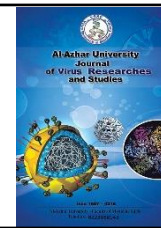




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Genotypic and Phenotypic Screening of Carbapenemase Mediated Resistance in *Klebsiella pneumoniae* Clinical Isolates: A Hospital Based Study.

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Abstract

Carbapenem-resistant *Klebsiella pneumoniae* (CRKP) has been reported increasingly all over the world and it is usually resistant to multiple antimicrobial agents. Is to detect the frequency and distribution of carbapenemase genes in CRKP at Al-Zahraa University hospital, Cairo, Egypt, and to compare between the performance of Rapidec Carba NP and modified carbapenemase inactivation (mCIM) phenotypic methods for early and reliable recovery of carbapenemase production in *Klebsiella pneumoniae* (KP) clinical isolates. Sixty *K. pneumoniae* isolates were recovered in our Microbiology laboratory. Carbapenemase production by these isolates was assessed using antimicrobial sensitivity testing, Rapidec Carba NP, modified carbapenemase inactivation methods (mCIM) and detection of carbapenemase genes by PCR. CRKP was implicated in several types of healthcare associated infections with frequency of 46.7 (28/60). The frequency of carbapenemase genes was 13 (54.2) for blaNDM, 7 (29.2%) for blaOXA-48 and 1(4.1%) for blaKPC. 3 (12.5%) isolates carried both blaNDM and blaOXA-48. All isolates were negative for blaIMP, the sensitivity and specificity of Rapidec Carba NP test were (100%) and (91.7%) respectively, while sensitivity and specificity of mCIM were (66.7%) and (88.9 %) respectively, in comparison to the PCR test which is used as the gold standard test. The Carbapenem resistance is frequently encountered in *K. pneumoniae* isolates at our hospital. Rapidec carba NP and mCIM tests have high performance and applicability in detection of carbapenemases. However, The Rapidec carba NP was found to be rapid and with higher sensitivity.

Keywords: *Klebsiella pneumoniae*, Carbapenem-resistance, Rapidec Carba NP, Carbapenemes Inactivation Method, Carbapenemase genes.

1. Introduction

Klebsiella pneumoniae (*K. pneumoniae*), is a significant frequently encountered nosocomial pathogen. Multidrug-resistant *K. pneumoniae* (MDRKP) is considered a dangerous and urgent threat in clinical settings [1]. Carbapenems were considered a reliable and effective antibiotic against *K. pneumoniae*, but during the last 10 years, carbapenemase-producing *K. pneumoniae* (CPKP) incidence was dramatically elevated worldwide [2]. The Ambler carbapenemase classes A (KPC), B (VIM, IMP, NDM) and D (OXA-48) are the most significant carbapenemases in clinical settings. They are frequently detected in *K. pneumoniae* isolates associated with life threatening hospital acquired infections [3].

Consistent and rapid identification of CRKP is necessary for therapeutic management of patients, for antibiotic stewardship practice and for infection control to ban the dispersal of these multidrug-resistant (MDR) bacteria [4,5].

Numerous procedures are present for rapid detection of CRKP as immunochromatographic assays, molecular techniques and matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) technology, but these techniques are not routinely available in microbiology laboratory and also require significant expertise and high cost [6,7].

Carba NP test was suggested by the Clinical Laboratory Standard Institute (CLSI), 2012 for the recovery of carbapenemases in Gram-negative bacilli, but the reagent preparation required for the in-house test is sophisticated and the reagents are not stable for long duration, decreasing its clinical applicability [8]. Rapidec carba NP test is a commercially available carba NP kit that was designed for speedy detection of different carbapenemases [9].

In 2017, CLSI has suggested the modified carbapenem inactivation method (mCIM) for the recovery of carbapenemases in *P. aeruginosa* and *Enterobacteriaceae* [8].

The purpose of our study is to identify the frequency of CRKP in our hospital setting, their carbapenemase gene profile and to compare the performance of Rapidec Carba NP and modified carbapenem inactivation (mCIM) phenotypic methods to settle a screening phenotypic tool for detection of these isolates

2. Materials and Methods

2.1 Study design:

This is a hospital based cross-sectional study that was done on sixty isolates of *K. pneumoniae*, isolated from 315 clinical specimens provided to Microbiology laboratory of Al-Zahraa University Hospital, Cairo, Egypt for routine culture and antibiotic sensitivity testing in the period from March to August 2020.

