ORIGINAL ARTICLE

Phenotypic and Genotypic Evaluation of Carbapenamase Producing *Klebsiella Pneumoniae* Isolates with Their Phylogenetic Analysis at an Egyptian University Hospital

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ABSTRACT

Background*: Widespread dissemination of carbapenem-producing Klebsiella pneumoniae (KPC) is of major concern in healthcare settings. Resistance to carbapenems involves multiple mechanisms such as the production of carbapenemases, impermeability of outer membrane and efflux pump mechanism.* ***Objective:*** *The aim of this study was to evaluate the prevalence of carbapenemase-producing K. pneumoniae strains among various clinical specimens obtained from different wards and to detect KPC as a mechanism of resistance.* ***Methodology:*** *100 samples (55urine and 45sputum) were collected from outpatients and inpatients attending urology and chest departments in Beni Suef University Hospital aiming to isolate K. pneumoniae during the period of December 2016 to January 2018. The isolates were tested for susceptibility to ertapenem using E test. Resistant isolates were subjected to phenotypic detection of carbapenemase production by Modified Hodge Test (MHT) and molecular assessment of KPC gene by PCR. Phylogentic tree analysis was used to detect their relationship by DNA sequencing reaction.* ***Results:*** *K.pneumoniae were isolated from 31(31%) of the samples taken. Out of them 19(61.8%) were resistant to ertapenem by E test. By phenotypic method,17/19 (89.4%) were positive for carbapenemase by MHT; and only 13 out of them (76.4%) were confirmed as KPC by PCR.* ***Conclusion:*** *High rate of carbapenem-resistance in K. pneumoniae by both phenotypic and molecular methods was observed. These results warrant more firm infection control measures along with a strictly implemented antibiotic stewardship program to prevent their spread.*

INTRODUCTION

Carbapenemase-producing Enterobacteriaceae (CPE) have been associated with hospital acquired infections (HAI), resulting in complicated health problems due to futility of antibiotics in treating such infections.

There is an obvious global increase in antimicrobial resistance (AMR) among Gram-negative pathogens (GNP). For more than 20 years high rates of AMR and outbreaks caused by GNP have been reported in Egypt.[1] In Egyptian hospitals AMR rates have been increasing; probably due to widespread abuse of antimicrobials including carbapenems and poor implementation of infection control practices.

Klebsiella pneumoniae is defined as one of the leading causes of hospital and community acquired infections, manifesting as urinary tract infections, pneumonia, septicaemia and abscesses.[2]

A current major threat is the increasing carbapenem resistance as they are one of the last effective choices available for antibiotic therapy against multi-resistant strains.[3] World Health Organization (WHO) has listed carbapenem-resistant *K. pneumoniae* (CRKP) as a critical priority pathogen due to high morbidity and mortality.[4]

Carbapenem resistance may be attributed to porin mutations, efflux pumps, and/or carbapenemases production. From an epidemiological point of view class A carbapenemases of the type *Klebsiella pneumoniae* carbapenemases (KPC) and class B carbapenemases of the type New Delhi metallo-beta-lactamases (NDM) are the most important.[5]

The modified Hodge test (MHT) and the susceptibility to ertapenem are the most indicated methods to reveal the production of these enzymes, especially in endemic areas.[6] The sensitivity of the test reaches almost the 100%; however, diversities in specificity values and false positivity of the results are

of concern. Therefore, molecular techniques are known to be the gold standard method to confidently confirm KPC production.[7]

Thus, the current study aimed to evaluate the prevalence of carbapenemase-producing *K. pneumoniae* strains among various clinical specimens obtained from different wards in Beni-Suef University Hospitals and to detect KPC as a mechanism of resistance.

METHODOLOGY

Patients and Methods:

A retrospective cross-sectional study was conducted on 100 clinical samples collected from Outpatient Clinics and hospitalized patients admitted to Beni Suef University Hospitals during the period from December 2016 through January 2018. All samples were collected after taking a written informed consent from all patients. Ethical approval (Number: FMBSUREC/05072022/ Sheemy) was obtained from the Ethical Committee, Faculty of Medicine, Beni-Suef University.

