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ORIGINAL ARTICLE

Evaluation of OXA-48 in Carbapenem Resistant Gram-Negative Isolates

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ABSTRACT

Background*: Carbapenems production represents the most important mechanism that affects carbapenem activity. Among the Enterobacteriaceae, three molecular types of carbapenems are of major clinical importance since they are frequently associated with severe nosocomial infections. These are KPC (Ambler class A), NDM (class B) and OXA-48 (class D).* ***Objective:*** *The aim of the work is to determine the prevalence of carbapenem resistance and carbapenems encoding genes among clinical K. pneumoniae isolates obtained from patients at Intensive Care Units (ICUs) of Mansoura Oncology Institute.* ***Methodology****: This study included twenty-four Carbapenem resistant gram-negative bacilli (CRG), samples were collected from the Oncology Center in Mansoura University, Egypt, between October 2014 and February 2017. Samples were collected from different body sites such as: swabs, blood, urine, pus aspirate.* ***Results:*** *OXA-48 gene was positive in 16 samples and negative in 8 samples. Among the OXA-48 positive samples, 8 samples were positive by Modified Hodge test- Ertapenem disk, 5 samples were positive by Modified Hodge test- Meropenem disk, 3 samples were positive by combined disk test-Meropenem boronic acid Class A and 5 samples were positive by combined disk test- Imipenem EDTA Class B. Among the OXA-48 negative samples, 2 samples were positive by Modified Hodge test- Ertapenem disk, 3 samples were positive Modified Hodge test- Meropenem disk and 3 samples were positive by combined disk test- Imipenem EDTA Class B.* ***Conclusion****: Antibiotic resistance is considered a global health problem which is caused by misuse of antibiotics with the lack of the new antibiotic's development.*

INTRODUCTION

Carbapenem resistance among *Enterobacteriaceae* has been extensively observed worldwide, seriously compromising the usage of carbapenems[1]. Extended-spectrum beta-lactamase genes, which are linked to multidrug resistance, may be present. As a result, while waiting for culture results, the therapy must be empirical, usually with a carbapenem[2]. Resistance to carbapenems is becoming more common over the world. *Pneumoniae*, carbapenem resistance is conferred primarily through the synthesis of carbapenem enzymes[3].

Carbapenems production represents the most important mechanism that affects carbapenem activity. Three molecular kinds of carbapenems are of great clinical concern among *Enterobacteriaceae*, these are KPC (Ambler class A), NDM (class B) and OXA-48 (class D), because they are usually associated with severe nosocomial infections[4].

In the 1970s and 1980s aminoglycoside resistance encoded by plasmids was common. Subsequently, expanded spectrum β-lactamases (ESBL) have emerged. This has been followed by resistance to fluoroquinolones and subsequently by resistance to carbapenems[5]. It has been reported that if reduced susceptibility to carbapenems is detected in routine susceptibility tests, phenotypic methods should be applied to detect carbapenems. modified Hodge test (MHT), combination disc methods (Mast discs, Rosco tablets), MBL gradient strip test and temocillin gradient strip tests are among the phenotypic verification methods used for the detection of carbapenems.

Many clinical laboratories use in-house PCR-based methods to identify carbapenems genes. There are also PCR and hybridization-based kits that determine the types of carbapenems gene in the market. However, there is no single method that has been put forward as ideal for the detection of all carbapenem resistance mechanisms[6,7].

Among the different carbapenems, OXA-48 has been particularly successful and is the most common carbapenems in Germany. It has spread swiftly over the world, starting in Europe and the Middle East. OXA-48 is found most commonly in *K. pneumoniae* and *E. coli*, although it can also be found in other *Enterobacter* species. The OXA-48 (blaOXA-48) -lactamase gene

(bla) is commonly found on plasmids (pOXA48) and bracketed by two identical insertion sequences, IS1999, generating the composite transposon Tn1999[8].