This study has been approved by the Research Ethics Committee of the Faculty of Medicine for Girls, AL-Azhar University, Cairo, Egypt (approval no. 20192287) and a waving from obtaining an informed patient consent was approved by the Committee.

2.2 Phenotypic identification of isolates and detection of carbapenem resistance:

2.2.1 Microbiological identification

Isolates have been identified as *K. pneumoniae* depending on Gram staining, characteristic colonial morphology, and routine biochemical reactions.

2.2.2 Antimicrobial sensitivity Testing:

Antibiotic sensitivity testing was done implementing the Kirby-Baur disk diffusion method in accordance with the CLSI [10]. The *K. pneumoniae* isolates were tested against ampicillin (10µg), ampicillin/sulbactam (10/10µg), amoxicillin/clavunate (20/10µg),

piperacillin (100µg), piperacillin-tazobactam (100/10µg), cefoperazone (75µg), ceftazidime (30µg), cefepime (30µg), cefotaxime (30µg), norfloxacin (10µg), ciprofloxacin (5µg), ceftaxime (30µg), amikacin (30µg), gentamicin (10µg), meropenem (10µg), and ertapenem (10µg). CRKP was determined as isolated *K. pneumoniae* strains with resistance to at least one of the carbapenem drugs. As a quality control, the *K. pneumoniae* ATCC700603 strain was used for the antibiotic sensitivity testing.

2.2.3 Double disk synergy method for confirmation of production of extended spectrum:

beta lactamase (ESBL): the resistant isolates to ceftazidime and or cefotaxime using disk diffusion technique were confirmed for ESBL production by the double disk synergy testing using cefotaxime /ceftazidime/clavulanic acid inhibitory disk. It was considered positive for ESBL if there is extension of the edge of the inhibition zone of ceftazidime/cefotaxime on the side exposed to the disc containing clavulanic acid [11].

2.2.4 AmpC disk test:

According to disk diffusion results, isolates with ceftaxime resistance were confirmed for ampC production by ampC disk test [12].

2.2.5 Rapid detection of carbapenemase activity by RAPIDEC CARBA NP test.

This assay was done as instructed by the manufacturer (bioMèrieux SAMarcy Etoile France, Lot No. 1005258372). Briefly, a loop full of bacterial colonies was taken from an overnight-incubated Muller Hinton agar Petri dish then mixed with API suspension medium given with the kit. Then the microbial suspensions were inoculated into wells in the provided test stripe and then incubated at 37°C. Reading of the results was carried on within 30- 40 min. A negative result was

indicated by a red color, while a positive test showed a color change from red to yellow orange.

2.2.6 Modified Carbapenem Inactivation Method (mCIM)

A suspension was prepared using one loop full of each isolate in 2 ml tryptic soy broth (Oxoid, England). Then, a sensitivity testing disk containing 10 µg meropenem (Oxoid, England) was placed in the bacterial suspension and then incubated for 4 hours at 37°C. Then the disk was removed from the bacterial suspension and placed on an agar plate of Mueller Hinton that was inoculated with a susceptible *E. coli* indicator strain (ATCC 25922) followed by incubation at 37°C. If the bacterial isolate produced carbapenemase, then the meropenem was inactivated while the carbapenemase nonproducing isolate yielded a clear inhibition zone [10].

2.3 Carbapenemase genes detection by PCR:

2.3.1. Extraction of DNA:

DNA extraction was done by heat shock technique in which fresh culture of the test organism was suspended in 200 ml of sterilized distilled H₂O and mixed by a vortex to acquire a homogenous suspension. Then the cells were lysed by incubation in a water bath at 100°C for seven minutes followed by placing in ice for two minutes. Then centrifugation at 10000 rpm was done for five minutes for removal of cellular debris. Finally, the supernatant containing the DNA was aspirated and stored at -70°C to be used for amplification [13].