Sample collection:

Urine and sputum samples were collected under complete aseptic conditions using sterile screw capped universal containers, labeled and then transported immediately to Microbiology Department Laboratory.

Identification of the isolates*:** Different samples were cultured on Blood agar and MacConkey's agar plates ***(Oxoid, UK). All plates were incubated aerobically at 37ºC for 24-48 hours. Then, ***K. pneumoniae isolates*** was identified using API 20E ***(BioMérieux, France).***

Screening methods for carbapenemase production:

***E-test*:**

A pure colony from overnight agar plate culture was emulsified in sterile saline equivalent to 0.5 McFarland Standard. A uniform streak was done over the surface of Muller-Hinton agar. A strip of ertapenem (**(BioMérieux, France)** was applied on inoculated agar then the plates were incubated for 24 hours at 37^0C. The results were interpreted according to the CLSI breakpoints.

Phenotypic confirmatory tests for carbapenemase production:

Resistant isolates to ertapenem were tested for carbapenemase production by Modified Hodge Test.[8]

The results of the modified Hodge test were interpreted according to the CLSI guidelines as follows: no distortion of the inhibition zone around the ertapenem disk indicated negative carbapenemase producing isolate and the positive was considered when any distortion of the *E. coli* ATCC 25922 (indicator strain) inhibition zone around the ertapenem disk.

Molecular detection of KPC genes:

Genomic DNA was extracted from 50 µl bacterial suspension using Wizard® Genomic DNA Purification Kit **(Promega, Madison, USA).** The extracted DNA was then diluted with water and the optical density (OD) was measured at 260/OD280 nm using **Nano Drop Technologies Inc. (USA)** to assess the integrity of the extracted DNA.

The sequence of KPC DNA primers used was 5' ATGTCACTGTATCGCCGTC 3' as Forward primer and 5' TTTTCAGAGCCTTACTGCCC 3' as Reverse primer **(Qiagen).**

Amplification was performed in a DNA thermal cycler **(Biometra)** programmed at 95ºC (1 min) for initial denaturation step followed by 35 cycles: and final extension step at 72ºC for 2 minutes.

Gel electrophoresis was performed in a 2.5% agarose gel at 100 volts for 10 min to visualize gel under UV light. Determination size of fragments was compared with 92 bp DNA ladder size marker.

DNA Sequencing Reaction

The procedure was performed on *K. pneumoniae* isolates that were resistant to ertapenem. The PCR products were sequenced with forward primer (ATGTCACTGTATCGCCGTC3') using a Big Dye Terminator 3.1 Cycle Sequencing Kit **(Applied Biosystems, USA),** according to the manufacturer's instructions.

Briefly; PCR sequencing cycling reaction with final total volume 20 µl which included 8µl big dye terminator, 3.2µl of 1 pmole diluted forward primer, 1µl PCR product and 7.8µl nuclease free water. The thermal profile conditions were 94^0C for 4 minutes and 95^0C for 15 sec, 55^0C for 30 sec and finally 60^0C for 4 minutes for 25 cycles. Fluorescent fragments are generated by incorporation of dye-labeled ddNTPs. Each ddNTP (ddATP, ddCTP, ddGTP, or ddTTP) will carry a different color of dye, and correspond to either A, C, G, or T at the 3´ end.

Data analysis:

The sequences obtained were analyzed using the GenBank BLAST tool. Subsequently, the sequences were edited and aligned using the BioEdit Sequence Alignment.

Phylogenic tree:

The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site.[9] The analysis involved 27 nucleotide sequences.

Statistical analysis:

Qualitative data were presented in the form of frequency distributions with percentages, while quantitative data were presented as means and standard deviation. Cross tabulation test was done in addition to *p*-values for the Chi-square test. *P*-values of <0.05 were considered as statistically significant. Analysis was conducted using Statistical Package for Social Science, (SPSS) version 20 ***(IBM, Amronk NY)***.

