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Prevalence of MBL-among the tested carbapenemase producing EB

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Dissemination of carbapenem resistant Enterobacteriaceae (CRE) poses a considerable threat to public health. The aim of the present work was to estimate the prevalence of metallo beta lactamases (MBL) among CRE isolated from Alexandria Main University Hospital, Egypt, to evaluate the performance of different phenotypic methods for the detection of MBL, and to investigate the local antimicrobial sensitivity profile of these isolates. Eighty CRE were tested for MBL production by Etest® MBL MP/MPI, EDTA double disc synergy test, and EDTA combined disc test. All isolates were confirmed as MBL producers by polymerase chain reaction (PCR). Antibiotic sensitivity testing was performed using disc diffusion method. Among the 80 CRE, 56 isolates (70%) were MBL by PCR. Fifty-four isolates were positive for NDM (96.4%). Meropenem EDTA CDT was the most sensitive test (94.6%). Blood was the most frequent sample from which MBL were isolated (51.7%). Majority of the isolates were isolated from intensive care units (82.1%). All MBL were multidrug resistant; Colistin and polymyxin B showed the lowest resistance rate (26.8 and 19.6%, respectively). The EDTA-CDT will provide a reliable, convenient, and cost-effective approach for detection of MBL in laboratories, which cannot afford to perform molecular tests.

Key words: Metallo beta lactamases, carbapenem resistant Enterobacteriaceae, New Delhi lactamase.

INTRODUCTION

Multidrug resistance is emerging worldwide at an alarming rate among a variety of bacterial species, including the family Enterobacteriaceae (EB) (IDSA et al., 2011). EB members have the tendency to spread easily between humans and to acquire genetic material through horizontal gene transfer, mediated by plasmids and transposons (Nordmann et al., 2011a).The rapid dissemination of extended spectrum beta lactamases (ESBL) in EB has led to the increased utilization of carbapenems in clinical practice. This is largely because ESBL-producers are capable of hydrolyzing all beta lactams except carbapenems, thus making carbapenems the last option for the treatment of serious infections associated with these organisms

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(Paterson and Bonomo, 2005).

As a consequence of increased use of carbapenems has been the emergence of isolates coding for carbapenemases (Nordmann et al., 2011a). Carbapenem-hydrolysing β-lactamases belong mainly to three clinically significant Amber classes, namely Ambler class A (*Klebsiella pneumoniae* carbapenemases; KPCs), class B (metallo-beta-lactamases; MBLs) and class D (oxacillinases) (Queenan and Bush, 2007).

MBL are broad-spectrum and hydrolyse all beta lactams except monobactams and they are not susceptible to therapeutic β-lactams inhibitors such as clavulanate, sulbactam, and tazobactam. MBLs require zinc-ions to catalyze the hydrolysis of beta-lactam antibiotics and due to the dependence on zinc ions; MBL catalysis is inhibited in the presence of metal-chelating agents like ethylenediaminetetraacetic acid (EDTA) (Bush and Jacoby, 2010). They are mostly found in *K. pneumoniae* isolates and are also frequently associated with serious nosocomial infections and outbreaks (Poirel et al., 2007).

Verona integron-encoded MBLs (VIM) and active on imipenem (IMP) were the common MBLs identified in EB (Nordmann and Poirel, 2002). New Delhi b-lactamase (NDM-1), which originated in India, was first reported in 2009 (Yong et al., 2009), and has been isolated in Europe, Asia, North America, Australasia, and Middle East (Grundmann et al., 2010; Wu et al., 2010; CDC, 2010; Poirel et al., 2010a; Shibl et al., 2013).

Although different phenotypic methods have been described, the Clinical and Laboratory Standards Institute (CLSI) currently does not include standardized recommendations for MBL screening. Carbapenemase gene detection by PCR is considered the gold standard, but its accessibility is often limited to reference laboratories (Behera et al., 2008). Other non-molecular based techniques have been studied; all depend on inhibition of carbapenemase activity by chelating agents e.g. EDTA, dipicolinic acid, and thiol compounds (Galani et al., 2008b).

