Egyptian Journal of Medical Microbiology Volume 25 / No. 1 / January 2016 109-116

ORIGINAL ARTICLE

Comparative Evaluation of Phenotypic and Genotypic Methods for Detection of Carbapenemases in Clinically Significant *Klebsiella pneumoniae* Isolates

Samar S. Morsi*

Medical Microbiology and Immunology Department, Faculty of Medicine, Zagazig University, Egypt.

Key words:

Carbapenemase, Modified Hodge test, combined disc test, Multiplex PCR, carbapenem-resistant K.pneumoniae

ABSTRACT

Background: *Carbapenems are the most active and reliable treatment options against ever more prevalent Klebsiella pneumoniae (K. pneumoniae) that produce extended-spectrum β-lactamases, but their efficacy is threatened worldwide by carbapenemase production which is frequently associated with serious infections and higher mortality.* ***Objectives:*** *The aims of this study were to detect occurrence of carbapenemase producing K. pneumoniae and to evaluate the use of Multiplex PCR for rapid detection of carbapenemase genes in comparison with phenotypic methods as modified Hodge test (MHT) and combined disc test (CDT).* ***Methodology:*** *Different clinical samples were obtained from 430 patients admitted to Zagazig University Hospitals over the period from June 2014-June 2015. They were cultured and K. pneumoniae isolates were identified. The antimicrobial susceptibility patterns were determined by disc diffusion test .Carbapenem-resistant isolates were selected and subjected to Modified Hodge test (MHT) for carbapenemase detection, combined disc test (CDT) for differentiation of Ambler classes of carbapenemases and multiplex PCR for detection of bla_{KPC}, bla_{NDM-1}, bla_{VIM}, bla_{IMP} and bla_{OXA-48} genes.* ***Results:*** *A total of non-duplicate 100 K. pneumoniae clinical isolates were collected from 430 clinical samples. By disc diffusion test, 42 Carbapenem-resistant K. pneumoniae isolates were detected. Thirty-four of the 42 isolates were MHT positive while 27 isolates were positive by CDT. MDT showed sensitivity & specificity of 100% & 47.06% respectively, while CDT showed sensitivity & specificity of 100% & 88.89% respectively. By PCR, it was found that out of 42 carbapenem-resistant isolates, 25 isolates harboring one gene. The most common resistance gene was bla_{OXA-48} (12/42) followed by bla_{KPC} (8/42) while the bla_{VIM} gene (4/42) while bla_{NDM-1} was detected only in one isolate. bla_{IMP} had not been detected in our isolates.* ***Conclusion:*** *Multiplex PCR is an accurate method for detection of carbapenemase production genes which overcomes the limitations of the phenotypic methods.*

INTRODUCTION

Klebsiella pneumoniae (K. pneumoniae) is an encapsulated, lactose-fermenting, facultative anaerobic, rod-shaped gram negative opportunistic pathogen that can cause a plethora of infections including urinary tract infections, pneumonia, meningitis and sepsis.[1] Although being not inherently resistant to antibiotics, *K. pneumoniae* is a "collector" of multidrug resistance plasmids commonly encoding resistance to aminoglycosides as well as plasmids encoding extended-spectrum β -lactamases (ESBLs) being the index species for ESBLs, along with the chromosomal mutations conferring resistance to fluoroquinolones, carbapenems were left as the first-choice drugs for the treatment of infections caused by *K. pneumoniae*. [2]

Then, global crisis of rapid dissemination of multidrug-resistant (MDR) *K. pneumoniae* strains producing carbapenemases encoded by transmissible plasmids had occurred. Again *K. pneumoniae* functions as a pool of potent β -lactamases.[3] Carbapenemases can be allocated to three of the four classes of β-lactamases, Based on amino acid homology, namely, Ambler classes A, B and D. They can also be differentiated based on the hydrolytic mechanism at their active sites. Class A and D carbapenemases are referred to as serine carbapenemases (serine dependent), whereas class B carbapenemases are referred to as (MBL) metallo-β-lactamases (zinc dependent).[2]

Ambler class A carbapenemases are partially inhibited by clavulanic acid, a β-lactamase inhibitor. The most frequently identified class A carbapenemases are Klebsiella Producing Carbapenemase (KPC). KPC

Corresponding Author:
Samar S Morsi
Medical Microbiology and Immunology Department,
Faculty of Medicine, Zagazig University, Egypt
Email: samarsaeed_76@yahoo.com; Tel.: 01228442139

was first identified in 1996 in the eastern United States then spread to Greece, China, South America and Italy, furthermore it has occurred in other countries in sporadic cases.[4]