RESULTS

One hundred and nine different clinical samples were collected from patients in Beni-Suef University Hospital from which 9 were culture negative (8.25 %). A retrospective cross-sectional study was conducted on the isolates recruited from the culture positive samples (100/109) 91.75 %. These samples were collected from 34 outpatients and 66 hospitalized patients (55 urine and 45 sputum samples).

Age of patients ranged from 10 to 65 years old. Both sexes were involved. Clinical isolates from male patients were 55(55%) 20 of them were identified as *Klebsiella pneumoniae* while female patients were 45 (45%) and 11 samples were identified as *Klebsiella pneumoniae*.

Out of 100 samples, 31(31%) were identified as *Klebsiella* by conventional methods, while 69 (69%) were diversity of other organisms; *Enterobacter*, *E. coli*, *Acinetobacter*, *Staphylococcus aureus and Staphylococcus epidermidis*.

Further identification to the species level of *Klebsiella* was done by API 20 E. (Figure 1).

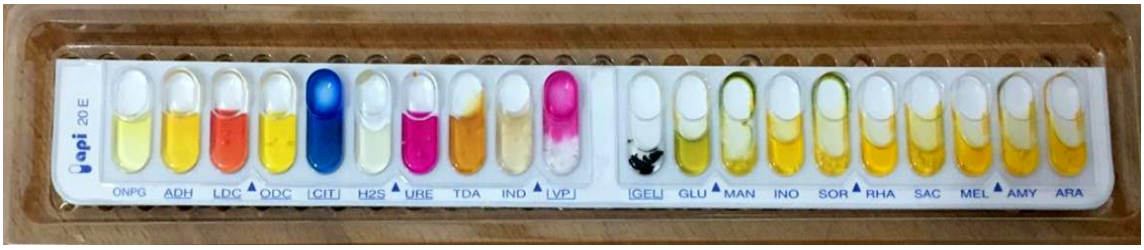

Fig. 1: API 20E test for identification

E-test for ertapenem MIC:

According to CLSI guidelines, 19/31 (61.8%) of *K. pneumoniae* isolates were ertapenem resistant (MICs ≥ 2 µg/ml), while 12/31 (38.2%) were sensitive to ertapenem (MIC < 0.5µg/ml). Figure (2) and figure (3) illustrate E test for ertapenem.

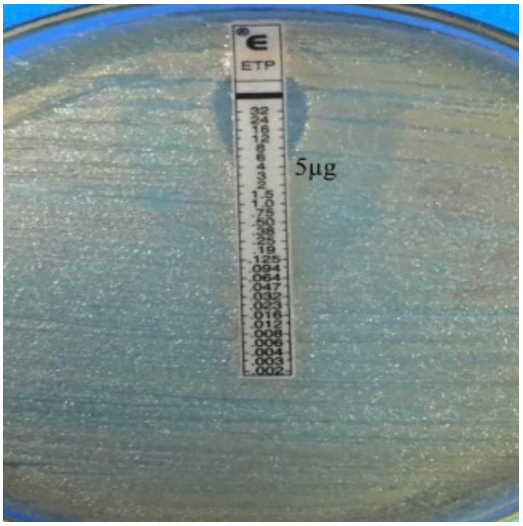

Fig. 2: resistant isolate (MIC 5 µg)

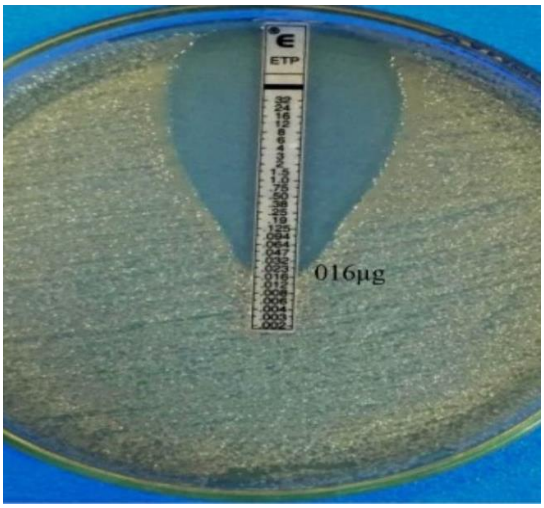

Fig. 3: sensitive isolate (0.016 µg)