The aim of the study was to: (1) Estimate the prevalence of MBL-among the tested carbapenemase producing EB isolated from Alexandria Main University Hospital (AMUH), Egypt. 2) Evaluate the performance of different phenotypic methods for the detection of MBL-producing EB in comparison with the gold standard PCR for MBL genes, in order to select a rapid, reliable, economical, and easy to set up workflow method for detection of MBLs. 3) Describe the local antimicrobial sensitivity profile of MBL producing EB isolates aiming to establish an appropriate empirical treatment.

MATERIALS AND METHODS

Clinical isolates

A total of 706 clinical isolates of EB isolated from blood, biological fluids, urine, bronchoalveolar lavage, pus and sputum specimens

from patients admitted to various wards and intensive care units in AMUH, were included in a descriptive cross sectional study. The study was conducted for a six-month period starting from January through July 2015.

All isolates were identified by conventional microbiological methods (Tille et al., 2013). All EB isolates were subjected to initial screening for carbapenem resistance byertapenem (ETP) 10 ug, meropenem (MEM) 10 ug, and imipenem (IPM) 10 ug discs, by disc diffusion method and by testing IPM MIC using the broth microdilution method according to CLSI guidelines (CLSI, 2015). Only 80 carbapenem resistant isolates (randomly selected covering the 6 months study duration) were tested for MBL production. Two criteria were used for selection of the 80 isolates: (i) an intermediate or resistant susceptibility to one of the carbapenems (MEM, IPM, ETP) and (ii) an IPM MIC of ≥2 ug/ml (intermediate or resistant). *Escherichia coli* ATCC 25922 was used as a control strain. The control strain was run simultaneously with the test organisms.

The 80 isolates were identified to the species level by the use of mass spectrometry, matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) (Seng et al., 2009) (Bruker Corporation) and were then subjected to the following.

Carbapenemase production confirmatory tests

Modified Hodge test (MHT)

It was performed in accordance with the CLSI guidelines (CLSI, 2015). A 0.5 McFarland standard suspension of *E. coli* ATCC 25922 was prepared in broth. A Mueller Hinton agar plate was inoculated as for the routine disk-diffusion procedure. The plate was allowed to dry for 10 min. MEM disk was placed in the center of the plate. Using a 10 µL loop, three to five colonies of the test organism grown overnight on a blood agar plate were picked and inoculated in a straight line out from the edge of the disk. The streak was at least 20 to 25 mm in length. Following incubation, Mueller Hinton agar was examined for enhanced growth around the test streak at the intersection of the streak and the zone of inhibition.

Result: Enhanced growth = positive for carbapenemase production.

No enhanced growth = negative for carbapenemase production.

The addition of 100 ug/ml of $ZnSO_4$ in Mueller Hinton agar was performed on the same isolates, to improve the limit of detecting MBL production, as previously described (Girlich et al., 2011).

RAPIDEC® CarbaNP test (BioMerieux)

Only ten randomly selected isolates were further tested by RAPIDEC® carbaNP test according to the manufacturer's instructions.

MBL production detection tests

Phenotypic tests

Test isolates' suspensions were adjusted to turbidity equivalent to that of a 0.5 McFarland standard and used to inoculate Mueller-Hinton agar plates.

(a) Etest® MBL MP/MPI was done according to the manufacturer's instructions (BioMérieux, Marcy l'Etoile, France) (Galani et al., 2008b).
(b) EDTA double disc synergy test (EDTA –DDST): Two antibiotic

(MEM 10 ug, IPM 10ug) discs were placed at a distance of 10 mm from a blank filter paper disc (6 mm in diameter, Whatman filter paper no. 2) to which 10 ul of 0.5 M EDTA solution (Sigma-Aldrich, Germany) was added. After overnight incubation, the presence of any synergistic inhibition zone was interpreted as positive (Galani et al., 2008b).

