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ORIGINAL ARTICLE

Molecular Detection of *bla* OXA-48 Carbapenemase in Uropathogenic *Klebsiella pneumoniae* Strains from Suez Canal University Hospital

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ABSTRACT

Background: *The emergence of multidrug resistant Klebsiella pneumoniae (K. pneumoniae) is a new storm of antibiotic resistant threats. With the rise of resistance to carbapenems, antibiotics of last choice, the problem is reaching life-threatening scopes.* ***Objective:*** *our aim of this study was to make recommendations for the identification and detection of Klebsiella Producing Carbapenemases (KPC) to improve patient management and antimicrobial stewardship.* ***Methodology:*** *this study included 120 consecutive and non-duplicate clinical strains of K. pneumonia isolated from cases of urinary tract infections. Phenotypic detection of ESBL and Carbapenemases production were performed. Identification of class D carbapenemases by 16S rRNA gene sequence was done. Carbapenem resistance bla OXA-48 gene was detected using conventional PCR.* ***Results:*** *Phenotypic tests show 57 strains (47.5%) were carbapenem resistance (p value = 0.002) and 47 strains (39.1%) were ESBL producers (p value = 0.001). By 16S rRNA sequencing analysis, most of class D carbapenemases were K. pneumonia (17 strains out of 20 strains). Two strains were K. oxytoca and the other strain was Bacillus cereus. Among class D carbapenemase resistant K. pneumonia identified by 16S rRNA sequencing, PCR for bla OXA-48 gene produced amplified product of 744 bp and was positive in 10 isolates (58.8%). All these isolates were ESBL producers by phenotypic tests and resistant to ciprofloxacin, levofloxacin, gentamycin and amikacin.* ***Conclusion:*** *There are high frequencies of both carbapenem resistance (47.5%) and ESBL production (39.1%) among K. pneumoniae isolates. Effective infection control and strict antimicrobial stewardship are decisive basics to limit the spread of resentence genes.*

INTRODUCTION

Multidrug-resistant organisms (MDR) are a universal clinical and public health problem. There is an increase in the *Enterobacteriaceae* resistant strains to beta-lactams, fluoroquinolones, aminoglycosides and polymyxins[1]. There is an universal increase of Carbapenem-resistance in *Enterobacteriaceae* (CRE) throughout the former decade and is considered a main threat to clinical patient care and public health with high rates of morbidity and mortality[2]. CRE strains are divided according to their resistance to all β-lactam antibiotics, into the cephalosporins and carbapenems, as well as to fluoroquinolones, aminoglycosides and co-trimoxazole [3]. CRE are resistant to carbapenems by diverse mechanisms as carbapenemase production or combinations of AmpC, extended spectrum beta lactamases (ESBLs) as well as lose outer membrane porin (OMP) proteins [4,5].

Carbapenem-producing *Enterobacteriaceae* (CPE) includes only those *Enterobacteriaceae* with a confirmed carbapenemase-producing gene coding for the enzymes "carbapenemases" [4]. CPE were originally chromosomally mediated resulting in resistance in a few specific species, but the mainstream is now plasmid mediated that emerged in the mid-1990s ensuing in horizontal transmission between several bacterial species and genera [6,7].

The carbapenemases fall into 3 Ambler classes (based on their amino acid sequences), class A (serine carbapenemases) includes *Klebsiella pneumoniae* carbapenemases (KPC) and Guiana extended-spectrum beta-lactamase (GES), class B (metallo carbapenamases) include metallo-beta-lactamases (MBLs), such as New Delhi MBLs (NDMs), imipenemases (IMPs) and Verona integron-encoded MBLs (VIMs), and class D (OXA carbapenemases) consists of BLA OXA-48 and its derivatives [8,9].

In 2003, the original BLA OXA-48 producer was acknowledged from a *Klebsiella pneumoniae* isolate [10], and then reported as sources of health care associated infections (HAIs) outbreaks in Turkey [11]. In India , a point-mutant derivative of BLA OXA-48 sharing identical hydrolytic properties, namely OXA-181, has been recognized in enterobacterial isolates [12, 13]. *bla* OXA-48 and OXA-181 carbapenemases producers

generally do not display high resistance levels to carbapenems. So, the detection of *bla* OXA-48-like enzymes can be difficult unless those isolates possess accompanying mechanisms of resistance like ESBL production and/or permeability defects. Consequently, it is plausible that the prevalence of *bla* OXA-48 or OXA-181 producers is underestimated [14].