OXA-48-like (e.g., OXA-181, OXA-232) variations have been described that differ from OXA-48 by few amino acids but have not spread as widely as OXA-48. However, the reasons for the successful dissemination of OXA-48 are currently not fully understood[8]. Interestingly, the presence of blaOXA-48 in clinical *E. coli* and *K. pneumoniae* isolates has been associated to virulence. Several investigations have reported on clinical isolates with unusually high mortality in mouse infection models, as well as the presence of genes linked to virulence or host colonisation, but the relevance of OXA-48 has not been investigated[9]. Because the dispersion is not driven by a single sequence type (ST), it is probable that OXA-48 or blaOXA-48-carrying plasmids contribute to clinical isolates' fitness and virulence, resulting in a survival advantage. We wanted to see if plasmids bearing the blaOXA-48 gene alter *E. coli* and *K. pneumoniae* fitness or virulence[10].

Whole genome sequencing (WGS) was used to examine OXA-48 generating clinical isolates of *E. coli* and *K. pneumoniae*, as well as single molecule real-time sequencing (SMRT). In addition, variables implicated in virulence and dispersion were studied in silico, in vitro, and in vivo[11]. These enzymes are widely distributed all over the world. However, specific reservoirs are identified for each type. KPC is mainly found in USA, China, Greece, and Italy. NDM is mainly present in India and Pakistan, while OXA-48 seems to be endemic in Turkey and in a lot of countries of the Mediterranean area. It is also largely found in North Africa[12].

Therefore this study aims to determine the prevalence of carbapenem resistance and carbapenems encoding genes among clinical *K. pneumoniae* isolates obtained from patients at Intensive Care units (ICUs) of Mansoura Oncology Institute, taking in consideration that carbapenems are frequently used as an empiric therapy.

METHODOLOGY

Population:

This study included twenty-four Carbapenem resistant gram-negative bacilli (CRG) selected from two hundred twenty patients receiving treatments during their hospitalizations and their information were completely analyzed and further studied. Samples were collected from the Oncology Center of Mansoura University, Egypt, between October 2014 and February 201٧ from different body sites such as: swabs, blood, urine, pus aspirate.

Ethical Approval

The Ethical Committee of Mansoura Oncology Institute approved this study. Additionally, we stated to confirm the patient data confidentiality.

Patients on antibiotic therapy before sample collection, infected by carbapenem sensitive bacteria or infected by any type of bacteria were not included and CRG were also excluded from this study.

Samples collection

Samples were collected before start of antibiotic therapy, Urine samples were collected as midstream urine in sterile containers, Pus samples were collected by syringe or sterile swab, Blood samples (5–10 ml) were collected under aseptic condition and inoculated into blood culture bottle.

Different samples (urine, pus, swabs) were cultured on different bacteriological media according to the type of samples and incubated for 24 hours at 37°C. Isolated colonies were identified by the routine bacteriological methods[13]. Blood samples were inoculated into blood culture bottle. CRG were kept in glycerol at -80°C to store until DNA extraction.

Anti-biotic sensitivity testing

Antimicrobial drug susceptibility was performed using two methods; the first one was the disc diffusion technique on Mueller–Hinton agar in which nine antibacterial agent disks were used ciprofloxacin (5 µg),Amikacin (30 µg) , Aztreonam (10 µg) , vancomycin (30 µg), imipenem (10 µg), ceftazidime (30 µg), Meropenem (10 µg) , cefotaxime (30 µg) and ceftriaxone (10 µg) (all from Bioanalyse, lot-150612G), inhibition zone for tested isolates with different disks was measured. The bacterium which showed resistance to one from three antibacterial groups were regarded as multidrug resistant. Isolated bacterium which displayed resistance to carbapenems was considered as carbapenems resistant bacterium. The resistance of the isolated bacterium was confirmed using (Vitec 2 system, Biomatrix Industry Inc., France) as automated microbial identification and drug sensitivity analytic system with reagent identification card related for Gram negative (GN) bacteria in which cards have 64 wells with a specific substrate for metabolic activities.