2.3.2 PCR amplification:

Amplification of blaKPC, blaNDM, blaIMP, and blaOXA-48 genes was performed by PCR technique. The total reaction volume was 25 µl containing 8.5 µl distilled water, 1 µl of each primer (Invitrogen, Germany) at a concentration

of 20 picomolar, 12.5 µl of Taq Master Mix Kit (QIAGEN Cat. No.201203), and 2 µl of the extracted DNA. Primers were prepared according to manufacturer's instructions. The specific forward and reverse primers for the amplification of blaKPC, blaNDM, blaIMP and blaOXA-48 genes are presented in Table. 1.

2.4 Statistical analysis:

The data were input in Microsoft Excel program, analyzed by SPSS (version 18) and the results were presented in charts and tables. Chi-square test was used to compare proportions among two qualitative parameters.

3. Results

The distribution of CRKP and CSKP in relation to the type of clinical specimens and the antimicrobial sensitivity pattern of the studied isolates are described in Table. 2 and Table. 3 respectively.

3.1 Phenotypic and Genotypic detection of carbapenemases:

Rapidec Carba NP test, mCIM and PCR were performed on the 60 isolates. Carbapenemase-producers were 27 (45%) isolates by Rapidec Carba NP test and 20 (33.3%) by mCIM test while 24 (40%) isolates were positive by PCR for carbapenemase genes as shown in Table. 4 and Fig.1.

Among the 24 PCR positive isolates, one (4.1%) isolate had blaKPC gene, 13 (54.2%) isolates were positive for blaNDM, 7 (29.2%) of isolates were positive for blaOXA-48 and 3 (12.5%) isolates were positive for both blaNDM and blaOXA48. All isolates were negative for blaIMP. The performance of phenotypic tests (Rapidec test, mCIM) in comparison to PCR is demonstrated in Table. 5.

Table (1): The primer sequences used for carbapenemase gene detection by PCR Al-Zahrani and Alasiri (2018) and Zarakolu et al., 2016 [14,15].

Gene	Primer	5'-sequence-3'	Amplicon size
blaKPC	KPC F	5'-CGTCTAGTTCTGCTGTCTTG-3'	798 bp
	KPC R	5'-CTTGTCATCCTTGTTAGGCG-3'	
blaNDM	NDM F	5'-GGGCAGTCGCTTCCAACGGT-3'	475 bp
	NDM R	5'-GTAGTGCTCAGTGTCGGC-3'	
blaOXA-48	OXA-48 F	5'-TGTTTTTGGTGGCATCGAT-3'	300 bp
	OXA-48 R	5'-GATCGCGATTCCAAGTGG-3'	
blaIMP	IMP-F	5'GGAATAGAGTGGCTTAAYTCTC-3'	188 bp
	IMP-R	5'-CCAAACYACTASGTTATCT-3'	

Table (2): The distribution of CRKP and CSKP in relation to the type of clinical specimens. CS= Carbapenem sensitive, CR= Carbapenem resistant, S= Significant (P value <0.05), NS= non-significant (p value >0.05).

	Total isolates	No. & % of CS	No. & % of CR	Test value	P-value	Sig.
	No. = 60	No. = 32	No. = 28			
Urine	41 (68.3%)	18 (56.3%)	23 (82.1%)	4.627	0.031	S
Sputum	10 (16.7%)	6 (18.8%)	4 (14.3%)	0.214	0.643	NS
Wound swab	4 (6.7%)	3 (9.4%)	1 (3.6%)	0.808	0.368	NS
Pus swabs	2 (3.3%)	2 (6.3%)	0 (0.0%)	1.810	0.178	NS
Blood	1 (1.7%)	1 (3.1%)	0 (0.0%)	0.890	0.345	NS
Peritoneal fluid	2 (3.3%)	2 (6.3%)	0 (0.0%)	1.810	0.178	NS

Table (3): Antimicrobial resistance pattern of the studied *K. pneumoniae* isolates. ESBL: Extended spectrum beta lactamases CSKP= Carbapenem-sensitive *Klebsiella pneumoniae*, CRKP= Carbapenem-resistant *Klebsiella pneumoniae*. MDR: multidrug resistant, ampC: ampler class C.