Class B MBL are able to hydrolyse all β-lactams except for aztreonam, a monobactam, and their hydrolytic activity is reduced or inhibited by EDTA; Ethylenediaminetetraacetic acid but not by clavulanic acid. The most common enzymes are VIM (Verona integron-encoded MBLs), IMP (active on imipenem) and NDM (New Delhi metallo-beta-lactamase). They have been detected Worldwide especially in Greece, Taiwan and Japan.[5] Class D enzymes are referred to as OXA (Oxacillin resistant) carbapenemases, they are not inhibited by clavulanic acid or EDTA. The first identified class D enzymes of the OXA-48 was in Turkey in 2003. OXA-48 is now endemic in countries around the Mediterranean and is rapidly spreading into other countries in Europe.[6]

Carbapenemases can be detected by the Modified Hodge Test (MHT) and the carbapenemase inhibition tests, in addition to detection of specific carbapenemase production genes by molecular methods as PCR.[7]

This study was conducted to determine the occurrence of carbapenem -resistant isolates of *K. pneumoniae* isolated from patients admitted to Zagazig University hospitals. As well as comparing phenotypic and genotypic methods for detection of carbapenemases produced in these isolates.

METHODOLOGY

Type of the study:

This is an observational cross sectional study that was conducted at the Medical Microbiology and Immunology Department, Faculty of Medicine, Zagazig University, Egypt for the period from June 2014 to June 2015. Patients who were admitted to the Intensive Care Unit and different wards of Zagazig University hospitals were enrolled. This study obtained an approval from the local institutional review board (Zagazig University IRB).

Sample Collection:

Mid-stream urine, sputa, wound, and blood specimens were collected from 430 patients. Consents were obtained from patients or guardians.

Cultivation and Identification of isolates:

Samples were collected and processed using standard Microbiologic procedures.[8] Mucoid lactose fermenting colonies were subjected to identification as *K. pneumoniae* by Gram staining and conventional tests[8]

Antimicrobial susceptibility testing of *K. pneumoniae* isolates

The antimicrobial susceptibility profiles of the isolates were determined by the disc diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [9], using amoxicillin-clavulinic acid, piperacillin-tazobactam, ceftriaxone, ceftazidime, cefoperazone, cefotaxime, cefepime, meropenem, ertapenem, azteronam, gentamicin, amikacin, Levofloxacin, ciprofloxacin, trimethoprim/ sulphamethoxazole, tigecycline and colistin. (Oxoid Ltd., Basingstoke UK).

Following the CLSI criterion, *K. pneumoniae* isolates showing resistance to one or more of the carbapenem discs and to one or more of the cephalosporins class III were selected for further testing by modified Hodge Test (MHT). [9] Furthermore, these isolates were subjected to combined disc test (CDT) and genes detection by multiplex PCR.

Modified Hodge test to detect carbapenemase production

Carbapenemase production was detected by using the MHT. This test was performed as per the CLSI guidelines.[9] The presence of an enhanced growth at the intersection of the streak and the zone of inhibition (notable indentation or "cloverleaf" appearance) indicated carbapenemase production (figure 1).

Combined disc test (CDT) to differentiate classes of carbapenemases:

CDT was performed as previously described by Tsakris et al. [10] The diameter of the growth inhibitory zone around the meropenem disc with PBA, EDTA, or PBA plus EDTA was compared with that around the plain meropenem disc and the data were interpreted (Table 1). The temocillin was added to detect OXA carbapenemase[11]

Table (1): Interpretation of phenyl boronic acid (PBA) and EDTA synergy tests and temocillin disc diffusion

Test	*Definition of positive test result*	*Class A carbapenemase*	*Class B carbapenemase*	*Class A plus B carbapenemase*	*Class D carbapenemase*
PBA synergy test	Meropenem± PBA ≥5 mm	+	-	-	-
EDTA synergy test	Meropenem± EDTA ≥5 mm	-	+	-	-
PBA+EDTA synergy test	Meropenem± PBA plus DPA ≥5 mm	+	+	+	-
Temocillin 30 µg disc [a]	Temocillin zone ≤10 mm	-	±	-	+

a :Interpreted only if PBA or EDTA synergy is absent.