Modified Hodge Test

Modified Hodge test is a type of disk diffusion method that usually used in detection of carbapenem resistance (Patrice Nordmann, Dortet, and Poirel 2012). It is a phenotypic assay used to detect carbapenems enzyme by carbapenems inactivation which leads to increase growth of the indicator strain. It has < 50 % sensitivity for class B carbapenems but addition of ZnSO4 increase it to 87 %.

Imipenem (IPM)-EDTA combined disc Test

The combined IPM-EDTA disc test was performed[14]. On the plate, two 10 µg imipenem discs (Becton Dickinson) were inserted and 10µl of EDTA solution was added to one of them to achieve a concentration of 750µg EDTA. An extra blank disc was placed to the plate for *Pseudomonas aeruginosa* isolates (n=10), to which 10µl of EDTA solution of the same concentration was added. After 16 to 18 hours of

incubation at 35 °C the inhibition zones of the imipenem and imipenem-EDTA disks were compared. In the combined disc test, if the increase in inhibition zone of an isolate, with the imipenem and EDTA disc was ≥ 7 mm as compared to the imipenem disc alone, the isolate was considered as MBL positive (CDC)[15]. The zones of inhibition around EDTA were also recorded separately to later compare it to the result of CD test (difference between the imipenem and imipenem-EDTA inhibition zones) in order to rule out false MBL positivity.

Automated Broth Dilution AST Systems

Vitek AST Automated System

Vitek 1 measures the turbidity changes over time (growth curve) and compare a growth control well with wells of other drug concentrations. Results as MIC and S-I-R category. They take from 4 to 18 hours and depend on the growth rate of the organism and its susceptibility. Another version of vitek 1 was developed, vitek 2 which is more automated[16].

MicroScan is a broth microdilution method that utilizes a standard 96 microwell panel. These microwells contain serial dilutions of dehydrated antimicrobial agents. Results are obtained after about 18 hours by turbidimetric readings of overnight conventional panels or after about 7 hours by fluorometric readings of rapid panels[16]. Fluorometric analysis depends on the degradation of fluorogenic substrates by viable bacteria[17]. The phoenix system detects the susceptibility results by gravity-based inoculation process. Monitoring of the growth depends on redox indicator systems which provide results in 8 to 12 hours[18].

Bacterial DNA extraction

The isolated *Klebsiella pneumoniae* species which illustrated resistant to carbapenems was re-cultured on MacConkey broth media for 48 hours aerobically followed by DNA extraction using fully automated system.

Plasmid extraction

For the detection of plasmid carbapenemase resistance genes from the isolates, the previously confirmed isolates were subjected to manual plasmid extraction using DNA-spin TM plasmid DNA purification Kit (Intron Biotechnology, Korea) which improves DNA extraction from (3 Kb) short to (34 Kb) long length plasmids. The isolates were re-cultured on MacConkey broth media containing 100 µg.ml-1 ertapenem as a selective pressure for improving recovery of plasmid.

Assessment of resistance genes using Polymerase chain reaction (PCR)

PCR analysis was performed on the extracted DNA to detect Carbapenem-Resistant genes (blaVIM, blaIMP, blaNDM-1, blaOXA-48 and KPC)[19]. The procedures was done according to manufacturer instructions using PCR primers as shown below:

Name	Sequence (5'-3')	Length	MW	Tm	GC %	OD	Nmol	Water/tube	Purification
KPC-F011	TGTCACTGTATCGCC GTC	18	5441.56	57.30	55.56 %	2	12.18	121.29	QPC
KPC-R-11	TATTTTTCGAGATGG GTGAC	21	6467.28	56.06	42.86 %	2	10.21	102.05	QPC
VIM-F011	TCTACATGACCGCGT CTGTC	202	6043.97	59.85	55.00 %	2	10.92	109.20	QPC
VIM-R-11	TGTGCTTTGACAACG TTCGC	20	6099.02	57.80	50.00 %	2	10.82	108.21	QPC
NDM-F011	GGTTTGGCGATCTGG TTTTC	20	6161.06	57.80	55.00 %	2	10.71	107.12	QPC
NDM-R-11	CGGAATGGCTCATCA CGATC	20	6102.02	59.85	50.00 %	2	10.82	108.16	QPC
OXA48-F011	GCGTGGTTAAGGATG AACAC	20	6206.11	57.80	50.00 %	2	10.63	106.35	QPC
OXA48-R-11	CATCAAGTTCAACCC AACCG	20	6014.97	57.80	50.00 %	2	10.97	109.73	QPC

