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A Study on Carbapenemase Producing Klebsiella Pneumoniae In Beni Suef University Hospital

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ABSTRACT

Background: Widespread dissemination of *Klebsiella pneumoniae* carbapenemase (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.

Methods: 100 samples of urine and sputum (55 urine and 45sputum) were collected from outpatients and inpatients attending urology and chest departments in Beni Suef University Hospital aiming to isolate *K.pneumniae* during the period of December 2016 through January 2018. The isolates were tested for susceptability to ertapenem using E test. Resistant isolates were subjected to phenotypic detection of carbapenemase production by MHT and molecular assessment of *KPC* gene by PCR. Phylogentic tree was used to detect their relationship.

Results*: K.pneumonia* were isolated from 31(31%) of the samples taken. Out of them 19(61.8%) were resistant to ertapenem. By MHT,17/19 (89.4%) were positive for carbapenemase; 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. Initiating appropriate infection control measures along with a strictly implemented antibiotic stewardship program are necessary to prevent their spread.

Keywords: Carbapenemase, Klebsiella Pneumoniae, Infections

I. INTRODUCTION

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

Widespread dissemination of Klebsiella pneumoniae carbapenemase (KPC) is ofmajor concern in healthcare settings. Resistance to carbapenems involves multiple mechanisms such as the production of carbapenemases, impermeability of outer membrane and efflux pump mechanism.

There is an obvious global increase in antimicrobial resistance (AMR) among Gram-negative pathogens (GNP). For more than 20 years, a high rate of AMR and outbreaks caused by GNP has been reported in Egypt (See et al., 2013). In Egyptian hospitals AMR rates have increased probably due to widespread abuse of antimicrobials including carbapenems and poor implementation of infection control practices.

A current major threat is the increasing carbapenemresistance as they are the last effective choice available for antibiotic therapy against multi-resistant strains (Pitout et al., 2015). WHO has been listed carbapenem-resistant K.pneumoniae (CRKP)as a critical priority pathogen due to high morbidity and mortality (Dong et al., 2018).

Carbapenem resistancemay be attributed to porinmutations, efflux pumps, and/or carbapenemases production. From an epidemiological viewpoint class A carbapenemases of the type Klebsiella pneumoniae carbapenemases (KPC) and class B carbapenemases of the type, New Delhi metallo-beta-lactamases (NDM) are most important (Pesesky et al., 2015).

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 (Tsakris et al., 2009).The sensitivity of the test reaches almost the 100% but diversities in specificity values and false positivity of the results are concerning. Molecular techniques are the most indicated methods to confidently confirm KPC production (Arnold et al., 2011).

II. Patients and Methods

A retrospective cross sectional study was conducted on 100 clinical samples collected from 34 outpatients and 66 hospitalized patients admitted to Beni Suef University Hospital. The sputum samples (45) were collected from chest department while urine samples (55) were collected from urology department. The study was carried out in the department of Medical Microbiology and Immunology, Faculty of Medicine, Beni Suef University during the period from December 2016 through January 2018.Age of patients ranged from 10 to 65 years old. Both sexes were involved. All samples were collected after taking a written consent from all patients.

I. Sample collection:

- Sputum samples were collected asmorning sputum samples in screw capped universal containers.
- Midstream urine sampleswere collected from outpatients and inpatients (25 samples) in sterile screw-capped universal containers.
- Thirty catheter specimens of urine (CSU) were collected from hospitalized patients admitted to urology department.

All samples were labeled with the date, patient's name, number, time of collection and specimen type, and then transported immediately to microbiology department.

II. Culture and identification of the isolates:

All samples were cultured on Blood agar and MacConkey's agar plates **(Oxoid Ltd., Basingstoke, and Hampshire, England),** incubated aerobically at 37°C for 24-48 hours

After incubation the growth was identified by conventional methods of identification.

Identification of *K.pneumoniae* up to the species level was done using API 20 E **(BioMérieux, France).**

III. Screening methods for carbapenemase production:

Ertapenem sensitivity:

A strip of ertapenem (BioMérieux, France), was applied to a Muller Hinton agar previously inoculated by *K.pneumoniae* isolate, incubated at 37◦ C for 24 hours.

The pointed end of the inhibition ellipse intersects the side of the strip is the MIC value.The results were interpreted according to the CLSI breakpoints.