On assessing some of the risk factors contributing to *K. pneumoniae* infections; male patients were 20/54 (37%) with *K. pneumoniae* compared to 11/45 (24.4%) isolates from female patients with no significant value (p>0.05). Hypertension can be considered a risk factor for infection by carbapenem resistant *K. pneumoniae* as (p value<0.05). Whereas, hospitalization and diabetes did not represent statistically significant risk factors (p value 0.2 and 0.16 respectively). Nevertheless, resistant isolates were observed in 7/10 patients with invasive devices (70%).

Modified Hodge test (MHT):

Seventeen out of 19 resistant *Klebsiella pneumoniae* isolates (89.4%) were positive by Modified Hodge test.

Molecular detection of *KPC* gene:

Out of 19 *K. pneumoniae*, 13 (68.4%) were found to have KPC by PCR, while 6 (31.5%) were negative **(**Figure 4).

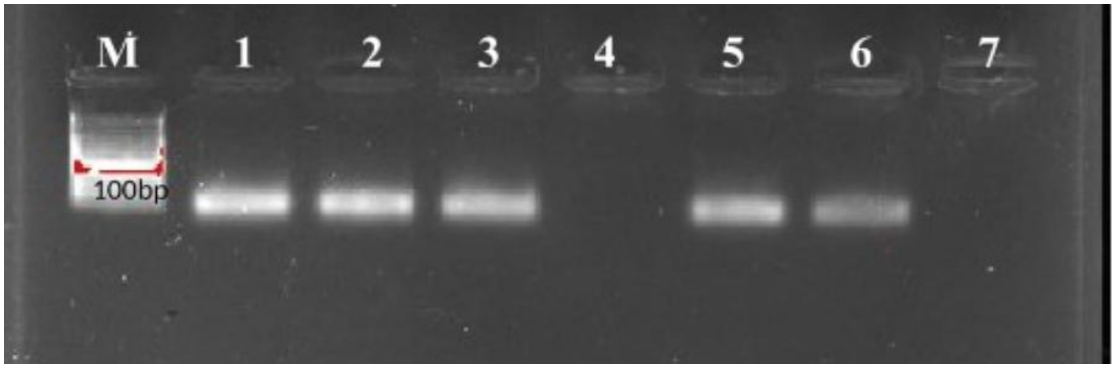

Fig. 4: Agarose gel electrophoresis showed PCR product of KPC gene: M: DNA ladder, 1 lane: positive control, 7 lane negative control, 2,3,5,6 positive samples, 4 lane negative sample

Considering PCR as a gold standard for detection of KPC, 13 isolates were carbapenemase producers. The sensitivity and specificity of MHT in relation to PCR were 100% and 33% respectively. The positive predictive value and negative predictive value were 40.26% and 100% respectively as shown in table (1).

Table 1: Correlation between phenotypic (MHT) and molecular methods (PCR) for detection of carbapenemase:

	PCR	MHT
Positive	13 (68.4%)	17(89.4%)
Negative	6 (31.5)	2 (10.5)
Sensitivity	100 % (CI 75.29% to 100%)	
Specificity	33.4 % (CI 4.33% to 77.72%)	
Positive predictive value*	40.26 % (CI 27.68% to 54.27%)	
Negative predictive value*	100%	
Accuracy	54% (CI 30.05% to 76.66%)	

Sequencing of *KPC* gene:

All strains resistant to ertapenem were submitted to gene sequencing and only seven isolates could be determined. The result obtained was analyzed through NCBI purposing for identifying similarities between strains. Sequencing confirmed the presence of the carbapenemase gene *bla* KPC in all 7 isolates with the accession numbers MT636778.1, DQ223685.1, MT452422.1.