Statistical analysis

Data were fed to the computer and analyzed using IBM SPSS software package version 20.0. (Armonk, NY: IBM Corp).The Kolmogorov- Smirnov was used to verify the normality of distribution of variables, Paired t-test was used to compare two periods for normally distributed quantitative variables while ANOVA with repeated measures was used for comparing the different studied periods for normally distributed quantitative variables and followed by Post Hoc test (Bonferroni adjusted) for pairwise comparison. Pearson coefficient to correlate between two normally distributed quantitative variables. Significance of the obtained results was judged at the 5% level.

RESULTS

The demographic characters of studied patients are shown in table 1. A total of 24 patients were enrolled in the current study. The mean age of the studied patients was 61.5 years (the age range was from 49 years to 77 years). In addition, the study included 16 males (66.7 percent) and 8 females (33.3 percent) as shown in Table 1.

Table 1: Demographic and Sample distribution of carbapenem resistant *klebsiella* of studied patients

Age (years)	Range 49-77	Mean ± SD 61.5 ± 8.3
Males	No. 16	66.7%
Females	No. 8	33.3%

The sample distribution of carbapenem resistant *klebsiella* is shown in table 2. A total of 24 samples were collected in the current study and were distributed as follows: 5 blood samples (20.8%), 5 urine samples (20.8%), 5 wound swabs (20.8%), 3 sputum samples (12.5%), 2 BAL (8.3%)and 4 other samples (16.8 %) (e.g. Pus aspirate, Mini2, ETT, Pleural samples) as shown in Table 2.

Table 2: Sample distribution of carbapenem resistant *klebsiella*

Type of samples	No.	%
Blood	5	20.8
Urine	5	20.8
Wound swab	5	20.8
Sputum	3	12.5
BAL	2	8.3
Others*	4	16.8
Total	24	100

Table 3 shows phenotypic detection in clinical samples. Based on Modified Hodge test, positive Ertapenem diskwas detected in 10 samples including 2 blood samples, 3 urine, 3 wound swabs, 2 sputum samples with no statistically significant difference between samples. Positive Meropenemdisk was detected in 8 samples including 4 blood samples, 1 urine, 1 wound swabs, 1 sputum samples and 1 other samples with no statistically significant difference between samples. Based on the combined disk test, positive Meropenemboronic acid Class A was detected in 3 samples including 1 blood, 1 wound swab and 1 other sample with no statistically significant difference between samples. Positive Imipenem EDTA Class B was detected in 8 samples including 3 blood samples, 1 urine, 1 wound swabs, 1 sputum samples and 2 other samples with no statistically significant difference between samples.

Table 3: Phenotypic detection methods

Genes	Blood N=5		Urine N=5		Wound swab N=5		Sputum N=3		BAL N=2		Others N=4		P value
	N	%	N	%	N	%	N	%	N	%	N	%	
Modified Hodge test													
Ertapenem disk (positive) N=10	2	40	3	60	3	60	2	66.7	-	-	-	-	0.3
Meropenem disk (positive) N=8	4	80	1	20	1	20	1	33.3	-	-	1	25	0.3
Combined disk test													
Meropenem N=3	1	20	-	-	1	20	-	-	-	-	1	25	0.9
Imipenem N=8	3	60	1	20	1	20	1	33.3	-	-	2	50	0.7