IV) Phenotypic confirmatory tests for carbapenemase production:

The Modified Hodge Test (MHT) (*CLSI, 2017*):

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

Principle:

- A standard strain of *E.coli*ATCC 25922 (obtained from Naval Medical Research Unit Three (NAMRU-3) was suspended in saline to 0.5 McFarland and then overlaid on Muller–Hinton agar plate.
- A disc of ertapenem 10µg (Oxoid, UK) was placed on the plate.
- From an overnight pure culture of a tested strain, three to five colonies were picked up and streaked from the edge of ertapenem disc to the periphery of the plate.
- Three isolates can be tested in the same plate.
- The plate was incubated aerobically for 18-24 hours at 35±2◦ C
- The results of the modified Hodge test were interpreted according to the CLSI guidelines ***(2013)*** as follows: no distortion of the inhibition zone around the ertapenem disk indicates negative carbapenemase producing isolate and any distortion of the *E. coli* ATCC 25922 (strain indicator) inhibition zone around the ertapenem disk indicates positive carbapenemase production.

Uninterpretable results for the isolate, when the inhibition zone of *E. coli* ATCC 25922 is parallel to the isolate streak.

VI) Molecular detection of KPC genes:

This work was done at Biochemistry Department, Faculty of Medicine, Cairo University and Faculty of Pharmacy, Beni Suef University.

For molecular detection of KPC genes, the following steps were followed:

A- DNA extraction.
B- Assessment of DNA integrity by Nano drop spectrophotometer.
C- Amplification of KPC gene by PCR.
D- Agarose gel electrophoresis to resolve amplified PCR products.

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.**

The sequence of KPC DNA primers used was5' 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 minto visualize gel under UV light.Determination size of fragments was comparingwith 92 bp DNA ladder size marker.

DNA Sequencing Reaction

The procedure was performed on 19 *K.pneunoniae* isolates that were resistant to ertapenem. The results of only seven could be determined. The PCR products were sequenced with forward primer (ATGTCACTGTATCGCCGTC3') using a Big Dye Terminator 3.1 Cycle Sequencing Kit **(Applied Biosystems, Foster City, CA, 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 94ºC for 4 minutes and 95ºC for 15 sec, 55ºC for 30 sec and finally 60ºC 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 ***(Kimura, 1980)***and are in the units of the number of base substitutions per site. The analysis involved 27 nucleotide sequences.

IV. RESULTS AND DISCUSSION

A retrospective cross sectional study was conducted on 100 different clinical samples (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* and *Acinetobacter*.

E-test for ertapenem MIC:

According to CLSI guidelines ***(2013)*** 19(61.8%) of *K. pneumoniae* isolates were ertapenem resistant (MICs ≥2 µg/ml) while 12 (38.2%) were sensitive to ertapenem (MIC<0.5µg/ml). Figure (1) and figure (2) illustrate E test for ertapenem.

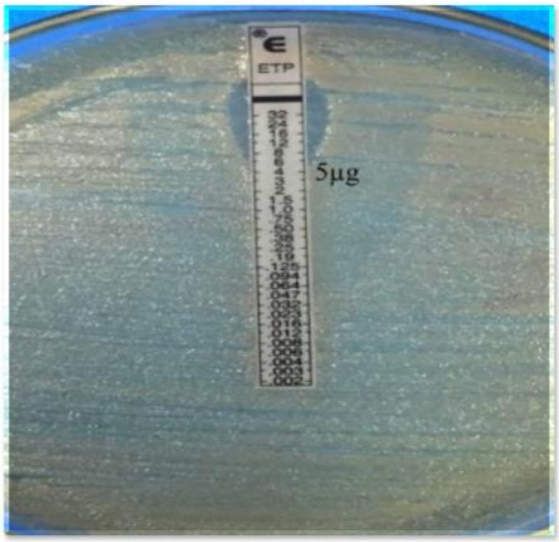

Figure (2) : resistant isolate (MIC 5 µg).

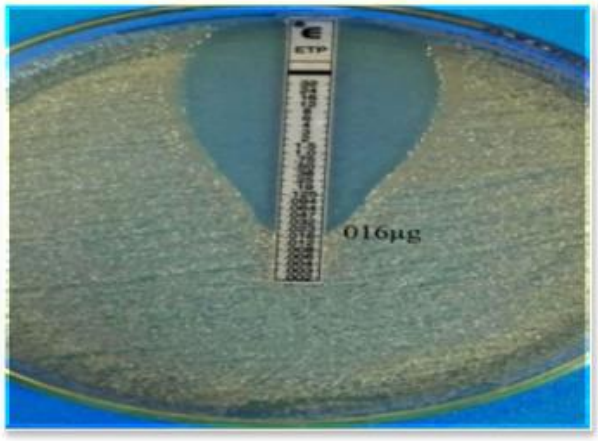

Figure (3) : sensitive isolate (0.016µg).