DNA Extraction and detection of bla $_{NDM}$, bla$_{VIM}$, bla$_{IMP}$, bla $_{KPC}$ and bla $_{OXA-48}$:

DNA extraction was carried out according to CDC; Center of disease control and prevention, protocol by the boiling method .[12]

NDM -1, VIM, IMP & KPC genes were amplified through the Multiplex PCR using specific primers (Table 2). The PCR reaction mixture contained: 0.5 µL DNA (50 ng) in 24.5 µL Multiplex PCR Master Mix; QIAGEN®, Germany (containing: HotStarTaq® DNA Polymerase, Multiplex PCR Buffer, dNTP Mix, Q-Solution, 5x, RNAse-Free Water) and 5 µl 10x primer mix; 2 µM each primer (Midland Certified Reagent Company Inc, USA). Each PCR program must start with an initial heat-activation step at 95°C for 15 min to activate HotStarTaq DNA Polymerase, then thermal cycling (Biometra T gradient, Germany) for 30 cycles was done at 94°C for 1 min, 54°C for 1 min and 72°C for one and half min. and the final extension step was performed for 5 min at 72°C [13]

OXA-48 was amplified through PCR using specific primers (Table 2) using the following conditions: 94ºC for 5 minutes; 35 cycles of 94ºC for 60 seconds; 55ºC for 45 seconds, and 72ºC for 60 seconds; and a final heating at 72ºC for 7 minutes.[14] An universal 16S rRNA gene was amplified with 1500 bp product in each PCR run as positive control.[15] Reagent control was included in each PCR run as negative control. PCR products were analyzed in parallel with a DNA MW-marker (iNtRON Biotechnology, Korea) by electrophoresis on 2% agarose gel (Figure 2)

Table 2: Primer sequences of 5 bla$_{NDM-1}$, bla$_{VIM}$, bla$_{IMP}$, bla$_{KPC}$ and bla $_{OXA-48}$ genes

Gene target	*Primers sequence (5'-3')*	*Product size (bp)*	*Reference*
NDM-1	GCATAAGTCGCAATCCCCG CTTCCTATCTCGACATGCCG	237	[13]
VIM	GTTTGGTCGCATATCGCAAC AATGCGCAGCACCAGGATAG	382	[13]
IMP	GAAGGCGTTTATGTTCATAC GTAAGTTTCAAGAGTGATGC	587	[13]
KPC	TCGAACAGGACTTTGGCG GGAACCAGCGCATTTTTGC	201	[13]
OXA-48	TTGGTGGCATCGATTATCGG GAGCACTTCTTTTGTGATGGC	734	[14]

Statistical analysis:

Collected data were computerized and statistically analyzed using SPSS program version 18.0. Qualitative data were represented as percentages. Accuracy was represented using the terms sensitivity, specificity, positive predictive value, negative predictive value. Chi square test was used to calculate difference between qualitative variables. *P*-value of <0.05 indicates significant results.

RESULTS

Out of 430 different clinical specimens, 100 (23.25%) *K. pneumoniae* isolates were identified over the study period. The sources of the isolates were: urine (46%), pus (32%), blood (14%), and sputa (8%).

Carbapenem- resistance was detected in 42 *K. pneumoniae* isolates (42%) by agar disc diffusion screening test. The most common source of carbapenem-resistant *K. pneumoniae* isolates came from medical ICU (59.6%) followed by surgical ICU (19%), the internal medicine wards (14.3%), and the least percentage was found in surgical ward (7.14%).

The susceptibility pattern of carbapenem resistant and sensitive *K. pneumoniae isolates* to antimicrobial agents is shown in table 3. In our study, carbapenem-resistant *K. pneumoniae* isolates comprised a large proportion of the isolates resistant to various antibiotic classes with significant association (Table 3). They were found to be 100% resistant to ceftriaxone, cefotaxime, ceftazidime, and cefoperazone. High resistance rates were also observed to cefepime (95.2%), azteronam (88.1%) amoxycillin-clavulanic acid (88.1%). However, only (4.8%) were found to be resistant to tigecycline and all the isolates were sensitive to colistin. (Table 3).

Out of the 42 carbapenem resistant isolates, 34 (80.95%) isolates were identified as carbapenemase producers by MHT; 27 isolates out of the 34 MHT positive isolates were positive by CDT, also by which different classes of carbapenemases were determined (Table 4).

Table 3: The antimicrobial resistance pattern among carbapenem resistant and carbapenem sensitive *K pneumoniae* isolates.