The distribution of carbapenemase producers according to Phenotypic detection and presence of OXA-48 gene is shown in Table 4. OXA-48 gene was positive in 16 samples and negative in 8 samples. Among the OXA-48 positive samples, 8 samples were Modified Hodge test- Ertapenem disk positive, 5 samples were Modified Hodge test- Meropenem disk positive, 3 samples were combined disk test-Meropenem boronic acid Class A positive and 5 samples were combined disk test- ImipenemEDTA Class B Positive. On the other hand, 8 samples were Modified Hodge test- Ertapenem disk negative, 11 samples were Modified Hodge test- Meropenem disk negative, 13 samples were combined disk test-Meropenem boronic acid Class A negative and 11 samples were combined disk test- ImipenemEDTA Class B negative. Among the OXA-48 negative samples, 2 samples were Modified Hodge test-Ertapenem disk positive, 3 samples were Modified Hodge test- Meropenem disk positive and 3 samples were combined disk test- ImipenemEDTA Class B Positive. In contrast, 6 samples were Modified Hodge test- Ertapenem disk negative, 5 samples were Modified Hodge test- Meropenem disk negative, 8 samples were combined disk test-Meropenem boronic acid Class A negative and 5 samples were combined disk test-ImipenemEDTA Class B negative.

Table 4: Distribution of carbapenemase producers according to Phenotypic detection and presence of OXA-48 gene

Test	OXA-48 gene		Total
	Positive (N) (N=16)	Negative (N) (N=8)	
Modified Hodge test- Ertapenem disk			
Positive	8	2	10
Negative	8	6	14
Modified Hodge test- Meropenem disk			
Positive	5	3	8
Negative	11	5	16
Combined disk test-Meropenem boronic acid Class A			
Positive	3	-	3
Negative	13	8	21
Combined disk test- Imipenem EDTA Class B			
Positive	5	3	8
Negative	11	5	16

The performance of Modified Hodge test and combined disk test for detection of OXA-48 gene is shown in Table 5. As regards the Modified Hodge test, Ertapenem disk had 50% Sensitivity, 75% Specificity, 80% PPV, 42.9% NPV and an overall accuracy of 58.3% whereas Meropenem disk had 31.3% Sensitivity, 62.5 % Specificity, 62.5 % PPV, 31.3 % NPV and an overall accuracy of 41.7%. As regards the combined disk test, Meropenem boronic acid Class A had 18.8% Sensitivity, 100% Specificity, 100% PPV, 38.1 % NPV and an overall accuracy of 45.8 % whereas ImipenemEDTA Class B had 31.3% Sensitivity, 62.5 % Specificity, 62.5 % PPV, 31.3 % NPV and an overall accuracy of 41.7.

Table 5: Performance of Modified Hodge test and Combined disk test for detection of OXA-48 gene

	Sensitivity	Specificity	PPV	NPV	Accuracy
Modified Hodge test					
Ertapenem disk	50	75	80	42.9	58.3
Meropenem disk	31.3	62.5	62.5	31.3	41.7
Combined disk test					
Meropenem boronic acid Class A	18.8	100	100	38.1	45.8
Imipenem EDTA Class B	31.3	62.5	62.5	31.3	41.7

Gel electrophoresis in chromosomes show 16 positive to OXA-48 *klebsiella pneumonia* at 281 base pair and 8 were negative as shown in figure 1

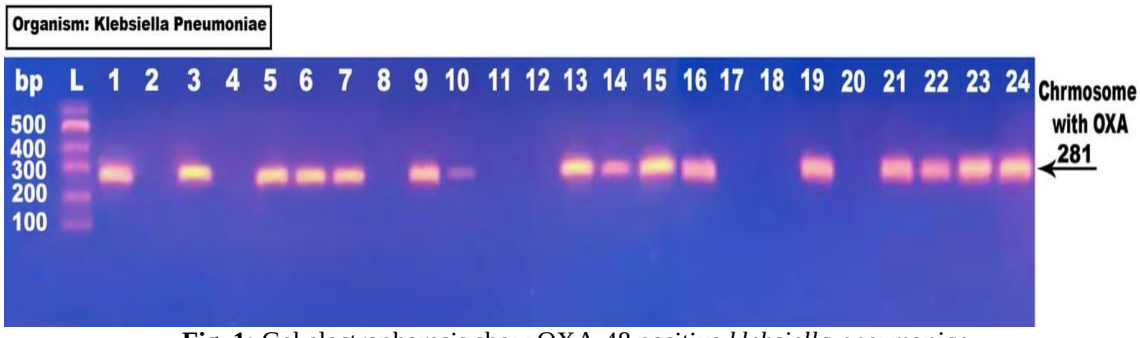

Fig. 1: Gel electrophoresis show OXA-48 positive *klebsiella pneumoniae*.