Adequate detection of carbapenemase-producing microorganisms in the routine laboratory diagnosis is indispensable for patient care to correct the choice of antibiotic therapy, decrease hospital stay and improve quality of life. As, the real prevalence of carbapenemase-producing bacterial strains remains unknown in our institution due to lack reporting and also the spread of CRE now characterizes a substantial risk for the public health and necessitates efforts toward detection and infection control policies, we decided to study the prevalence of *bla* OXA-48 *K.pneumoniae* in our institution to improve the antibiotic policy and quality of life. So, our aim of study was to make recommendations for the identification and detection of KPC to improve patient managing and antimicrobial stewardship.

METHODOLOGY

This study is a cross-sectional descriptive study. From May 2017 to June 2018, 120 consecutive and non-duplicate clinical strains of *K.pneumonie* were isolated from patients with clinical presentation suggestive of health care associated UTIs in Suez Canal University Hospital, Ismailia city, Egypt, for the detection of carbapenem-resistance among *K. pneumoniae* strains. This study was approved from the ethics committee in our institution.

Identification of K. pneumoniae:

Urine specimens were collected from all patients and handled to isolate and identify *K. pneumoniae* conventionally by gram stain, culturing on Mac Conkey agar media and Blood agar (Himedia, India) and biochemical tests using in-house, prepared media tubes [15]. Suspected isolates were screened using catalase test, oxidase test, indole production test, sugar fermentation tests, nitrate reduction to nitrite, citrate utilization test, Voges-Proskauer test (VP), Ornithine decarboxylation, Lysine decarboxylation and urease test [16]. These isolates were stored in vials containing nutrient broth 15% glycerol at -80°C until used.

Antimicrobial susceptibility:

Susceptibility profiles of *K. pneumoniae* isolates were determined using disk diffusion technique conferring to the Clinical and Laboratory Standards Institute (CLSI) guidelines[17]. Eleven different antimicrobial agents were used in this study and included Ampicillin, Cefazolin, gentamycin, amikacin, amoxicillin clavulanate, cefuroxime, cefepime, cefotaxime, ciprofloxacin, levofloxacin, imipenem, meropenem, colistin, Fosfomycin, nitrofurantoin and trimethoprim.

Phenotypic detection of ESBL-production in *K. pneumoniae* strains [17]:

Screening test:

The isolated strains were considered as potential producer of ESBLs if the inhibition zone diameter was ≤ 22mm for ceftazidime and ≤ 27mm for cefotaxime antibiotic disks on Muller Hinton (MH) agar plate inoculated with 0.5 McFarland, saline suspension made from fresh overnight blood agar culture of the test strains.

Confirmatory test:

A combination of ceftazidime (30 µg), ceftazidime clavulanic acid (30/10 µg), and cefotaxime (30 µg), cefotaxime- clavulanic acid (30/10 µg) disks were used for validation of ESBL production. The disks were placed on Mueller-Hinton plates at a distance of 20 mm (center to center) from each other. More than 5 mm increase in the inhibition zone diameter of ceftazidime-clavulanic acid or cefotaxime-clavulanic acid versus their zones when tested alone was considered as an indication of ESBL production**.**

Phenotypic detection for carbapenemase production in *K. pneumoniae* strains:

Screening test:

Isolates were considered as potential producer of carbapenemase when showed inhibition zone diameter 16–21 mm around meropenem antibiotic disk (10µg) and/or 19–21 mm around imipenem disk (10µg).

Confirmatory test:

Modified Hodge test (MHT) was performed for isolates that were positive by screening test, as well as those resistant to one or more agents in cephalosporins subclass III (cefotaxime – ceftazidime). A diluted saline suspension (1:10) of E. coli ATCC 25922 (0.5 McFarland), was spread on MH agar plate (15 cm). Meropenem disk (10µg) was placed at the center of the plate. The test organism (grown over-night on blood agar plate) were inoculated in a straight line from near the edge of the disk outwards till ~5 mm from the edge of the plate and then incubated at 35^{o}C±2 in ambient air for 16-20 hours. Positive and negative control strains (*K. pneumoniae* ATCC BAA-1705 and *K. pneumoniae* ATCC BAA-1706 respectively) were inoculated in the same way on each plate. The isolates were considered positive for carbapenemase production when enhanced growth was noticed.