Antibiotic	*Carbapenem-Resistant N=42*		*Carbapenem-Sensitive N=58*	
	No.	**%**	**No.**	**%**
Colistin	0	0	0	0
Tigecycline	2	4.8	0	0
Levofloxacin*	11	26.2	2	3.44
Pipracillin/tazobactam	15	35.7	12	20.6
Gentamicin*	21	50	5	8.6
Amikacin*,	22	52.4	6	10.34
Ciprofloxacin *	27	64.3	11	18.96
Trimethoprim */sulphamethoxazole	35	83.3	28	48.27
Amoxycillin-clavulanic acid	37	88.1	51	87.9
Azteronam*	37	88.1	19	32.75
Cefepime*	40	95.2	32	55.17
Ceftazidime*	42	100	11	18.9
Cefotaxime*	42	100	11	18.9
Ceftriaxone*	42	100	11	18.9
Cefoperazone *	42	100	15	25.8

* Significant difference ($P < 0.05$, by chi-square test)

Table 4: Carbapenemases classes in carbapenem resistant *K. pneumoniae* isolates as detected by CDT

Classes of Carbapenemase	*No. of K.pneumoniae isolates*	*% of K.pneumoniae isolates*
Class A only	6	25.2
Class B only	7	16.66
Class A+B	2	4.76
Class D only	12	28.57

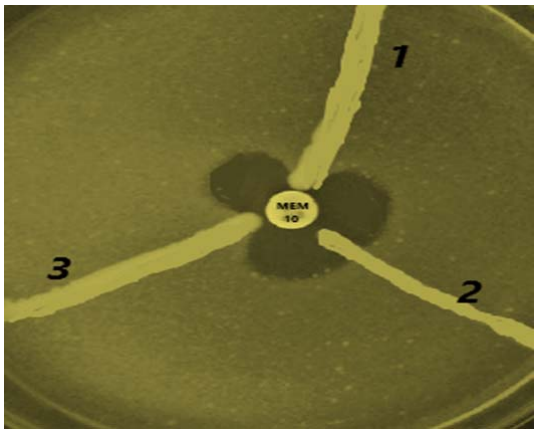

Fig. 1: Modified Hodge test: "Clover leaf"- shaped inhibition zones indicating carbapenemase production by the tested isolates (1&2), while isolate 3 gave negative result.

The results of the Multiplex PCR for five target genes are shown in (Fig-2). Out of 42 carbapenem resistant isolates, 25 isolates harbored one gene, while in 17 isolates none of the gene was detected. The most common resistance gene was bla $_{OXA-48}$ (12/42) followed by bla$_{KPC}$ (8/42) while the bla$_{VIM}$ gene (4/42) while bla $_{NDM-1}$ was detected only in one isolate. bla$_{IMP}$ had not been detected in our isolates. Performance of MHT and CDT in comparison to Multiplex PCR among carbapenemase producing isolates was assessed (table 5). MHT showed sensitivity and specificity of 100% & 47.06% respectively, while CDT showed sensitivity and specificity of 100% and 88.89 respectively

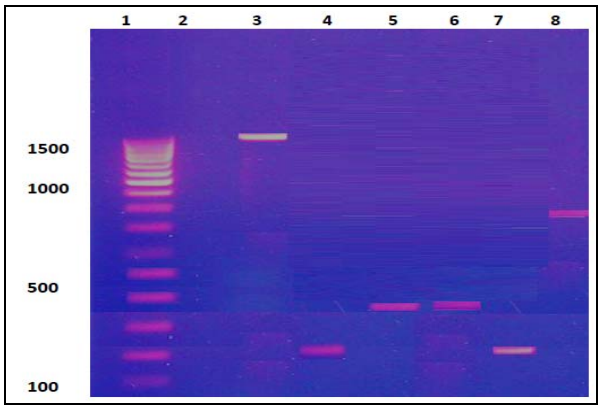

Fig. 2: Gel electrophoresis results of PCR amplicons of carbapenem-resistant *K. pneumoniae.* Lane 1: molecular weight marker, Lane 2: Negative control, Lane 3: 16S rRNA gene product as positive control (1500 bp), Lane 4&7:-bla$_{KPC}$ positive (201bp). Lane 5&6: bla $_{VIM}$ (382 bp) and Lane 8: bla $_{OXA-48}$ positive (734 bp)

Table 5: Performance of MHT and CDT in comparison to Multiplex PCR among carbapenemase producing *K. pneumoniae*