Table (1) : Distribution of *K. pneumoniae* isolates in relation to age, gender and risk factors:

		NO. of resistant isolates	%	*P* value
sex	**Male**	11	57.8	0.2
	female	8	42.1	
Risk factors				
Inpatient		16	84.2%	0.2
Diabetes		15	36.8%	0.16
Hypertension		5	26.3%	0.016

From male patients, 20 (36.4%) were identified as *K. pneumoniae* compared to 11 (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.Resistant isolates were found in 7 patients with catheter (70%).

Modified Hodge test:

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 negativeFigure (4).

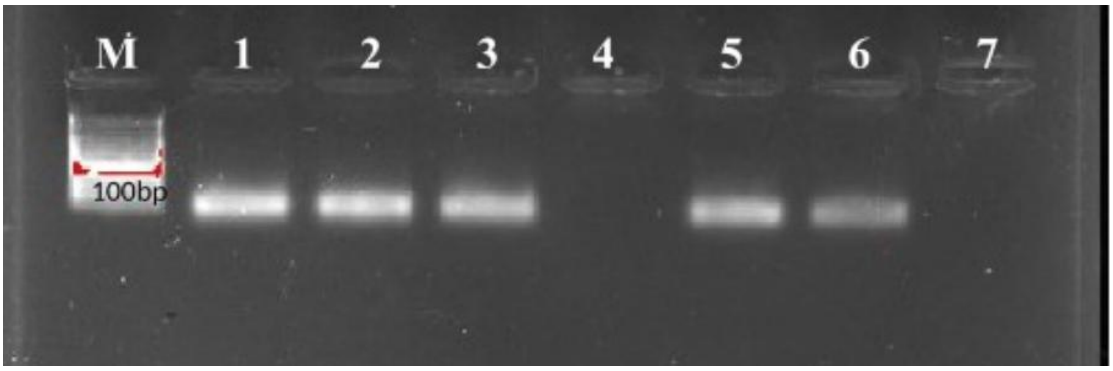

Figure (4): Agarose gel electrophoresis showed PCR product of KPC gene.
M: DNA ladder,1 lane: positive control,7 lane negative control,2,3,5,6 positive samples, 4 lane negative sample

Table (2): Correlation between phenotypic (MHT) and molecular methods for detection of carbapenemase (PCR)

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 (2).

	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 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 MEGA7are summarized in figure (5).

