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Full Length Article

Prevalence and characterization of carbapenem-resistant *Klebsiella pneumoniae* isolated from intensive care units of Mansoura University hospitals

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1. Introduction

Klebsiella pneumoniae (*K. pneumoniae*) is a Gram negative, facultative anaerobic bacilli that has great potential for significant morbidity and mortality in acute care settings, particularly in immunocompromised patients [1]. Moreover, *K. pneumoniae* is one of the most common organisms showing multiple antibiotic resistance worldwide [2]. These bacteria easily acquire and transfer drug resistance genes through plasmids and transposons [3]. Acquisition of these genes leads to production of β-lactamases of which extended spectrum β-lactamases (ESBLs) are the most common [3]. ESBLs are capable to hydrolyze extended spectrum penicillins, cephalosporins and monobactams, leaving the carbapenem group of β-lactam antibiotics as the only choice for therapy, hence carbapenem antibiotics are used as a last resort to treat infections caused by these multidrug resistant organisms [4,5]. However, there has been emergence of carbapenem resistant *Enterobacteriaceae*, most commonly carbapenem resistant *K. pneumoniae* (CRKP), which have a worldwide prevalence [6], due to high antibiotic use, self-medication by patients and lack of implementation of antibiotic policies in hospitals [7]. Mechanisms described for carbapenem resistance include, production of different classes of carbapenemase, hyperproduction of AmpC β-lactamase with an outer membrane porin mutation, and production of ESBL with a porin mutation or drug efflux. Production of carbapenemases is the most commonly reported mechanism of carbapenem resistance in *K. pneumoniae* [8]. Carbapenemases are β-lactamase enzymes that are capable of hydrolyzing all beta-lactam antibiotics, including monobactams, extended spectrum cephalosporins and carbapenem [9]. The most common carbapenemases include Verona integron metallo-beta-lactamases types (VIM), imipenemase (IMP) types, *Klebsiella pneumoniae* carbapenemase (KPC), oxacillinase-48 (OXA-48), and New Delhi metallo-beta-lactamase-1 (NDM-1), encoded by carbapenem resistance determining genes *bla*VIM, *bla*IMP, *bla*KPC, *blaOXA-48-like*, and *bla*NDM, respectively [10]. Phenotypic assays are used to identify activity of carbapenemase while molecular assays have developed to identify carbapenemase encoding genes [10,11]. Analysis of hospital surveillance data by the Center for Disease Control and Prevention (CDC) suggested that 8% of all *Klebsiella* isolates are carbapenem resistant [12]. Other studies showed that it accounted for 5–24% of *Klebsiella* isolates identified in hospitalized patients [13]. In Egypt, carbapenem resistance is emerging and alarming, one study reported that carbapenem resistance was detected in 44.3% of *K. pneumoniae* isolates in Suez Canal university hospitals [14]. The detection of carbapenem resistance is essential for the proper choice of antibiotic therapy as well as infection control measures to prevent dissemination of resistant strains in hospital settings. Therefore we set out this study to determine the prevalence of carbapenem resistance and carbapenemase encoding genes among clinical *K. pneumoniae* isolates obtained from patients at intensive care units (ICUs) of Mansoura university hospitals (MUHs), taking in consideration that carbapenems are frequently used as an empiric therapy in ICUs at our institution.

2. Subjects and methods

2.1. Study design

This was a cross-sectional study, between January 2015 and March 2016. All patients admitted to different ICUs of MUHs and had confirmed infection by *K. pneumoniae*, were enrolled in this study. The ICU bed numbers range from 4 to 27, with a median of 10. This study was conducted with approval from the Medical Research Ethics Committee, Mansoura University.

2.2. Case definition

A patient with a culture positive for *K. pneumoniae* was deemed to have an infection if *K. pneumoniae* was isolated from a sterile site (e.g., blood, peritoneal fluid, cerebrospinal fluid, or pleural fluid) in combination with clinical signs and symptoms of infection. Pneumonia was diagnosed on the basis of clinical signs and symptoms (cough, dyspnea, fever), the appearance of infiltrate on chest radiography and heavy growth of organisms in purulent tracheal secretions or bronchoalveolar lavage fluid (>10^4 colony forming units/ml). These samples were examined after Gram staining for the detection and quantification of leukocytes and organisms.

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A diagnosis of urinary tract infection required the isolation of at least 10000 microorganisms/ml associated with at least two of the following: dysuria; frequency; and/or pyuria (>10 white blood cells per high power field) [15].

2.3. Sample collection and processing

Samples differed according to site of infection; they included blood, peritoneal fluid, urine, respiratory secretions, wound swabs, and cerebrospinal fluid. Only one isolate per patient was included. All collected samples were processed in the Microbiology Diagnostics and Infection Control Unit (MDICU), within 1–2 h of collection.