Carbapenemase detection test	*Class of carbapenemase*	*Sensitivity*	*Specificity*	*PPV*	*NPV*
PBA synergy test	A	100	100	100	100
EDTA synergy test	B	100	94.59	71.43	100
Synergy test with PBA plus EDTA	A & B	100	100	100	100
No synergy with PBA or EDTA and temocillin zone ≤10 mm	D	100	100	100	100
Overall CDT	A,B&D	100	88.89	100	100
Modified Hodge test	-	100	47.06	73.53	100

*PPV: Positive Predictive value, NPV: Negative Predictive value

DISCUSSION

The prevalence of carbapenem-resistant *K pneumoniae* is on the rise worldwide, causing serious opportunistic infections resulting in a difficult situation to manage for clinical microbiology laboratories, clinicians and infection preventionists. Resistance is conferred by carbapenemases, which are β -lactamases that can breakdown essentially all β –lactams.[2]

In our study, *K. pneumoniae* was isolated from 100 (23.25%) isolates from 430 infections. In Al-Mansoura University Hospital, Egypt, *K. pneumoniae* represented 15.4% [16], and in Al-Azhar University Hospital, Egypt, *K. pneumoniae* represented 14.2%. [17]

In the current study, 42 *K. pneumoniae* isolates (42%) were carbapenem-resistant. This result agreed with other results varying from 20 to 40 % in New York [18], Greece. [19] Higher results were shown in other studies reaching 83% in USA.[20]

El-Sweify et al[21] reported that 44.3% of *K. pneumoniae* isolates were carbapenem-resistant, while other Egyptian studies showed lower incidence reaching 13.9% in the Egyptian National Cancer [22]and 14.2% in Al- Azhar University Hospital. [17] The high frequency of carbapenem – resistant *K. pneumoniae* in the current study could be attributed to the excessive empirical use of carbapenems in our hospital, and the improper application of the infection control measures.

The highest percentage of carbapenem-resistant *K. pneumoniae* isolates came from ICUs which was 78.6% (medical and surgical ICUs). This is in agreement with other researchers who found most of their isolates detected from ICUs concluding that ICU stay was a risk factor for carbapenem-resistant *K. pneumoniae a*cquisition. [23,24]

Regarding antibiotic activity, it was found in our study that all carbapenem-resistant *K. pneumoniae* were susceptible to colistin and 95.2% were susceptible to tigecycline. This is in agreement with other studies [25,26]

Carbapenem-resistant isolates showed significant resistance to amikacin and gentamycin compared with carbapenem sensitive isolates (52.4% vs 10.34%) and (50% vs 8.6%) respectively (P <0.05) These results were coincided with other studies. [26,27]

In the present study, although MHT was positive in 34 isolates, only 27 isolates were confirmed to produce carbapenemase by the carbapenemase inhibition tests. Kandeel [23] reported only 10 isolates were confirmed to be carbapenemase producers out of 24 MHT positive isolates. Pasteran et al [28] observed that false-positive isolates produced CTX-M-type ESBLs. Conversely, Miriagou et al [7] reported false negative results on using MHT and it was explained by presence of MBL-producing isolates exhibiting weak carbapenemase activity.

The evaluation of MHT comparing to PCR was assessed showing sensitivity, specificity, PPV and NPV of 100%, 47.06%, 73.53% and 100% respectively. Our study concluded that MHT was simple, low cost, having high sensitivity (100%) and could be applied in routine microbiological laboratories, however, there are some limitations as being time consuming, specificity problems (false positive results) as well as, sensitivity problems (false-negative results shown in other studies).

As regards to carbapenemase classes, 28.57% of our isolates produced carbapenemase class-D, 25.2% produced class A enzyme, 16.66% produced class-B, and 4.76% produced combination of class-A and B enzymes (Table 4). Sonia et al.[29]; found class D-carbapenemase in 13.7% of the strains. In the Asia-Pacific region, the SMART (Study for Monitoring Antimicrobial Resistance Trends) global surveillance program found that between 2008 and 2009, 42.7% of carbapenem – resistant *K. pneumoniae* strains produced class A, 23.6% produced class B, and 11.8% produced class D carbapenemases [30]

The most frequent carbapenemase genes among our isolates was OXA-48 gene; Class D. It was detected in 12 (28.57%) of carbapenam- resistant isolates. Since first detection in Turkey, the gene emerged in the Middle East and North Africa and those countries are considered as reservoirs expanding to India, Senegal, and Argentina [31] In Saudi Arabia, Memish et al[32] declared that OXA-48 and NDM-1 are the dominant carbapenemases across the kingdom.