(c) EDTA combined disc test (EDTA-CDT): Two 10 ug MEM discs and Two 10 ug IPM discs were placed on a plate inoculated with the test organism, and 10 ul of 0.5 M EDTA solution was added to one disc of MEM or IPM. The inhibition zones of the MEM and MEM+EDTA or IPM and IPM+EDTA disc were compared after overnight incubation. A zone diameter difference between any of the discs alone and with EDTA ≥7 mm was interpreted as positive (Galani et al., 2008b).

Genotypic tests (Multiplex PCR)

DNA extraction and multiplex PCR amplification for the simultaneous detection of NDM, VIM, and IMP MBL genes was carried out, as previously described, on a thermal cycler instrument (Techne Genius, Cambridge, UK) (Doyle et al., 2012).

Three primer pairs were used to target 3 MBL genes: NDM F: 5'-GCAGCTTGTCGGCCATGCGGGC-3', NDM R: 5' GGTCGCGAAGCTGAGCACCGCAT-3', VIM F:5'-GTTTGGTCGCATATCGCAAC-3', VIM R: 5'AATGCGCAGCACCAGGATAG-3', IMP F: 5'-GAAGGCGTTTATGTTCATAC-3', IMP R: 5'-GTACGTTTCAAGAGTGATGC-3'(Invitrogen by life technologies, Thermo Fisher Scientific Inc. USA).

Multiplex PCR amplification for the simultaneous detection of NDM, VIM, and IMP MBL genes was carried out on a thermal cycler instrument (Techne Genius, Cambridge, UK) using the following cycling conditions: initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 45 s, annealing at 55°C for 45 s and extension at 72°C for 60 s, then final extension of 72°C for 8 min. The PCR reaction consisted of 12.5 µl DreamTaq™ Green PCR Master Mix (2X) (Fermentas), 0.4 uM NDM primer, 0.3 uM each VIM and IMP primer, and PCR grade water to a final volume 25 ul. *E. coli* ATCC 25922 was used as negative control. The PCR products were electrophoresed for 45 min and visualized under UV light.

Antimicrobial susceptibility testing

Antibiotic susceptibility testing of the 80 CRE isolates was performed by the disc diffusion method using Mueller–Hinton agar. The following antibiotics were tested: ampicillin, piperacillin, amoxicillin/clavulanic acid, ampicillin/sulbactam, piperacillin/ tazobactam, cefoperazone/sulbactam, cefaclor, cefuroxime, ceftriaxone, ceftazidime, cefotaxime, cefepime, aztreonam, ciprofloxacin, levofloxacin, ofloxacin, amikacin, gentamicin, tobramycin, chloramphenicol, sulfamethoxazole-trimethoprim, tetracycline, doxycycline, minocycline, tigecycline, tienam, meropenem, ertapenem, colistin and polymyxin B. Norfloxacin, nitrofurantoin, and fosfomycin were only used for urinary isolates. All the antibiotic discs were procured from oxoid, UK. The results were interpreted as per CLSI guidelines (CLSI, 2015), except for colistin and tigecycline. The results for colistin were interpreted by following the criteria proposed by Galani et al. (2008a), and for tigecycline by the breakpoints for EB as suggested by The European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2015). *E. coli* ATCC 25922 was used as a control strain.

ESBL producers were identified by CLSI confirmatory test using a disc of cefotaxime (30 ug) and a disc of cefotaxime/clavulanate (30/10 ug). ESBL production was confirmed if the zone given by the cefotaxime/clavulanate disc was ≥5 mm larger than the zone given

by cefotaxime alone (CLSI, 2015).

Statistical analysis

Data were fed to the computer and analyzed using IBM SPSS software package version 22.0. Analysis of data was done using count percentage. The sensitivity, specificity, positive and negative predictive values and accuracy of the phenotypic MBL detection methods was evaluated using PCR as the gold standard.

Sensitivity = a ×100/a+c,
Specificity = d ×100/b+d,
Positive predictive value = a ×100/a+b,
Negative predictive value = d×100/c+d,
Accuracy = (a+d) ×100/a+b+c+d,

where a= True positives, b=False Positives, c=False Negatives, d=True Negatives (Ilstrup et al., 1990).