The phylogenetic analyses of the available data using MEGA7 are summarized in figure (5).

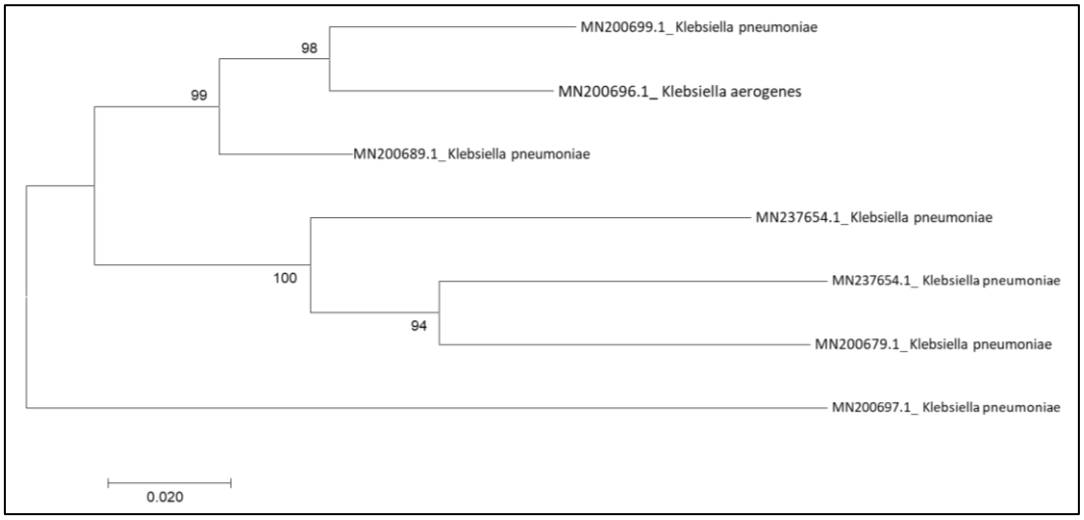

Fig. 5: Evolutionary relationships of taxa

DISCUSSION

A current major threat, in most hospitals in Egypt, is the emergence of increasing resistance to carbapenems which complicates the management of infections caused by *K. pneumoniae*.[10] Carbapenems were considered the last effective options available for antibiotic therapy against multi-resistant strains.[11] The mortality rate associated with infections caused by Carbapenem-resistant *Klebsiella pneumoniae* may reach up to 75%.[12]

KPC is one of carbapenemases which hydrolyse carbapenems and it shows rapid and widespread dissemination among susceptible strains.[11]

In the present study, *Klebsiella pneumoniae* have been identified in 31 isolates (31%) out of 100 samples collected from patients attending Beni-Suef University Hospital. A study done in Egypt estimated that *K. pneumoniae* was isolated from 902 samples out of 3836 with a percentage of 23.5% which is slightly lower than the present finding.[13]

In the current study, 10 out of 19 CRKP strains (52.6%) were isolated from urine and 9 from sputum (47.3%). This finding was comparable with a study where urine was 9 isolates (32%); and from sputum it was 5 strains (18%).[14] Meanwhile, resistant strains were more frequently associated with urinary catheter samples taken from 7/10 hospitalized patients (70%).

The univariate analysis in the present study showed that the risk factors as hypertension was significantly associated with CRKP (*p* value = 0.016), while diabetes (p value=0.169) and hospitalization (*p* value =0.219) had no significant association. Similar findings have been noted in a study where diabetes was associated in 30.6% of patients and was not a significant associated risk factors for CRKP (*p* value = 0.89) whereas invasive procedure as catheter was proved to be associated with CRKP (*p* value was 0.001).[15]

Assessing resistance to ertapenem, it was a sensitive screening test for detecting carbapenem resistance.[12] Nevertheless, resistance to this carbapenem is not a direct indicator for the production of carbapenemases.[16]