In our study, KPC activity was detected in 8 isolates by boronic acid test and all of them were confirmed by PCR to harbor bla $_{kpc}$ gene, showing 100%

sensitivity and specificity. Similar results with 100% sensitivity and specificity were obtained in other studies.[10,33] Mathers et al. [33] stated that CDT had a higher specificity compared to MHT for the detection of KPC-producing bacteria.

CDT used for detection of MBL showed sensitivity of 94.59 % and specificity of 71.43% as 5 isolates yielded positive PCR results (4VIM and 1 NDM-1). This may be explained by presence of rarer carbapenemases, such as bla_{NMC-A}, and bla_{GES} as the PCR targets only specific carbapenemase genes with a possibility of potentially missing genes not amplified by the primers used. Similar results were observed by Singh et al. [34].

Pournaras et al.[35] stated that CDT exhibited high sensitivity (94.8%) and excellent specificity equal to those of the PCR assay while effectively differentiating KPC and/or MBL production.

Our study demonstrated the co- production of KPC and MBL in 2 isolates. The production of both enzymes might contribute to their hydrolytic activity and levels of resistance to broad- spectrum β lactams, as well as to the possible co-migration of both enzymes.[36]

OXA-48 (class D) was detected in 12 isolates by adding temocillin to CDT . On evaluated with PCR detection of bla_{OXA-48}, it showed 100% sensitivity and specificity. These results were in line with van Dijk et al. [11] Tsakris et al. [37] concluded that this OXA-48 disc test is an accurate phenotypic surrogate marker for the direct differentiation of OXA-48 producing isolates showing sensitivity and specificity of 96.3% and 97.7% respectively.

Compared to MHT, CDT is a satisfactory and inexpensive method for detection and characterization of different classes of the carbapenemases. The proposed CDT protocol exhibited 100% sensitivity and excellent specificity (88.89%). These results are in accordance with results obtained by Solanki et al[13] who stated that CDT has 100% sensitivity and specificity comparing to PCR, however they recommended molecular methods like Multiplex PCR for the optimal detection of carbapenemase. Kaase et al.[38] stated that multiplex PCR is more reliable test for carbapenemase detection over CDT.

Memish et al. [32] evidenced that a combination of phenotypic tests for carbapenemase production in conjunction with PCR detection of carbapenemase genes had substantially improved the quality of the data.

CONCLUSION

Our study concluded that carbapenem resistant *K. pneumoniae* in Zagazig University Hospitals is an increasing problem, caused by the selective pressure exerted by intensively used carbapenems. This requires more attention to rationalize the antibiotic usage and strengthen the application of infection control precautions. It is very important to detect and report carbapenemase production. The more rapid turnaround time of Multiplex PCR suggest this method might be more suitable as an initial screening test for carbapenemase production that overwhelms the limitations of the phenotypic methods, it would improve clinical consequences.by optimizing the selection and beginning of appropriate antibiotic therapy in a timely manner.

REFERENCES

1. Chen LF, Anderson DJ, Paterson DL. Overview of the epidemiology and the threat of *Klebsiella pneumonia* carbapenemases (KPC) resistance. Infect Drug Resist 2012; 5: 133 – 41.).
2. Tzouvelekis LS, Markogiannakis A, Psichogiou M, Tassios PT, Daikos GL. Carbapenemases in Klebsiella pneumoniae and Other Enterobacteriaceae: an Evolving Crisis of Global Dimensions. *Clinical Microbiology Reviews*. 2012;25(4):682-707.
3. Hrabák J., Chudáčkova E. , Papagiannitsis C.C.: Detection of carbapenemases in Enterobacteriaceae: a challenge for diagnostic microbiological laboratories Clinical Microbiology and Infection , 2014;20 :839 – 853
4. Nordmann P, Naas T, Poirel L Global spread of Carbapenemase-producing Enterobacteriaceae. Emerg Infect Dis 2011;17:1791-8.
5. Toleman MA, Bugert JJ, Nizam SA. Extensively Drug-Resistant New Delhi Metallo-β-Lactamase–Encoding Bacteria in the Environment, Dhaka, Bangladesh. *Emerging Infectious Diseases*. 2015;21(6):1027-1030.
6. Rendani I. Manenzhe, Heather J. Zar , Mark P. Nicol and Mamadou Kaba1, J The spread of carbapenemase-producing bacteria in Africa: a systematic review. Antimicrob. Chemother. ;2014: 12-16
7. Miriagou V1, Cornaglia G, Edelstein M, Galani I, Giske CG, Gniadkowski Met al.. Acquired carbapenemases in Gram-negative bacterial pathogens: detection and surveillance issues. Clin. Microbiol. Infect. 2010;16:112–122
8. Bergey's Manual of Systematic Bacteriology. (2005): 2nd edn. Edited by Brenner, D,J., Kreig, N.R., Staley, J.T., and Garrity, G.M. Springer, New York
9. Clinical and Laboratory Standards Institute,. Performance standards for antimicrobial susceptibility testing; 24th Informational Supplement; Wayne, Pennsylvania. 2014 CLSI Document M100-S24.Volume 34, No.1
10. Tsakris A, Poulou A, Themeli-Digalaki K, et al. Use of Boronic Acid Disk Tests To Detect Extended- Spectrum β-Lactamases in Clinical