RESULTS

During the study period, 706 EB isolates were delivered to the Microbiology Laboratory of AMUH. A total of 240 isolates (33.9%) were initially identified as carbapenem resistant on the basis of their reduced susceptibility to MEM, IPM or ETP by disc diffusion test. Eighty carbapenem resistant EB (CRE) isolates were randomly selected for testing MBL production.

Out of 80 CRE isolates, 75 isolates (93.75%) were *K. pneumoniae*, three isolates (3.75%) were *E. coli* and two isolates (2.5%) were *Enterobacter cloacae* by MALDI-TOF. The highest rate of CRE isolates was isolated from blood cultures (41/80; 51.2%), followed by respiratory cultures (bronchoalveolar lavage, mini BAL, sputum) (25/80; 31.2%), urine (11/80; 13.8%), and pus cultures (3/80; 3.8%). Most of isolates (71/80; 88.75%) were from ICUs followed by internal medicine wards (7/80; 8.75%), then surgical wards (2/80; 2.5%). The mean age of patients was 46 years. Thirty five (44%) were males and 45 (56%) were females (male: female ratio; 1:1.3).

All CRE isolates were at least resistant to one of the carbapenems (IPM, MEM, ETP) by disc diffusion test. Three isolates (3.75%) were sensitive to each of IPM and MEM, while only one isolate (1.25%) was sensitive to ETP. Nine isolates (11.25%) were intermediate to IPM, while only one isolate (1.25%) was intermediate to MEM. Regarding the IPM MIC results, 72 isolates (90%) had an IPM MIC of 8 µg/ml (resistant) and three isolates (3.75%) had an MIC of 4 µg/ml (intermediate), while five isolates (6.25%) were sensitive by MIC (≤1 ug/ml). All sensitive and intermediate isolates were resistant to at least one of the carbapenems by disc diffusion.

The MHT revealed 72 isolates (90%) as positive and eight isolates (10%) as negative for carbapenemase production. Out of the ten isolates tested by RAPIDEC® CarbaNPtest, nine (90%) gave a positive reaction (Table 1 and Figure 1).

Presence or absence of MBL genes was considered the reference method for detection of MBL producing

Table 1. The results of the ten CRE isolates tested by RAPIDEC® CarbaNP test.

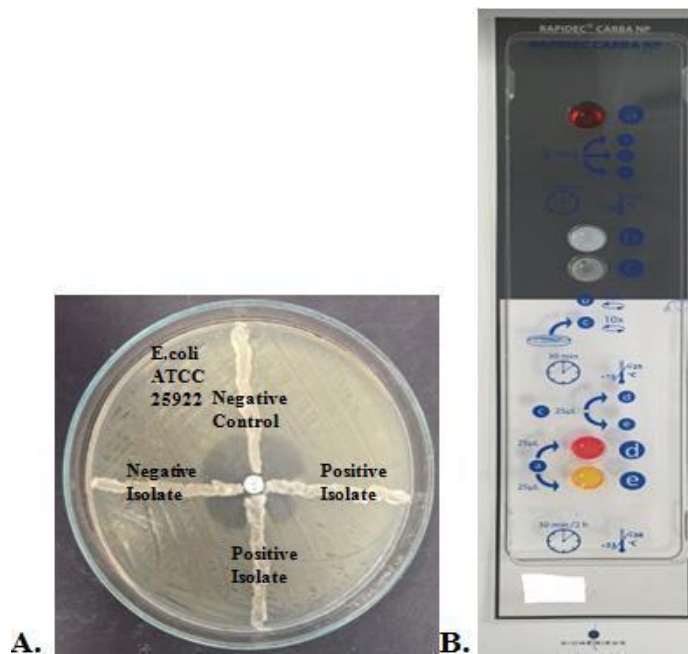| Isolate number	MHT	E-test® MBL	MEM EDTA DDST	IPM EDTA DDST	MEM EDTA CDT	IPM EDTA CDT	MBL PCR	RAPIDEC® CarbaNP test
1	+	-	+	+	+	+	+VIM	+
2	+	+	+	+	+	+	+NDM	+
3	+	-	+	+	+	+	+NDM	+
4	+	+	-	-	-	-	+NDM	+
5	-	+	+	+	+	+	+NDM	-
6	+	-	-	-	-	-	-	+
7	+	+	-	-	-	-	-	+
8	+	-	-	+	-	-	-	+
9	+	-	-	+	-	-	-	+
10	+	-	-	-	+	-	-	+

+: Positive, -: negative.