Resistance	CRKP (28)	CSKP (32)	P value	Significance
ESBL	26 (92.9%)	12 (37.5%)	19.705	<0.001
ampC	4 (14.3%)	-----	4.898	0.027
Ciprofloxacin	28 (100%)	18 (56.3%)	12.857	<0.001
Norfloxacin	27 (96.4%)	10 (31.3%)	27.265	<0.001
Gentamicin	25 (89.3%)	7 (21.9%)	23.907	<0.001
Amikacin	22 (78.6%)	5 (15.6%)	29.921	<0.001
MDR	28 (100%)	15 (46.9%)	14.725	<0.001

Table (4): Detection of carbapenemase production among the studied *K. pneumoniae* isolates by Rapidec carba NP test, mCIM test and PCR. *CRKP*= Carbapenem-resistant *k. pneumoniae*, *CSKP*= Carbapenem-sensitive *k. pneumoniae*, *mCIM*=modified carbapenem inhibition method.

Tested isolates	Rapidec Carba NP		mCIM		PCR	
	Positive	negative	positive	negative	positive	negative
CRKP (28)	27 (96.4%)	1(3.6%)	20 (71.4%)	8(28.6)	24 (85.7%)	4(14.3%)
CSKP (32)	0	32(100%)	0	32(100%)	0	32(100%)
Total (60)	27	33	20	40	24	36

Table (5): The performance of phenotypic tests (Rapidec test, mCIM) for detection of carbapenemase genes in comparison to PCR. *TN*= true negative, *TP*=true positive, *FN*=false negative, *FP*=false positive, *NPV*= negative predictive value = *PPV*, positive predictive value, *mCIM*=modified carbapenem inhibition method

Parameter	TP	TN	FP	FN	Accuracy	Sensitivity	Specificity	PPV	NPV
Rapidec Carba NP	24	32	4	0	95 %	100 %	91.7 %	88.8 %	100 %
mCIM	16	32	4	8	80 %	66.7 %	88.9 %	80 %	80 %

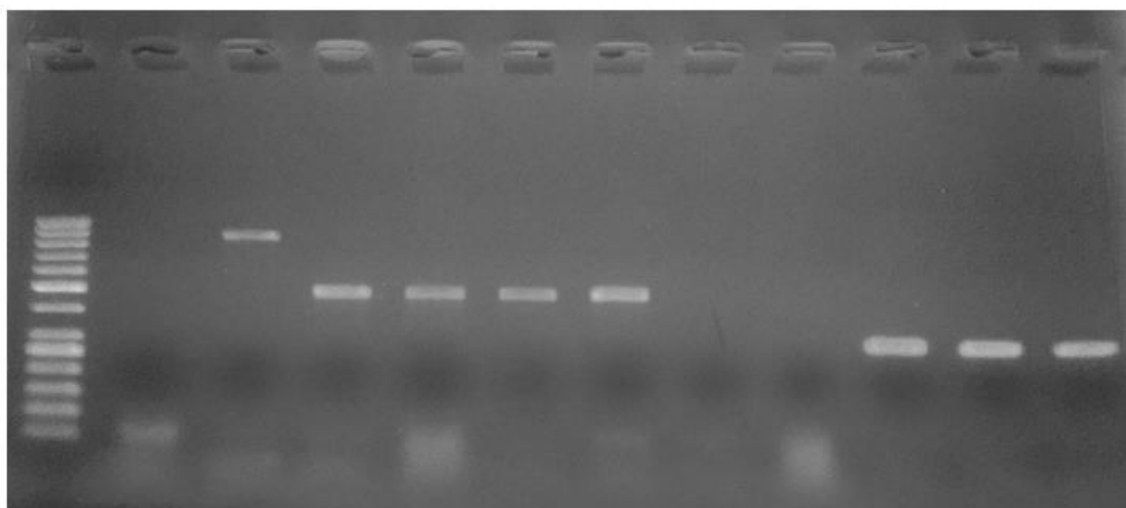


Figure (1): Carbapenemase genes detection in *K. pneumoniae* isolates by PCR. Lane 1: molecular weight ladder (50 bp), Lane 2: control negative, Lane 3: blaKPC positive isolate (798 bp), Lanes 4, 5, 6 and 7: blaNDM positive isolates (475 bp) Lanes 8 and 9: negative isolates, Lane 10, 11 and 12: blaOXA-48 positive isolates (300 bp).