2.4. Isolation and identification of K. pneumoniae

Bacterial identification to the species level were carried out by colonial morphology on blood and MacConkey's agar plates (oxoid, UK), Gram stained films, biochemical reactions including oxidase, motility, indole, methyl red, voges-proskauer, citrate and urease tests and confirmed with API 20E (BioMerieux, Inc., Hazelwood, MO).

2.5. Antibiotic susceptibility testing

The disk diffusion method was employed to determine the susceptibility profile, carried out on a Muller-Hinton agar, as recommended by CLSI M100-S24 [16]. The following disks, provided by (Oxoid, UK) were used: imipenem (IPM) (10 μg), meropenem (MEM) (10 μg), ertapenem (ERT) (10 μg), amoxicillin/clavulanic acid (AMC) (30 μg), piperacillin/tazobactam (TZP) (110 μg), ceftazidime (CAZ) (30 μg), cefepime (FEP) (30 μg), ciprofloxacin (CIP) (10 μg), gentamicin (CN) (10 μg), amikacin (AK) (30 μg). CRKP was defined as *K. pneumoniae* isolates that test intermediate or resistant to ertapenem, according to breakpoints defined by the CLSI [16].

2.6. Detection of carbapenemase production

Carbapenemase production was confirmed by the following tests: a) Modified Hodge test (MHT): a 1:10 dilution of 0.5 McFarland standard suspension of *E. coli* ATCC 25922 was made (adding 0.5–4.5 ml of saline) and swab streaked all over the plate. Then, 10 μg ertapenem disk was placed in the center of the test area. The test isolate was streaked in a straight line from the disk to the edge of the plate. Interpretation of negative and positive tests was done according to CLSI, (2014) [16]. b) Boronic acid synergy test: was done by streaking 0.5 McFarland standard suspension of the test isolate on a plate. 10 μg ertapenem and 400 μg of phenylboronic Acid (PBA) disks were then placed on the inoculated plate 15 mm apart center to center, and incubated for 24 h. The presence of enhanced growth inhibition zone between the carbapenem disk and boronic acid disk was considered positive for KPC enzyme production [17] c) Ethylene diammine tetra acetic acid (EDTA) test: 0.5 McFarland standard suspension of the test isolate was spread on the surface of a Mueller Hinton Agar plate. Two 10 μg ertapenem disks were placed on the agar 15 mm apart center to center. 10 μl of 0.5 M EDTA was added to one of the ertapenem disk to get 750 μg concentration and incubated at 37 °C overnight. Increase of inhibition zone diameter of more than 5 mm in the disk potentiated with the EDTA was considered positive for metallo-β-lactamase production [18].

2.7. Detection of carbapenemase encoding genes

DNA extraction was performed by the boiling method using the CDC protocol [19]. Polymerase chain reaction (PCR) was carried out in a thermal cycler machine (MJ Research, Inc., USA). The genes *bla*IMP, *bla*VIM, *bla*KPC, *blaOXA-48-like* and *bla*NDM-1 were amplified using primers and conditions as described by Karuniawati et al. (2013) [7]. A volume of 1 μL of template DNA was added to a final volume of 25 μL PCR mixture comprising 12.5 μL of Taq PCR Master Mix (Fermentas, UK), including, 1× PCR buffer, 1.5 mmol/L MgCl2, 0.15 mmol/L dNTP, and 1.25 IU Taq DNA polymerase, 1 μL of 0.8 μmol/L each primer (except OXA 0.4 μl) and 9.5 μL of sterile distilled water. The primer pairs were tested in simplex PCR (only one gene screened, for blaNDM-1 gene) and with a multiplex approach. The amplicons were analyzed by electrophoresis in a 1.5% agarose gel.

2.8. Data analysis

Data analysis was done in STATA version 12. Out comes were presented as proportions and percentages in a tabular form.

3. Results

3.1. Patients and isolates characteristics

This study included 125 patient with confirmed *K. pneumoniae* infections, among which 42 had CRKP infections. CRKP isolates were obtained from 23 male and 19 female patients. Patients were generally elderly (median age was 60 years, with a range of 43–77 years). Respiratory samples (62%) was the predominant source of CRKP, followed by urine (14%), wound (9.5%), blood (9.5%), and catheter tip (5%) samples.