DISCUSSION

Carbapenem resistance mediated by carbapenemases is an important issue in infection control programmers. BlaOXA-48 represents the main enzyme among OXA â-lactamases isolated around the world[12].

The study was conducted on 24 cases who were infected with CRKP included 16males and 8females. With an average age of 61.5 years old selected from two hundred twenty patient received treatments during their hospitalizations. This study was performed on CRKP isolates which were identified by phenotypic methods and VITEK (bio-Merieux). DNA extraction and PCR amplification were used for determination of Carbapenem resistant genes in Chromosomal and plasmid.

Recent study conducted a cross-sectional study between late April and September in 2015. A total of 54 *Klebsiella pneumoniae* (*K. pneumoniae*) isolates with reduced carbapenem sensitivity were collected from diverse clinical specimens at the province's two main hospitals. The E-test was used to confirm carbapenems' minimum inhibitory concentrations (MICs). Multiplex-polymerase chain reaction was used to discover the

most frequent carbapenemase genes (blaIMP, bla-carbapenem-hydrolyzing oxacillinase [OXA-48], blaVIM, bla-New Delhi metallo-ß-lactamas [NDM], and blaKPC)[20].

As regards the demographic data sample distribution of carbapenem resistant *klebsiella*, The 24 samples were distributed as follows: 5 blood samples (20.8%), 5 urine samples (20.8%), 5 wound swabs (20.8%), 3 sputum samples (12.5%), 2 BAL (8.3%)and 4 others . In the same line, a study demonstrated that, carbapenem-resistant *Klebsiella* were collected from blood (n=13), sputum (n=10), urine (n=8), wound swab (n=6), tracheal aspirate (n=5) and body fluid (n=5)[20]. In addition, other study reported the distribution by biological source of carbapenem-resistant *Klebsiella* and revealed that, urine, bronchoalveolar lavage, and blood were the most frequent sources of CRKP strains with an incidence of 26% (n=25), 24% (n=24), and 24% (n=24), respectively[21].

As regards, the performance of Modified Hodge test and combined disk test for detection of OXA-48 gene, Ertapenem disk had 50% Sensitivity, 75% Specificity, 80% PPV, 42.9% NPV and an overall accuracy of 58.3% whereas Meropenem disk had 31.3% Sensitivity, 62.5% Specificity, 62.5% PPV, 31.3% NPV and an overall accuracy of 41.7%. As regards the combined disk test, Meropenem boronic acid Class A had 18.8% Sensitivity, 100% Specificity, 100% PPV, 38.1% NPV and an overall accuracy of 45.8% whereas ImipenemEDTA Class B had 31.3% Sensitivity, 62.5 % Specificity, 62.5% PPV, 31.3% NPV and an overall accuracy of 41.7[22]. Similarly, a study demonstrated that, the modified Hodge test has an excellent sensitivity for detecting enterobacterial isolates producing Ambler class A (KPC) and class D (OXA-48) carbapenemases. Its sensitivity is low for NDM-1 producers (50%) but is increased to 85.7% by adding ZnSO4 (100 µg/ml) in the culture medium. However, this test has a low specificity and is time-consuming[23].

CONCLUSION

In the present work, the significant high frequency of bla Oxa-48 resistance gene among the resistance strains may indicate that it is a main source of initiating resistance among these strains, while its presence among carbapenem sensitive isolates suggesting the occurrence of silent gene, that encoded in a movable genetic element (plasmid), therefor, it plays a key role in the transfer of horizontal resistance gene from one bacterium to another. Therefore, prevention and control programs of carbapenem resistant should be performed to prevent the spread of carbapenems producers

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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