4. Discussion

Carbapenemase- producing *K pneumoniae* (CPKP) has been an important issue in terms of public health and clinical outcome as it is usually resistant to almost all antimicrobial agents and due to its rapid worldwide spread [16,17]. The CRKP prevalence is variable between countries and even between medical health care settings in the same country. In our study, 46.7% (28/60) of isolated *k. pneumoniae* were carbapenem-resistant.

Carbapenem resistance in Egypt, is alarming, as it was identified in 44.3% of *K. pneumoniae* isolates in the hospitals of Suez Canal University, which is similar to its prevalence in our hospital [18]. However, lower prevalence rates of (33%) and (13.9%) were reported at Mansoura university hospitals and at the Egyptian National Cancer Institute respectively [19,20]. The high frequency of CRKP in this study may be due to the frequent use of carbapenems empirically at our hospital.

In the present study, carbapenem-resistant *K pneumoniae* were more prevalent in urine (82%), followed by sputum specimens (14%). Li et al., 2019 reported that *K pneumoniae* was isolated from respiratory specimens (62.9%) followed by blood [21]. However, they found no significant differences between CRKP and CSKP as regard the source of samples. In an Egyptian study, respiratory specimens (62%) were the major source of CRKP, followed by urine samples (14%) [19].

All 28 CRKP isolates in the present study were MDR, 93% were ESBL producers, while 14.3% were ampC producers. This significant association of carbapenem-resistance with multidrug resistance among our *K. pneumoniae* studied isolates was also reported in several studies. Li et al., 2019 demonstrated that the tested antibiotics in the group of CSKP showed better sensitivity (65%) than in CRKP, except for ampicillin-sulbactam as it was

(55.9%) while for piperacillin it was (55.9%) [21]. It was reported that carbapenem-resistant isolates often carry more resistance determinants and shows complex phenotypes of MDR. Moreover, higher-level of carbapenem-resistance are regularly accompanied with expression of mobile genetic elements within carbapenemase genes [22].

Reliable and rapid identification of carbapenemases is crucial for development of infection control strategies. To date, phenotypic methods are the tools available in the routine Microbiology laboratory. In this study, we assessed the performance of two phenotypic techniques for detection of carbapenemase in *K. pneumoniae* and their applicability in the clinical Microbiology laboratory. The RAPIDEC® CARBA NP assay is a fast phenotypic test that does not require expensive devices or special expertise, furthermore, it is considered to have a low cost in relation to molecular tests [22].

This study revealed that all isolates of *K. pneumoniae* carrying carbapenemase genes were detected by Rapidec carba NP test with (100 %) sensitivity. This was in agreement with results obtained by Dortet et al. (2015) who reported a sensitivity of 99 % among Enterobacteriaceae isolates [23].

Lower sensitivities were reported in several studies [6, 22, 24, 25]. However, in these studies the lower sensitivities were attributed to greater proportion of class D producing *A. baumannii* complex among the tested isolates which were not included in our research and when the sensitivity was calculated only for *Enterobacteriaceae* isolates, it raised from 70 % to 100% [22]. In this study, the Rapidec Carba NP test specificity was 91.7 %. Higher specificities (98 % and 100 %) were reported in other studies [22,7]. However, the false-positive results observed in our study were also reported elsewhere [6,26,27], and may be

attributed to a decreased stability of the imipenem substrate toward strains producing enzymes with weak carbapenemase activity but are not true carbapenemases (e.g., AmpCs, CMY, CTX-M-type producers) and may be due to using the recommended inoculum in the Producer's directives. Dortet et al. (2015) discovered a serious effect of the bacterial inoculum in the test performance and suggested using a greater amount of the inoculum to decrease false positive results [23].

The mCIM is a straightforward and cost-effective test available in many clinical laboratories and is recommended by CLSI 2018 for detection of carbapenemases. The present study showed that the sensitivity and specificity of this assay for detection of carbapenemase production in *K. pneumonia* were (66.7%) and (88.9 %) respectively. The test failed to detect the blaKPC producing isolate and all blaNDM positive isolates.