Figure 1. A. Results of MHT. B. Results of RAPIDEC® CarbaNP test (BioMerieux): a positive carbapenemase test.

CRE. Accordingly, among the selected 80 CRE, 56 isolates (56/80; 70%) were identified as MBL producing CRE. The distribution of MBL genes was as followed: 54 isolates were positive for NDM (67.5% from all CRE and 96.4% from MBL producing CRE), two positive for VIM (2.5% of all CRE and 3.6% of MBL producing CRE), and none of the isolates harbored IMP gene. Fifty-four MBL isolates (96.4%) were *K. pneumoniae* and two (3.6%) were *Enterobacter cloacae* (one harboring VIM gene and the other the NDM gene (Figure 2).

Out of the 56 PCR positive isolates, 50 (89.3%) were MHT positive and six (10.7%) were negative. Eight of the positive isolates (14.3%) gave a weakly positive reaction. The six negative MHT isolates were retested after addition of zinc and three isolates showed a positive test increasing the sensitivity of the test from 89.3 to 94.6%.

Regarding the results of phenotypic tests, the E-test® MBL MP/MPI identified 46 isolates (57.5%) as MBL producers, while the EDTA-DDST identified 60 isolates (75%) by IPM EDTA-DDST, and 54 isolates (67.5%) by MEM EDTA-DDST. The EDTA-CDT identified 67 isolates (83.75%) by MEM EDTA-CDT, and 55 isolates (68.75%) by IPM EDTA-CDT (Figure 3 and Table 2) summarizes the results of all phenotypic tests in comparison with MBL
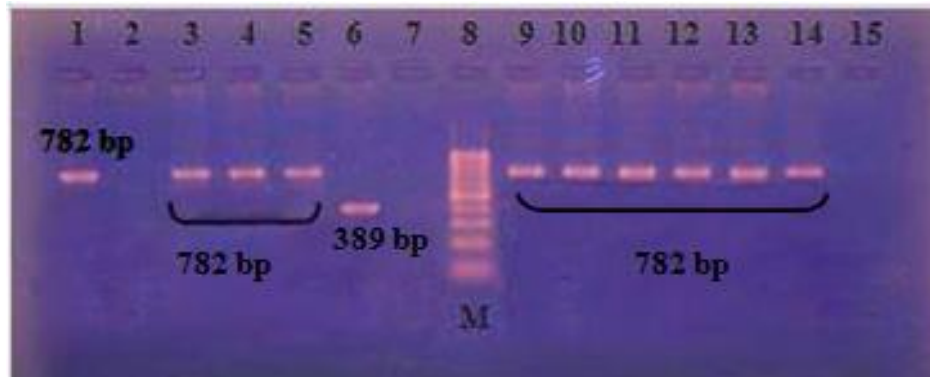
Figure 2. Agarose gel stained with ethidium bromide of MBL isolates. Lane 1, 3-5, 9-14: show positive amplified NDM gene (782 bp). Lane 6: shows VIM gene (389 bp). Lane 2 and 7: show negative results. Lane 15: negative control. Lane 8: 100-1000 bp DNA ladder.
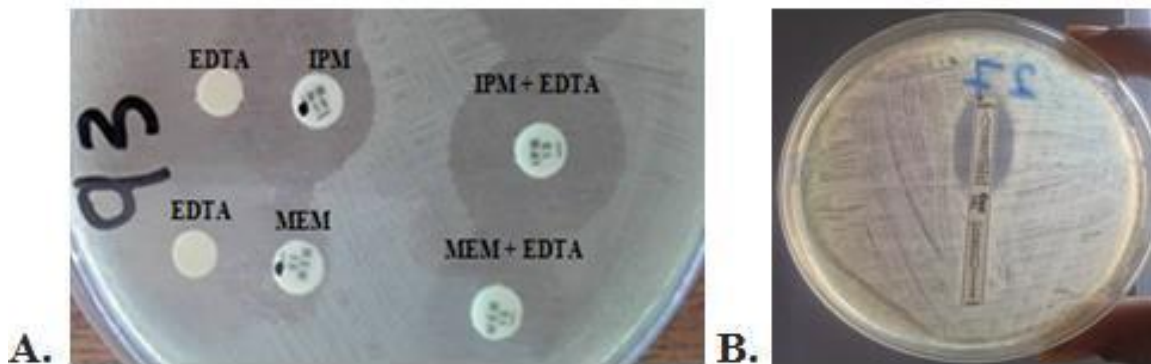