Determination of class of carbapenemase in *K. pneumoniae* strains

The isolates that showed positive carbapenemase production by MHT were tested to determine the specific class of carbapenemase they possess as following:

Class-A carbapenemase:

Three meropenem antibiotic disks (10µg) were used on MH agar plate, 70µL solution (containing 600µg

phenylboronic acid dissolved in water) were added to the first one meropenem disk, 10µg clavulanic acid were added to the second meropenem disk (30 µg amoxicillin/10 µg clavulanic acid) that was placed on the plate for half an hour before the meropenem disk is placed) and the third disk was left as it is.

A rise in the inhibition zone diameter by ≥5mm around the meropenem + phenylboronic acid and/or meropenem + clavulanic acid disks more than the meropenem disk alone, was considered positive for the presence of class-A carbapenemase.

Class B carbapenemase:

Two meropenem antibiotic disks (10µg) were placed on MH agar surface, 10µl of a solution containing 292µg EDTA dissolved in water were added to the first meropenem disk and the other disk was left as it is. An increase in the diameter of the zone of inhibition around the meropenem-plus-EDTA disk by ≥5mm, more than the meropenem disk alone indicated the presence of class B metallo-carbapenemase.

Class D carbapenemase:

Isolates that showed positive MHT, Class A-negative, and Class B-negative results were considered –potentially- as producing class D carbapenemase.

Genotypic characterization of class D carbapenemases producing isolates:

DNA extraction:

The DNA was extracted using GeneJET genomic DNA purification kit (Fermentas) according to manufacturer instructions. Extracted DNA was stored at -80oC prior to PCR amplification.

Identification of class D carbapenemases by 16S rRNA gene sequence analysis PCR gene fragment was performed using 16S rRNA amplification from the purified genomic DNA [18]. Cycling parameters were at 94°C for 3 min, 94°C for 1.5 min, 55°C for 1 min, 72°C for 1 min, 36 cycles of steps 2 through 4 inclusive and72°C for 10 min. Aliquots of the amplification products were analyzed by agarose gel electrophoresis using 1.0% agarose containing 0.5 µg of ethidium bromide per ml. All PCR products were sequenced in Elim Biopharmaceuticals lab. United States. The results of BLASTn for 16S rRNA DNA sequences were retrieved and aligned with the sequences of bacterial isolates using ClustalW embedded in MEGA 6 (Molecular Evolutionary Genetics Analysis) software[19].

Molecular detection of carbapenem resistance *bla* OXA-48gene [10]

Carbapenem resistance *bla* OXA-48 gene was detected using conventional PCR using the following primers:

- Forward: TTGGTGGCATCGATTATCGG
- Reverse: GAGCACTTCTTTTGTGATGGC

The PCRs were carried out in reaction volumes of 25 µL with 8 µL DNAse/RNAse free water (Gibco, Invitrogen), 12.5 µL PCR Master Mix M752 (Promega, Fitchburg, WI, USA) and 0.5 µL of each primer. The PCR cycling conditions were as follows: one cycle at 95°C for 5 minutes, 30 cycles of 95°C for 30 seconds,58°C for 30 seconds, and 72°C for 60 seconds, followed by one cycle at 72°C for three minutes, and holding stage at 4°C.

Agarose gel electrophoresis.

The resulting amplicons were separated using a 1.5% agarose (Promega, USA) gel containing 0.5 µg/µL Ethidium Bromide. The gel was electrophoresed in 1x TBE buffer at 100 V for 55 min in an electrophoresis system (Bio-Rad, USA). A 50 bp ladder was used as a molecular size marker. DNA bands were visualized with a gel documentation system from Syngene (UK).

Statistical analysis

Collected data were entered into a database file. All statistical analyses were done using Statistical Package for Social Science program (SPSS version 22 for windows). Descriptive data were achieved according to its type; mean, standard deviation and range summarized continuous data; while qualitative data were summarized by frequencies. Chi square test was used for analytical data to detect the difference between qualitative data. Statistical significance was considered at P value ≤ 0.05.

RESULTS

Phenotypic characterization of *K.pneumoniae*:

One hundred and twenty strains of *K.pneumoniae* were isolated from cases of urinary tract infections from different wards in the hospitals. Forty-one strains were isolated from Intensive Care Unit (ICU) followed by Neonatal ICU (29 strains), urology (21 strains), Surgery Department (17 strains) and Medicine Departments (12 strains).

Phenotypic tests show 57 strains (47.5%) were carbapenem resistance (p value = 0.002) and 47 strains (39.1%) were ESBL producers (p value = 0.001). Greater than one third of these KPC isolates were recovered from ICU (25 isolates, 43.9%) followed by Urology Department (21 isolates, 36.8%) with a statistical significance difference (P value <0.005).