Better performance of mCIM was achieved in other studies, (Aktaş et al., 2017) reported 100% sensitivity and specificity when evaluating the test using *Enterobacteriaceae* isolates [7]. Tijet et al., 2015 compared the performance of both carba NP test and mCIM and reported better sensitivity and specificity of mCIM which is inconsistent with our results [28]. However, the authors in that study used the carba NP test and not our commercially available Rapidec Carba NP test which may have better performance.

In our study, again, the four isolates that were falsely positive by Rapidec carba NP test, were falsely positive upon testing with mCIM. In a study by (Jing et al., 2018), false positive result in a *K. pneumoniae* strain making CTX-15 was reported [8]. Similarly, Pierce et al. (2017) stated that TEM-1 and TEM-52 enzymes may produce false positive results in mCIM tests [29].

Molecular techniques remain the gold standard that are reliable for detection of carbapenemases-producing bacteria more than phenotypic methods. However, they

have some limitations as the results may be false-negative due to mutations affecting annealing of primers or the occurrence of a carbapenemase gene that is not tested for in the PCR test or it may give false positive results as in cases of detecting silent genes (i.e. no expression of carbapenemase) [25]. In the present study, carbapenemase coding genes in *K. pneumonia* isolates were detected in 24 out of the 28 (85.7%) carbapenem resistant isolates, while in the four isolates none of the tested carbapenemase genes (blaIMP, blaKPC, blaOXA-48, and blaNDM) were found. These four isolates were positive by both Rapidec carba NP and mCIM methods. Carbapenem- resistance shown in these isolates may be due to a presence of both ESBLs and outer membrane proteins changes (ESBL/Omp) or may be mediated by other carbapenemase genes that were not tested in our study.

This is the first study that reported the frequency and distribution pattern of carbapenemase genes in CRKP clinical isolates in our hospital. In our study, the most prevalent gene among the 24 carbapenemase positive *K. pneumoniae* isolates was blaNDM 13/24 (54.2) % which differs from a previous study in Egypt, where blaOXA-48-like types were the most predominant (28.6%) while 2.4% only of CRKP carried bla NDM [30]. In another Egyptian study, the most prevalent gene among the 42 CRKP isolates tested was blaKPC at 43.5% while blaNDM-1 genes was detected in only 4.3% [19].

In our study, one (1.6%) isolate of *K. pneumoniae*, identified as blaKPC producer. This low frequency was also reported in Saudi Arabia, where no blaKPC producers were detected among isolates investigated [14]. While a much higher prevalence (89 %) was reported in another study [31].

In the present study, the blaOXA-48 producer's prevalence among CRKP was 29.2% (7/24). While other studies identified blaOXA48 as the most prevalent carbapenemase [32]. Also, a much higher

prevalence was reported by (Al-Zahrani and Alasiri, 2018) who reported that (81.5%) isolates of *K. pneumoniae* were harboring blaOXA-48 [14].

The presence of several carbapenemase genes in the same isolate raises the concern about the likelihood of multiple carbapenemase genes crossing onto the same plasmid. This study detected three isolates (12.5%) that were positive for both blaNDM and blaOXA-48. Another study showed that the first isolate of *K. pneumoniae* carrying both blaOXA-48 and blaNDM-1 in Turkey was isolated from a patient living nearby the border of Syria [33]. Dual carbapenemase prevalence was fortunately lower (1.5%) in another study [34].

5. Conclusion:

In conclusion, both Rapidec carba NP and mCIM tests have a high performance and applicability in the carbapenemases detection. The Rapidec carba NP is a simple rapid test with high sensitivity but at a low cost than molecular methods. However, the mCIM method also represents a simple valuable tool for district

microbiology laboratories especially in low-income countries due to its simplicity, availability and cost effectiveness.

High rate of resistance to carbapenems is found in *K. pneumoniae* clinical isolates in our hospital. The most prevalent gene is blaNDM, and dual carbapenemase genes were detected, these findings necessitate the immediate implementation of antibiotic stewardship program and infection control strategies.

Further studies for profiling of all resistance gene determinants in the CRKP isolates is recommended to direct a proper antibiotic policy.

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