3.2. Susceptibility to antibiotics

Using current breakpoints recommended by CLSI (M100-S24) for carbapenem interpretation, 42 out of 125 (33.6%) *K. pneumoniae* isolates were nonsusceptible (intermediate and resistant) to ertapenem. 25.6% and 20% were nonsusceptible to meropenem and imipenem respectively. The full antibiogram for the tested drugs is seen in Table 1. Two isolates were resistant to all tested antibiotics (panresistant), however these isolates clinically respond well to the last-resort antimicrobials colistin and tigecycline.

3.3. Prevalence of carbapenemase activity based on phenotypic tests

Carbapenamase activity was detected in 26/42 (61.9%) by MHT method, 22/42 (52.4%) by Boronic acid screen and 5/42 (11.9%) by the EDTA test. 18 of the isolates were positive for both the MHT and Boronic acid methods. Overall, 35 (83.3%) of the 42 CRKP were positive for the production of one or more carbapenemases. Details of the carbapenemase activity among the isolates are shown in Table 2.

3.4. Prevalence and distribution of carbapenemase genes

Based on the PCR assays, 39/42 of the CRKP isolates were positive for one or more carbapenemase genes, while none of the carbapenemase genes tested (blaKPC, blaNDM, blaVIM-1, blaIMP, and blaOXA-48-like) were detected in three isolates tested. Furthermore, these three isolates did not phenotypically express a carbapenemase, according to the MHT, Boronic acid and EDTA tests. Carbapenem resistance of these isolates is likely due to a combination of ESBLs and changes in outer membrane proteins (ESBL/Omp) [20]. Five of the 39 (12.8%) carbapenemase gene carrying isolates harbored two or more genes. As shown in Table 3, the most prevalent gene was *bla*KPC 47.8% followed by *bla*VIM-1

Table 1
Susceptibility pattern of CRKP isolates.

Antibiotic	CRKP isolates (*n.* = 42)					
	Susceptible		Intermediate		Resistant	
	n.	%	*n.*	%	*n.*	%
Ertapenem	0	0	3	7.1	39	92.9
Meropenem	10	23.8	2	4.8	30	71.4
Imipenem	17	40.5	0	0	25	59.5
Cefuroxime	3	7.1	7	16.7	32	76.2
Ceftazidime	1	52.7	0	0	41	97.6
Cefotaxime	0	0	0	0	42	100
Cefepime	17	40.5	12	28.6	13	30.9
Piperacillin/tazobactam	0	0	1	2.4	41	97.6
Gentamicin	31	73.8	3	7.2	8	19.0
Amikacin	19	45.2	1	2.4	22	52.4
Ciprofloxacin	2	4.8	0	0	40	95.2

Table 2
CRKP isolates positive for phenotypic tests.

Test	CRKP isolates (*n.* = 42)	
	n.	%
MHT	26	61.9
Boronic acid	22	52.4
MHT and Boronic positive	18	42.9
EDTA	5	11.9
Total	35	83.3

Table 3
Distribution of carbapenemase encoding genes in the CRKP isolates.

Carbapenemase-encoding genes	Total genes	
	n.	%
*bla*KPC	22	47.8
*bla*VIM-1	10	21.7
*Bla*IMP	7	15.2
blaOXA-48-like	5	10.9
*bla*NDM-1	2	4.3
Total genes	46	100

21.7%, *bla*IMP 15.2%, *bla*OXA-48-like 10.9% and *bla*NDM-1 4.3%. Number of genes per isolate were shown in Table 4.

3.5. Correlation of the phenotype and genotype of carbapenem resistance

Out of 26 MHT positive isolates, only 18 were positive for KPC by PCR and 5 were positive for OXA-48. Of the 22 Boronic acid positive isolates, 18 were positive by the MHT, and 20 were positive for KPC by PCR assay. Out of the 5 isolates detected by EDTA test as positive for metallo-β-lactamases, all were positive by PCR (4 IMP,1 NDM-1). The EDTA test was more accurate in detection of metallo-β-lactamases, specially IMP and NDM-1.

Table 4
Number of genes per isolate.

Number of CRKP isolates	Genes per isolate	Genes present
1	3	NDM-1, VIM-1, KPC
1	3	VIM-1, OXA-48, KPC
2	2	VIM-1, OXA-48
1	2	IMP, NDM-1
34	1	20 KPC, 6 VIM, 6 IMP, 2 OXA-48