Figure 3. (A) Results of double disc and combined discs for testing of MBL. (B) Etest® MBL MP/MPI showing positive result.

Table 2. Results of all phenotypic tests in comparison to PCR for the 80 CRE isolates.

Phenotypic tests	MBL +ve (n=56)		MBL –ve (n=24)		Sensitivity	Specificity	PPV	NPV	Accuracy
	True +ve	False -ve	True -ve	False +ve	(%)	(%)	(%)	(%)	(%)
E-test® MBL	45	11	23	1	80.3	95.8	97.8	67.6	85
MEM EDTA DDST	45	11	15	9	80.3	62.5	83.3	57.7	75
IPM EDTA DDST	45	11	9	15	80.3	37.5	75	45	67.5
MEM EDTA CDT	53	3	10	14	94.6	41.6	79.1	76.9	78.75
IPM EDTA CDT	51	5	20	4	91.1	83.3	92.7	80	88.75

MBL +ve: MBL positive, MBL –ve: MBL negative. PPV: positive predictive value, NPV: negative predictive value.

gene detection by PCR. None of the tests was 100% sensitive orspecific.

The results of screening tests for carbapenemases (carbapenem disc diffusion, IPM MIC, MHT, carbaNP test) could not be included in the comparison to gold standard PCR results as they are used for screening of all types of carbapenemases and they are not specific for MBL only.

Concerning the antibiotic susceptibility profile, all MBL isolates (100%) were ESBL producers as detected by the CLSI confirmatory test, and all isolates (100%) were multidrug resistant (resistant to three or more antibiotic classes). All isolates (100%) were resistant to penicillins and cephalosporins except one isolate (98.2%) which

Table 3. The distribution of antimicrobial resistance of the 56 MBL isolates.

Antibiotic	Resistance	
	No	%
menepereM	45	96.4
mnnepereM	44	98.2
mpereMmM	45	91
nMpmimppmr	45	100
nmpeneimppmr	45	100
releipen	45	100
relxneumMe	45	100
Ceftriaxone	45	100
relnezmtmMe	45	100
releneumMe	45	100
relepmMe	44	98.2
nznneereM	44	98.2
nMeumimppmrvipe/xpermi eimt	45	100
nMpmimppmrvixpieineM	45	100
Piperacillin/tazobactam	45	96.4
Cefoperazone/sulbactam	45	94.6
Gentamicin	49	87.5
Amikacin	50	89.3
Tobramycin	51	91
Tetracycline	56	100
Doxycycline	44	78.6
Minocycline	44	78.6
Tigecycline	19	33.9
Ciprofloxacin	44	78.6
Levofloxacin	25	44.6
Ofloxacin	56	100
Trimethoprim-sulphamethoxazole	51	91
Chloramphenicol	40	71.4
Colistin	15	26.8
PolymyxinB	11	19.6
For urine isolates only (n=7)		
Norfloxacin	7	100
Nitrofurantoin	3	42.8
Fosfomycin	0	0
Combined Resistance		
Cephalosporins + fluoroquinolones + carbapenems	5	10.7
Cephalosporins + aminoglycosides + carbapenems	82	50
Cephalosporins + fluoroquinolones + aminoglycosides + carbapenems	51	33.9

was sensitive to each of cefepime and aztreonam. Antibiotic resistance pattern is as shown in Table 3.