Among the 57 KPC isolates, 40 strains (70%) showed a high resistance to levofloxacin, 38 strains (66.67%) showed resistance to ciprofloxacin, 34 strains showed resistance to gentamycin and amikacin (59.6%), 13 strains (22.8%) were resistant to colistin and 30 strains (52%) were carbapenems resistant and ESBL producers.

Class A carbapenemase was detected as the only mechanism in 9 isolates (15.8%). Class B carbapenemase and class D were detected alone in 23 strains (40.4%) and 20 strains (35%) respectively. Combined resistance with class A and B was detected in 5 isolates (8.7%).

Genotypic characterization of 16S rRNA and Carbapenem resistant *K. pneumoniae* class D:

PCR for 16S rRNA of isolates produced amplified product of 1500 bp as shown in Figure (1). By 16S rRNA sequencing analysis, most of class D carbapenemases were *K. pneumonia*e (17 strains out of 20 strains). Two strains were *K. oxytoca* and the other strain was *Bacillus cereus* (data not shown). Among class D carbapenemase resistant *K. pneumonia*e confirmed by 16S rRNA sequencing, PCR for *bla*OXA-48 gene produced amplified product of 744 bp and was positive in 10 isolates (58.8%) as shown in Figure (2). All these isolates were ESBL producers by phenotypic tests and resistant to ciprofloxacin, levofloxacin, gentamycin and amikacin.

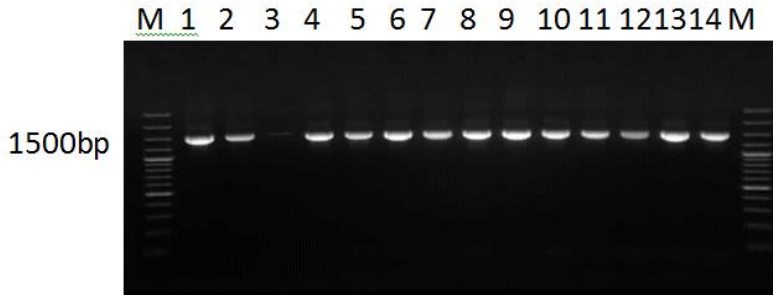

Fig. 1: Agarose electrophoresis. PCR products of the *16S rRNA* of some tested isolates. Lanes: M, molecular weight marker (Axygen Biosciences); All lanes (1-14) are positive strains for this reaction.

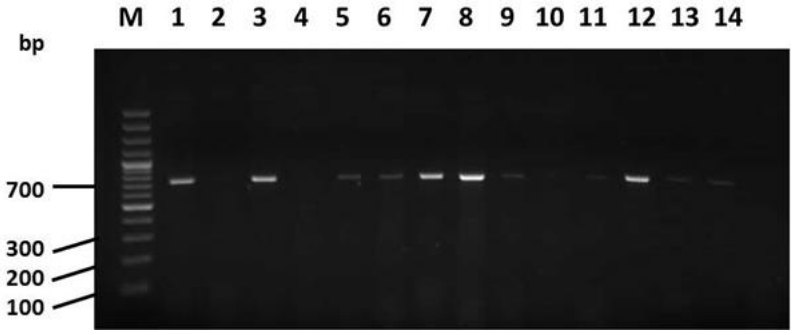

Fig. 2: Agarose electrophoresis. PCR products of the *blaBLA OXA-48* of some tested isolates. Lanes: M, molecular weight marker (Axygen Biosciences); lane (1) is the positive control; lane (2) is the negative control. All lanes (3-14) are positive strains for this gene except lanes (4) and (10).

DISCUSSION

In Egypt, the actual incidence of carbapenemase-producing bacterial strains is still unidentified due to the absence of reporting systems for the rates of antibiotic susceptibility. Consequently, the blow out of carbapenemase-producing organisms nowadays characterizes a substantial threat for the public health and necessitates efforts toward detection and infection control policies. Early identification of carbapenemase-producing strains both in clinical infections and/or carriers is a significant matter to decrease morbidity, mortality and hospital stay.

In our study, greater than one third of isolates were from ICU (43.9%). This finding is consistent with those of Al-Zahrani and Alasiri in Saudia Arabia which reported that ICU patients are more vulnerable population to *K. pneumoniae* infections representing 42.6% [20]. A recent Egyptian study reported that the prevalence of CRE species was 19.9% in Sohag and *Klebsiella Pneumoniae* was the most common isolated species representing 51.4% of the isolates [21]. Previous studies have also reported that ICU admission was associated with KPC colonization and infection [22, 23]. **Carrer *et al.*** reported that the most common hospital location in Turkey was the ICU followed by the urology and general surgery department, equally [11]**.** The high prevalence of carbapenem resistant *K. pneumoniae* isolates among the ICU can be attributed to the nature of patients admitted, immune-compromised, with disturbed consciousness or end-stage cases.