4. Discussion

Our results present a worrying trend of antimicrobial resistance in the middle east region, as the prevalence of CRKP was 33.3% among *K. pneumoniae* isolates. Previous Egyptian literature showed a prevalence of 44.3% of CRKP isolates [14], others reported lower incidence at 13.9% in the Egyptian National Cancer Institute [21] and 14.2% in Al-Azhar University Hospital [22]. Similarly other studies showed varying prevalence rate from 20 to 40% in New York and Greece [23,24]. Higher result of 83% was shown in a study in USA [25]. The high trend of CRKP in the current study could be attributed to the frequent use of carbapenems as an empiric therapy in ICUs at our institution, as well as lack of implementation of antimicrobial stewardship program. Our study subjects were ICU-admitted patients, since it was documented in previous studies that ICU stay was a major risk factor for CRKP acquisition [26]. In this study, sputum samples were the predominant sources of CRKP. These finding are similar to those observed by a study in Indonesia in which sputum had the largest number of bacteria carrying carbapenemase-encoding genes [7]. This may be due to cross-infection with multi-resistant clones or long-term exposure of respiratory tract microbiota to antibiotics, causing accumulation of resistance determinants. These resistant organisms may later cause respiratory tract infection [27]. In the current study, non susceptibility to ertapenem, based on the updated CLSI criteria was used in the surveillance of CRKP isolates. The use of ertapenem has been suggested to screen for carbapenem resistance among *Enterobacteriaceae* [28]. Endimiani et al. (2009) reported that nearly 60% of CRKP isolates are susceptible to IPM or MEM, and resistant to ertapenem [29]. The isolates collected in this study were highly resistant to β-lactams, fluoroquinolones and variably to aminoglycosides. Antibiotic susceptibilities of CRKP showed a worrisome trend, since two patients presented with panresistant isolates. Therefore there is an urgent need to develop new antimicrobials for CRKP, together with strict infection control measures in particular hand-hygiene to control the cross-infection with *K. pneumoniae.* The most prevalent gene among the 42 CRKP isolates genes was *bla*KPC at 43.5% which differs from what was seen in a previous study in Egypt, where *blaOXA-48-like* types were the most predominant at 28.6% *and bla*KPC accounted for only for 19% [30]. OXA-48 gene was detected in 10.9% genes among our isolates. Since the emergence of this gene in Turkey, the Middle East and North Africa had become reservoirs expanding to India, Senegal, and Saudi Arabia [31,32]. Our results detected only two (4.3%) *bla*NDM-1 genes. The low prevalence of blaNDM-1 gene observed in our study agrees with that of Morsi, et al., where it was seen in 2.4% of CRKP isolates [30]. Moreover, the blaNDM-1 genes co-existed with other carbapenamase genes in the two isolates among which it was detected, this explained why these isolates

were pan resistant. When comparing phenotypic tests to PCR results, we noted that out of the 26 MHT positive isolates, 18 were positive for blaKPC and 5 for blaOXA-48-like by PCR. False-positive results (3 isolates) may be due to carbapenem hydrolysis by ESBLs, coupled with disrupted porin expression as reported by others [33,34]. Conversely, false negative results (4 isolates) may be explained by presence of metallo-betalactamse producing isolates with weak carbapenemase activity as reported by Miriagou et al. (2010) [11]. MHT was more efficient at detection of OXA-48 than KPC carbapenemases. This observation is in agreement with others in which, MHT has a high sensitivity for detection of OXA-48 carbapenemases [35]. As regards to Boronic acid test, out of the 22 isolates detected by Boronic acid, 20 were positive by PCR. Absence of KPC in 2 isolates could be explained by that Boronic acid detects other class A carbapenemases such as IMI, Sme, NMC, and GES as described previously [36]. In our study, among the 5 isolates detected by the EDTA test, all were positive by PCR, whereas most of the metallo-β-lactamases detected by PCR were missed by EDTA test. This result is consistent with what was reported in a study by Khosravi. et al. (2012) [37], in which EDTA test showed 100% sensitivity, as all isolates detected by EDTA test were positive by PCR. However, they observed a low specificity of 43.1%. The main limitation of MHT is being time consuming and unable to distinguish blaKPC and ESBL producers with Omp changes isolates from each other. This observation has been reported by others but without clear explanation [20,34]. Boronic acid may provide some accuracy over the MHT but still time consuming. These limitations suggest the use of multiplex PCR for optimal detection of carbapenemase and early selection of proper antibiotics.

5. Conclusion

The spread of CRKP isolates represents a serious threat to our hospitals, which prompt developing new antimicrobials for CRKP, together with strict infection control measures including hand hygiene promotion, patients' isolation or cohorting, contact precautions, environmental cleaning, active surveillance and antibiotics stewardship programs. Using the updated CLSI breakpoints for ertapenem, susceptibility testing by disk diffusion method detected most carbapenem-nonsusceptible *K. pneumoniae* isolates without the need for other phenotypic tests. Multiplex PCR is an effective method for detection of carbapenemase genes which overcomes the limitations of the phenotypic tests.

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None.

Competing interests

None declared.

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