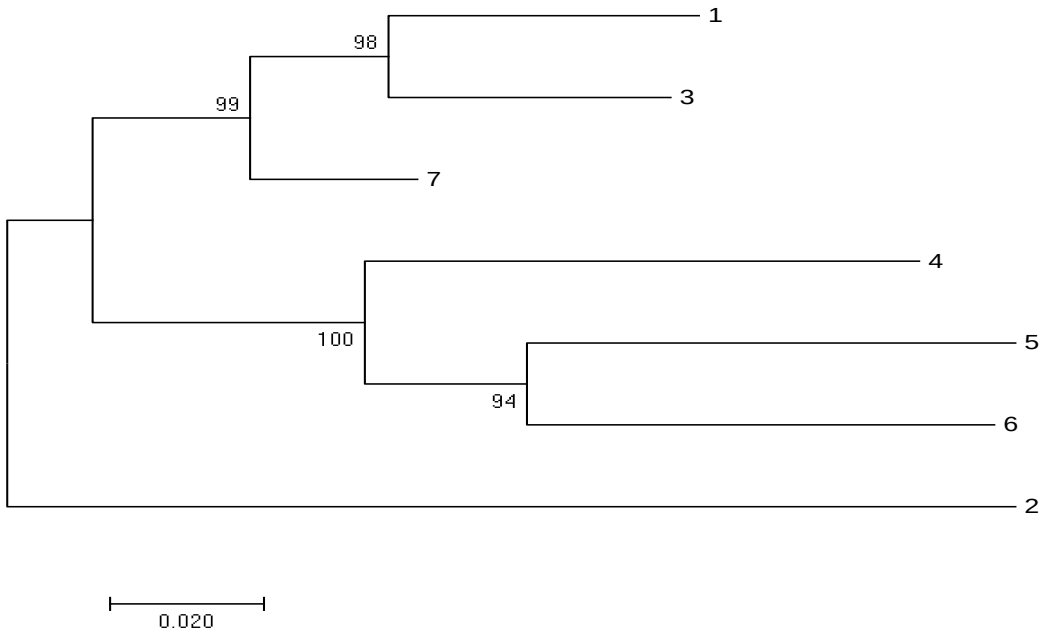

Figure (4): Evolutionary relationships of taxa

Discussion

A current major threat is the emergence of increasing resistance to carbapenems which complicates the management of infections caused by *K. pneumoniae*. Carbapenems were considered the last effective options available for antibiotic therapy against multi-resistant strains *(Pitout et al., 2015).*The mortalityrate associated with infections caused by Carbapenem-resistant *Klebsiella pneumoniae* may reach up to75%. KPC is one of carbapenemases which hydrolyse carbapenems and it shows rapid and wide spread dissemination.

In this study, *Klebsiella pneumoniae* have been identified in 31 isolates (31%) of out of 100 samples collected from patients attending Beni Suef University Hospital. While *Kotb et al., (2020)* estimated that *K. pneumoniae* was isolated from 902 samples out of 3836 with percentage of 23.5% which is higher than our result.

Resistance to ertapenem is a sensitive screening test for detecting carbapenemases. However, resistance to this carbapenem is not a direct indicator for the production of carbapenemases *(El Mahallawy et al., 2018).*

In this study, ertapenem resistance was found in 19 isolates (61.3%) while 12 (38.7%) were sensitive (MIC≤ 0.5µg/ml). In contrast,*Kotb et al., (2016)* reported that 19 (19%) of studied isolates were ertapenem resistant.The resistance to ertapenem was lower than that of *Vivas et al.*,*(2020)* who proved that ertapenem is a good marker for the suspected production of carbapenemases as the isolate was considered resistant to ertapenem with MIC of >0.5 µg/ml. These discrepancies in frequency of carbapenem resistant *K. pneumoniae* may be due to geographical differences, patterns of antibiotic use and the population selected in different studies.

Among 19 resistant strains, 11(57.8%) were isolated from male and 8 (42.1%) from female patients with no statistically significant difference. This is supported by *Hatem et al., (2012)* who identified 53.2% of isolates from men, and 46.8 % were from women, with no statistically significant difference.

As regards specimen; 10 out of 19 CRKP strains (52.6%) were isolated from urine and 9 from sputum (47.3%). This finding was similar to that of *Jayakumar et al., (2017)* where urine was 9(32%), sputum was 5 (18%).Meanwhile, resistant strains were more frequently associated with urinary catheter taken from 7 hospitalized patients (70%).

In this study, univariate analysis showed that the risk factors as hypertension was associated with CRKP as (*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 by *Yuan et al., (2020)* who found that diabetes was associated in 30.6% of patients and was not significant associated risk factors for CRKP (*p v*alue=0.89) whereas invasive procedure as catheter was proved to be associated with CRKP as (*p* value was 0.001).

The modified Hodge test was recommended by*CLSI* in detecting KPC producers among *Enterobacteriaceae (CLSI, 2017).* 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 KPCgene when investigated by PCR. This means that MHT gave false positive results in 4 (23.5%) isolates, in agreement with the explanation of *Wozniak et al., (2012)* who reported that these strains act *via* other mechanisms of resistance; these negative strains most likely produced CTX-M or produced AmpC in association with porin loss. Another possibility is that these isolates produced carbapenemases other than KPC.

The present study revealed that MHT showed sensitivity of 100% and specificity of 31.6%. The results of this study was close to that of *Morsi, (2016)* where MHT was positive in 34 isolates; only 27 isolates were confirmed to produce carbapenemase by molecular methodsgiving sensitivity, specificity,

PPV and NPV of 100%, 47.06%, 73.53% and 100% respectively.

In Egypt, a recent study of Elkholy et al., (2020), KPC gene was isolated from only 2 isolates of *Klebsiella pneumoniae* indicating low prevalence of the gene in tertiary hospitals in Egypt. Whereas, Metwally, (2016) determined the prevalence as 31%. WHO (2011) explained the high rate of antibiotic resistance in Egypt is essentially due to inappropriate use of antimicrobials in human and animal health care.

Bacterial typing is an important method to identifythe route of pathogen transmission. In the current study, all isolates identified in clusters presented an average genomic similarityratio of >89.45%. The isolates were significantly differentfrom 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.

V. 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 byCRE.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.

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