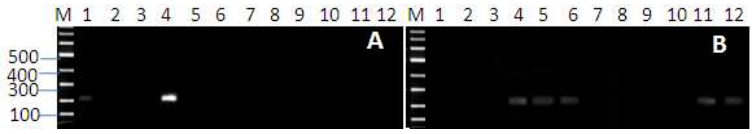


Fig. 6. Agarose gel electrophoresis showing: PCR detection of FOX gene on DNA (A) and plasmid (B).

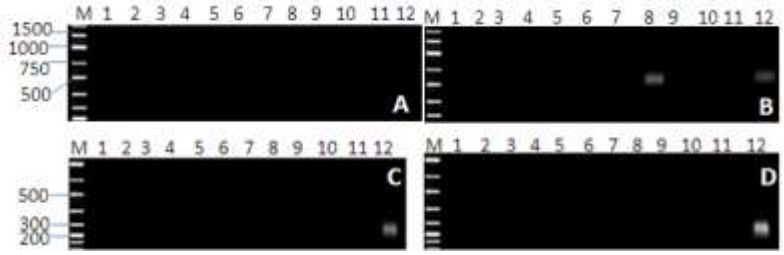


Fig. 7. Agarose gel electrophoresis showing: PCR detection of OXA-10 gene on DNA (A) and plasmid (B), OXA-24 on DNA (C) and plasmid (D).

4. Discussion

Recently, Labib *et al*. (2018) reported that 291 isolates of Gram-negative bacteria out of 1420 patients admitted to the PICU with a mortality rate of 37.1% were detected in 2 PICUs at the Cairo University Hospitals, Egypt. However, *Klebsiella* sp. (36.0%) was the most frequently isolated microorganism. Similarly, Amer *et al*. (2017) detected the distribution of carbapenem resistance in a survey study within the different patient's samples (BAL, blood, CVP and skin and soft tissues). The recorded percentage of carbapenem resistance distribution in the *K. pneumoniae* strains was 39%. Previously, Ejaz, (2013) illustrated that in a total number of 710 *K. pneumoniae*; only 214 (30.1%) were ESBL positive, while Saied *et al*., (2011) revealed that ESBL was detected in 79% of the *K. pneumonia*, and 39% of *E. coli* using the DDST test.

In a comparative study for detection of the accuracy between DDST and CLSI confirmatory tests, Amin *et al*., (2013) reported that 25 (75.75%) out of 33 isolates were identified by the DDST, whereas, 33 (100%) were identified using the CLSI confirmatory assay. Gupta *et al*., (2014) reported that AmpC β-lactamase detection may be difficult. In the study of Amjad *et al*., (2011), 138 isolates out of 200 were carbapenemase positive detected by using Modified Hodge test, and were distributed as follows: *E. coli* (38%), *P. aeruginosa* (30%), *K. pneumoniae* (17%).

The TEM-type gene has an average Mw of 1100 bp, while SHV-gene has 868 bp as reported by Polse *et al*., (2016). However, Trung *et al*., (2015) reported that KPC gene has an average Mw of 412 bp, while the BETA gene (686) detection was done by using a specific primer designed according to *Klebsiella* sp. information documented in the GenBank. On the other hand, the VIM-, NDM- and IMP-type genes have an average Mw of 500, 475 and 432 bp; respectively, which were similar to the findings of Shanthi *et al*., (2014). The FOX-type gene has an average Mw of 190 bp, which was similar to the molecular weight recorded by Sorour *et al*., (2008). Similar to the current findings, Najar *et al*., (2013) reported that OXA-24 and OXA-58 genes have average Mw of 564 and 246 bp, respectively.

Conclusion

The current results of incidences of the ESBL and CRKP of *K. pneumoniae* in the ICU's at Al-Shatby Pediatric Hospital, Alexandria, Egypt, may pave the way for a surveillance program to monitor the resistance patterns of this pathogenic bacterium, thus

provide a clear vision towards the correct treatment options.

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Conflict of interest

No conflict of interests exists between the authors of this study.

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