The present results showed that ertapenem resistance was evident in 19 isolates (61.3%) while, 12 (38.7%) were sensitive (MIC≤ 0.5µg/ml). In contrast to these findings, a study reported that, 19 (19%) of the studied isolates were ertapenem resistant.[17] In another study, out of 230 *K. pneumoniae* isolates, 50 isolates (21.7%) were found resistant to carbapenem (meropenem).[18]

In the same context, a survey concerning infections from medical and surgical units of hospitals in Egypt between 2004 and 2020, carbapenem-resistant *K. pneumonia ranged between* 35– 100%.[10]

These discrepancies in frequency of carbapenem resistant *K. pneumoniae* may be due to difference in geographical distribution, patterns of antibiotic used, and the population selected in different studies.

The modified Hodge test was recommended by ***CLSI*** in detecting KPC producers among *Enterobacteriaceae*.[8] In the present study, 17 (89.4%) of resistant isolates to ertapenem were proved to be carbapenemase producer by MHT. However, only 13 (76.4%) were positive for KPC gene when investigated by PCR. This means that MHT gave false positive results in 4 (23.5%) isolates. This could be justified by an explanation that these strains acted *via* other mechanisms of resistance; these negative strains most likely produced CTX-M or hyperproduced AmpC in association with porin loss.[19] Another possibility is that these isolates produced carbapenemases other than KPC.

The current findings revealed that MHT showed sensitivity of 100%, specificity of 31.6%, positive predictive value of 38.68% and negative predictive value of 90.49%. These results were close to that of another study, where MHT was positive in 34 isolates; only 27 isolates were confirmed to produce carbapenemase by molecular methods giving sensitivity, specificity, PPV and NPV of 100%, 47.06%, 73.53% and 100% respectively.[20] While, in Uganda phenotypic carbapenem resistance in *kl.pneumoniae* was 23.3% (53/227) with significantly higher genotypic resistance prevalence of 43.1% (98/227).[21]

In a recent study in Egypt, KPC gene was isolated from only 2 isolates of *Klebsiella pneumoniae* indicating low prevalence of the gene in tertiary hospitals in Egypt.[22] Whereas, a previous study in Egypt determined the prevalence as 31%.[23] WHO explained the high rate of antibiotic resistance in Egypt is essentially due to inappropriate use of antimicrobials in human and animal health care.[24]

Bacterial typing is an important method to identify the route of pathogen transmission. In the current study, all isolates identified in clusters presented an average genomic similarity ratio of > 89.45%. The isolates were significantly different from each other in the percentage of similarity. This suggests that dissemination of KPC resistance is due to horizontal gene transfer rather than clonal spread which may require plasmid extraction for mapping.

CONCLUSION

Antimicrobial resistance resulting from continuous selective pressure due to wide spread of antibiotic use is a growing health problem. KPC producing *Klebsiella pneumoniae* is emerging as an important mechanism of resistance. Phenotypic detection of KPC by MHT is of great importance to guide the clinicians and help to control the spread of infections caused by CRE. Molecular methods including PCR for KPC genes can

be used as a confirmatory gold standard test. Antimicrobial policy and its strict implementation with regular surveillance of KPC producing isolates are needed along with appropriate infection control measures to curtail its emergence and spread.

Authors' contributions statement:

MOH collected the data, carried out the laboratory work and performed the background literature review for the manuscript. MAA and AAM designed and supervised the study. MF collected and analysed the clinical data. AOE carried out and analysed the DNA sequencing laboratory work. MSS supervised the study, supervised the microbiological laboratory work conducted the statistical analyses and drafted the manuscript. All authors reviewed and approved the final version of the manuscript.

This manuscript has not been previously published and is not under consideration in the same or substantially similar form in any other reviewed media. I have contributed sufficiently to the project to be included as author. To the best of my knowledge, no conflict of interest, financial or others exist. All authors have participated in the concept and design, analysis, and interpretation of data, drafting and revising of the manuscript, and that they have approved the manuscript as submitted.

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