Isolates of KPC Carbapenemase-Possessing *Enterobacteriaceae* . *Journal of Clinical Microbiology*. 2009;47(11):3420-3426.
11. Van Dijk K, Voets GM, Scharringa J, et al. Disc diffusion assay for detection of class A, B and OXA-48 carbapenemases in Enterobacteriaceae using phenyl boronic acid, dipicolinic acid and temocillin. Clin Microbiol Infect 2014;20:345-349.
12. Center for disease control and Prevention. Multiplex Real-Time PCR Detection of *Klebsiella pneumoniae* Carbapenemase (KPC) and New Delhi metallo-β-lactamase (NDM-1) genes, CDC Available at http://www.cdc.gov/HAI/settings/lab/kpc-ndm1-lab-protocol. accessed April 20, 2013.
13. Solanki R, Vanjari L, Subramanian S, B A, E N, Lakshmi V: Comparative Evaluation of Multiplex PCR and Routine Laboratory Phenotypic Methods for Detection of Carbapenemases among Gram Negative Bacilli. J Clin Diagn Res. 2014; 8(12):23-26.
14. Aktas Z, Kayacan ÇB, Schneider I, Can B, Midilli K, Bauernfeind A. Carbapenemhydrolyzing oxacillinase, OXA-48, persist in Klebsiella penumoniae in Istanbul, Turkey. Chemotherapy 2008; 54: 101-6.
15. Jenkins C1, Ling CL, Ciesielczuk HL, Lockwood J, Hopkins S, McHugh TD, et al. Detection and identification of bacteria in clinical samples by 16S rRNA gene sequencing: comparison of two different approaches in clinical practice. J Med Microbiol. 2012 ;61:483-8.
16. Kandeel, A.Y.A.. Genotyping of nosocomial methicillin-resistant *Staphylococcus aureus* isolates in Mansoura University Hospital by restriction endonuclease digestion. *J. Mansoura Uni.,* 2000;51(2): 561 577.
17. Khaleid, M., Ibraheim, Z., Eman, M. 2010. Surgical site infections and associated risk factors in Egyptian orthopedic patients. *J. Amer. Sci.,* 6(7): 272 280.
18. Bratu, S., Landman, D., Haag, R., Recco, R., Eramo, A., Alam, M., et al.. Rapid spread of carbapenem resistant *Klebsiella pneumoniae* in New York City: a new threat to our antibiotic armamentarium. *Arch. Intern. Med.,* 2005;165(12): 1430 1435.
19. Giakkoupi P, iakkoupi P, Pappa O, Polemis M, Emerging *Klebsie lla pneumoniae*isolates coproducing KPC-2 and VIM-1 carbapenemases. Antimicrob Agents Chemother 2009;53:4048-50.
20. Marquez, P., Terashita, D., Dassey, D., Mascola, L.. Population based incidence of carbapenem resistant *K. pneumoniaee* along the continuum of care, Los Angeles County. *Infect. Cont. Hosp. Epidemiol.,* 2013;34(2): 144 150.
21. El-Sweify M A., Nahed I. Gomaa, Nermine N. El-Maraghy and Hasnaa A. Mohamed Phenotypic Detection of Carbapenem Resistance among Klebsiella pneumoniae in Suez Canal University Hospitals, Ismailiya, Egypt Int. J. Curr. Microbiol. App.Sci 2015;4(2): 10-18
22. Hossam, M.A., Amany, E. 2009. Species distribution and antimicrobial susceptibility of gram-negative aerobic bacteria in hospitalized cancer patients. J. Trans. Med., 7(4): 7 14.
23. Kandeel A, Epidemiology of carbapenemase producing Enterobacteriaceae in a general hospital Journal of Microbiology and Infectious Diseases / 2015; 5 (2): 57-62
24. Maragakis LL. Recognition and prevention of multidrug-resistant Gram-negative bacteria in the intensive care unit. Crit Care Med. 2010;38(Suppl 8):S345-351.
25. Karaiskos L & Giamarellou H Multidrug-resistant and extensively drug-resistant Gram-negative pathogens: current and emerging therapeutic approaches Expert Opinion on Pharmacotherapy 2014;15(10):1351-1370
26. Shanmugam P, Meenakshisundaram J, Jayaraman P. bla_{KPC} gene Detection in Clinical Isolates of Carbapenem Resistant *Enterobacteriaceae* in a Tertiary Care Hospital. *Journal of Clinical and Diagnostic Research□: JCDR*. 2013;7(12):2736-2738.
27. Bina M, Pournajaf A, Mirkalantari S, Talebi M, Irajian G. Detection of the*Klebsiella pneumoniae* carbapenemase (KPC) in K. pneumoniaee Isolated from the Clinical Samples by the Phenotypic and Genotypic Methods. *Iranian Journal of Pathology*. 2015;10(3):199-205.
28. Pasteran F, Mendez T, Rapoport M Controlling false-positive results obtained with the Hodge and Masuda assays for detection of class A carbapenemase in species of Enterobacteri-aceaeby incorporating boronic acid. J Clin Microbiol 2010;48:1323-1332.
29. Sonia K, Basma Mnif, Fadoua Louati,Sonia Rekik, Sonda Mezghani, Fouzia Mahjoubi,. Spread of *Klebsiella pneumoniae* isolates producing *OXA*- 48 -lactamase in a Tunisian University Hospital. *J. Antimicrobiol. Chemother.,* 2011;10(1): 181 189.
30. Christine, L., Gisele, E., Meredith, H., Kevin, B., Johann, D.. Surveillance and Molecular Epidemiology of *Klebsiella pneumoniae* isolates that produce Carbapenemases: First report. *Antimicrob. Agents Chemother.* 2012*;* 57(1): 130 136.
31. Poirel L, Bonnin RA, Nordmann P. Genetic Features of the Widespread Plasmid Coding for the Carbapenemase OXA-48. *Antimicrobial Agents and Chemotherapy*. 2012;56(1):559-562.
32. Memish Z A., Assiri A, Almasri M, Roshdy H, Hathout H, Kaase M, et al. Microbial Drug Resistance.2015;21(3): 307-314.