Regarding the distribution of MBL positive isolates among the different clinical specimens, blood was the most frequent sample from which MBL were isolated (29 isolates, 51.7%), followed by respiratory samples (16 isolates, 28.6%), urine (8 isolates, 14.3%) and pus (3 isolates, 5.4%). Majority of MBL isolates were isolated from ICUs (46 isolates) representing 82.1%. Five isolates (8.9%) were from hematology ward. One isolate (1.8%) was isolated from each of the following wards (rheumatology, diabetes and metabolism, hepatology, plastic and burn unit). The mean age of patients was 46 years with male: female ratio 1:1.3.

DISCUSSION

According to data from the European Antimicrobial Resistance Surveillance Network (EARS Net, formerly EARSS, 2014), the population-weighted mean for carbapenem resistance was 8.3% in 2013 (ECDC, 2014). The percentages of resistant isolates in the reporting countries ranged from 0% (Bulgaria, Finland, Iceland, Latvia, Lithuania and Sweden) to 59.4 % (Greece).

Out of 594 *K. pneumoniae* isolates, 5.6% were carbapenem resistant, according to US Naval Medical Research Unit No 3, Global Disease Detection Program, conducted in Egypt as part of the National surveillance 2002 to 2010 (WHO, 2014). A higher prevalence was in the present study (33.9%). This could be explained by the continuous use of carbapenems in treatment due to high prevalence of ESBL strains in our hospital.

In the present study, a very high rate of MBL (56/80; 70%) was reported among EB, of these 96.4% was *K. pneumoniae* and 3.6% was *E. cloacae*. Several studies also demonstrated increasing incidence of MBL production in EB isolates (Yong et al., 2006; Datta et al., 2012).

In the present study, the MHT had a sensitivity of 94.6% for detecting MBL producers. This sensitivity is higher than that reported by Doyle et al. (2012) (only 12%). The MHT and MHT added with $ZnSO_4$ showed a positive result, respectively, for 72 and 75 of the 80 CRE isolates. Similarly, Ambretti et al. (2013) showed better MHT results after addition of zinc. This finding should be taken into consideration while detecting MBL in routine work.

For financial reasons, CarbaNP was performed for only ten CRE isolates, the sensitivity of the test (90%) was less than reported elsewhere (Nordmann et al., 2012). The negative carbaNP isolate (NDM MBL PCR positive) was also negative for MHT, this could explain negative results, however, the results obtained from this isolate need further assessment. The positive results obtained with MBL PCR negative isolates could be explained by the presence of other types of carbapenemases as positive results were obtained in less than 30 min with the five negative isolates.

According to the results of multiplex PCR, 56 out of 80 (70%) CRE were MBL producers, NDM gene was detected in 96.4% of the isolates. Similarly, in a study conducted in Pakistan, gene for NDM-1 enzymes was detected in 94% of clinical isolates and none of the clinical isolates were found positive for IMP, VIM and KPC enzymes (Sultan et al., 2013). On the other hand, *K. pneumoniae* isolates with VIM-MBLs have been found as causes of country wide epidemics in the USA, several Latin American countries, China and Europe (Grundmann et al., 2010).

NDM-1 is the latest carbapenemase to be discovered. It was first described in 2008 in *K. pneumoniae* and *E. coli* isolated in Sweden from an Indian patient transferred

from a New Delhi hospital (Yong et al., 2009). Most positive NDM bacterial isolates have shown epidemiological links to India and Pakistan (Nordmann et al., 2011b). It has been suggested that the Middle East region might be a secondary reservoir for the spread of NDM-1 isolates as there is a high frequency of population movement between the region and the Indian subcontinent (Nordmann et al., 2011b). NDM CRE have been reported in Oman (Poirel et al., 2010b), Kuwait (Jamal et al., 2011), Saudi Arabia (Shibl et al., 2013) and Morocco (Poirel et al., 2011). Of interest, the majority of the cases could not be directly linked to the Indian subcontinent nor had a history of foreign travel.