In the present study, 57 strains (47.5% of the isolates) were carbapenem resistance (p value = 0.002) and 47 strains (39.1%) were ESBL producers phenotypically. The majority of these strains were class B carbapenemase (23 strains, 40.4%), where Class A and class D carbapenemase were detected in 9 isolates (15.8%) and 20 strains (35%) respectively. Combined resistance with class A and B was detected in 5 isolates (8.7%). Probably, this high prevalence is associated to the lower controlled use of antibiotics in Egypt and the improper application of the infection control measures by the hospital personals.

Bouchillon *et al* [24] reported that 38.5% of *Enterobacteriaceae* isolates are an ESBL producer. A lower ESBL prevalence rate (16%) was found among 120 isolates collected between May 2007 and August 2008 at the Theodor Bilharz Research Institute, Cairo, Egypt [25]. In 2009, **Ahmed *et al*** [26] reported 64.7% ESBL-producing *Enterobacteriaceae* amongst strains isolated from patients suffering from Blood stream infections in the Intensive Care Unit in Assuit University Hospital . These results were markedly high when compared to **Bratu and colleagues** [27], where only 3.3% of *K. pneumoniae* isolates were carbapenem-resistant.

Carbapenems have served as the first-choice drugs for the treatment of ESBL [28]. The emergence of CPE is causing a public health threat leaving few treatment options. In Egypt, data about the prevalence of ESBL and CPE is inadequate. Conferring to the Pan European Antimicrobial Resistance Local Surveillance (PEARLS) study (2001–2002), Egypt was one of the countries with the uppermost rate of ESBL between *Enterobacteriaceae* (38.5%) [24]. Study for Monitoring Antimicrobial Resistance Trends) global surveillance program between 2008 and 2009 –conducted in the Asia-Pacific region– found that among 110 carbapenemase producing *K. pneumoniae* isolates; 42.7% were class A, 23.6% were class B and 11.8% were class D producing strains [29].

In the present study the percentage of class B metallo-β lactamases was higher than that of other classes, this can be explained as –in contrast to the chromosomal metallo-β-lactamases– there has been a huge increase in the detection and spread of the transferable families of these metallo-enzymes which are located within a variety of integron structures, so that their transfer between bacteria is readily facilitated[30]. On the other hand, the production of class D enzyme in the present study is still high (despite being second after class B), this may be due to the excessive potential for spread of this enzyme due to its position on plasmids; besides, it is most commonly found in *K. pneumoniae*, a notorious organism for its capacity to accumulate and transfer resistance factors.

BLA OXA-48 manufacturers have been found global, especially in Europe and in countries neighboring the southern and eastern parts of the Mediterranean Sea [31]. In the present study, among class D carbapenemase resistant *K. pneumonia* (17 strains), *bla*BLA OXA-48 was positive in 10 isolates (58.8%). All these isolates were ESBL producers and resistant to ciprofloxacin, levofloxacin, gentamycin and amikacin. In 2018, Moodley and Perovic [32] reported about 75.2% of the isolates harbored at least one or a mixture of carbapenemase-producing genes with the most chief carbapenemase type was *bla* OXA-48 and its variants (*n*=978, 36.5%) with the majority of the carbapenemase-producing isolates were *K. pneumoniae* (*n*=1 413, 52.8%), followed by *E. cloacae* (*n*=170, 6.3%). Also, **Moquet *et al*** [33] reported a recent emergence of bla OXA-48 in Senegal signifying the spread of the identical main carrying plasmid between Africa and Middle East.

CONCLUSION

There are high frequencies of both carbapenem resistance (47.5%) and ESBL production (39.1%) among *K. pneumonia*e isolates. Effective infection control and strict antimicrobial stewardship are crucial elements to limit the spread of resentence genes.

Recommendations:

Colonization rate can be better assessed by active surveillance by patient screening than cultures gained from clinical samples alone to minimize the spread of resistance genes among patients. Effective infection control and strict antimicrobial stewardship are crucial elements to limit the spread of resentence genes. Measures should be taken to raise awareness of Health care Personnel about MDR transmission, prevention and control. Improvement of laboratory facilities for detection and reporting such MDR organisms

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- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

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