33. Mathers A J., Carroll J, . Sifri CD and . Hazen KC Modified Hodge Test versus Indirect Carbapenemase Test: Prospective Evaluation of a Phenotypic Assay for Detection of Klebsiella pneumoniae Carbapenemase (KPC) in Enterobacteriaceae J. Clin. Microbiol. 2013; 51:4 1291-1293
34. Singh K, Mangold KA, Wyant K, Schora DM, Voss B, Kaul KL, Hayden MK, et al. Rectal screening for Klebsiella pneumoniae carbapenemases: comparison of real-time PCR and culture using two selective screening agar plates. J. Clin. Microbiol. 2012;50:2596–2600
35. Pournaras S, Zarkotou O, Poulou A A Combined Disk Test for Direct Differentiation of Carbapenemase-Producing Enterobacteriaceae in Surveillance Rectal Swabs. *Journal of Clinical Microbiology*. 2013;51(9):2986-2990.
36. Chmelnitsky, I., Navon-Venezia, S., Strahilevitz, J.. Plasmid-mediated qnrB2 and carbapenemase gene blaKPC-2 carried on the same plasmid in carbapenem-resistant ciprofloxacinsusceptible Enterobacter cloacae isolates. *Antimicrob. Agents Chemother.* 2008*;* 52:2962 5.
37. Tsakris A, Poulou A, Bogaerts P, Dimitroulia E, Pournaras S, Glupczynski Y. Evaluation of a New Phenotypic OXA-48 Disk Test for Differentiation of OXA-48 Carbapenemase-Producing Enterobacteriaceae Clinical Isolates. Bourbeau P, ed. *Journal of Clinical Microbiology*. 2015; 53(4):1245 1251.
38. Kaase M, Szabados F, Wassill L and Gatermann SG Detection of Carbapenemases in Enterobacteriaceae by a Commercial Multiplex PCR J. Clin. Microbiol. 2012;50:3115-3118