The first reported case of NDM MBL CRE in Egypt was in a cancer patient in Cairo, in 2012, by Abdelaziz et al. (2013). There was no apparent epidemiological link to an endemic area. The study reinforced the hypothesis of an autochthonous presence of the NDM resistance determinant in the Middle East and North African area. To the best of our knowledge, this is the first report of NDM carrying *K. pneumoniae* in Alexandria, Egypt.

In the present study, blood was the most frequent sample from which MBL were isolated (51.7%). Majority of MBL isolates were isolated from ICUs (82.1%). Our findings are in accordance with several studies, which found that the majority of clinical isolates were yielded from blood cultures and from patients in the ICU (Bora et al., 2014; Sultan et al., 2013; Shibl et al., 2013).

Out of three phenotypic methods used for confirmation of MBL production, the overall sensitivity of EDTA-CDT was better than that of E-test and EDTA-DDST. Previous studies also found that the combined disc test was a highly sensitive (100%) method among the analyzed procedures (Picao et al., 2008; Galani et al., 2008b; Bora et al., 2014). This could be attributed to the subjective interpretation of DDST results. It should be mentioned that a 7 mm was used as a cutoff in CDT (Galani et al., 2008b) and that our results of CDT would be definitely better (much sensitive test) if relied on a smaller diameter difference between EDTA free and EDTA combined discs.

The MBL IPM E-test was designed to detect the presence of MBLs in *P. aeruginosa* (Walsh et al., 2002). This E-test is often difficult to interpret when investigating the presence of MBLs in EB. This is due to the fact that the MICs of IPM are often low in EB that produce MBLs (Doyle et al., 2012). For this reason and as recommended by BioMérieux, the E-test was performed using MEM and not IPM EDTA strips. These results of this study were much better in term of sensitivity (80.3%) and specificity (95.8%) than that reported previously by authors using IPM EDTA strips (Doyle et al., 2012; Galani et al., 2008b).

MBL inhibitor (EDTA) may possess their own bactericidal activity, which may result in expanded inhibition zones not associated with true MBL production and hence false positive results (Chu et al., 2005). On the

other hand, authors reported that false-negative results might arise from carbapenem hydrolysis or inactivation caused by EDTA (Picao et al., 2008). Also, previous studies reported that phenotypic tests failed to identify the presence of MBL in isolates harboring more than one carbapenemase gene (Bartolini et al., 2014). This could justify false positive and negative results of all phenotypic tests encountered in our study.

NDM gene is carried on plasmids which also carry a number of other genes conferring resistance to aminoglycosides, macrolides and sulphamethoxazole, thus making these isolates multidrug resistant (Franklin et al., 2006). This is in agreement with the findings of the present study, which revealed that all isolates were multidrug resistant. Combined resistance to cephalosporins, aminoglycosides and carbapenems was the most frequent resistance phenotype (50%) encountered in the study. The antibiotic susceptibility profile of our isolates is in keeping with the reported multidrug-resistant phenotype associated with isolates harboring NDM (ECDC, 2014; Shibl et al., 2013; Poirel et al., 2011).

Treatment of patients infected with MBL producers is challenging due to the currently limited options. The tested isolates showed the lowest resistance to fosfomycin, polymyxin B, colistin and tigecycline (0, 19.6, 26.8, and 33.9%, respectively), but these antibiotics also have limitations and adverse effects (El-Herte et al., 2012). Although Colistin and polymyxin B seem to be the last treatment choice for these isolates; our finding of colistin and polymyxin B resistant MBL-producers is of major concern.

On the basis of our study findings, it was concluded that EDTA-CDT could be a sensitive, easy to perform, and interpret phenotypic rapid method for the detection of MBLs in Enterobacteriaceae. It could be introduced into the workflow of any clinical Microbiology laboratory that routinely performs antibiotic sensitivity by disc diffusion test. The liability of subjective interpretation of EDTA-DDST makes it a bad choice. Although the E-test has a higher specificity, it is not considered a cost-effective test.

Conflict of Interests

The authors have not declared any conflict of interests.

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