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Emergence of carbapenem resistant enterobacteriacae coharboring New Delhi metallo-beta-lactamase (NDM-1) and *Klebsiella pneumoniae* carbapenemase (KPC) isolated from a tertiary hospital in Egypt

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ABSTRACT

Carbapenem-resistant Enterobacteriaceae (CRE) is considered a significant problem in the healthcare facility. Resistance to carbapenems in Enterobacteriaceae is mediated by different mechanisms; one of them is due to the production of carbapenemases. This study was aimed to detect the prevalence of *bla*KPC and MβLs encoding genes (*bla*VIM and *bla*NDM-1) production among CRE isolates isolated from Egypt Children's Hospital. Additionally, isolates were evaluated via phenotypic methods of detection. One hundred Enterobacteriaceae isolates were isolated from different clinical samples and screened for carbapenem resistance with imipenem 10 µg and meropenem 10 µg discs. The resistant isolates were tested for antibiotic susceptibility, modified Hodge test (MHT) and imipenem-ethylene diamine tetra-acetic acid (EDTA) combined disc diffusion (IPM-EDTA CDT). Finally, conventional polymerase chain reaction (PCR) was done to detect carbapenemase genes (*bla*VIM, *bla*KPC and *bla*NDM-1). Thirty isolates were initially identified as carbapenem resistant based on resistance to imipenem 10 µg and meropenem 10 µg discs. *Klebsiella pneumoniae* (*K. Pneumoniae*) was the most common isolated species (73.33%) followed by *Escherichia coli* (*E. coli*) (26.67%). The majority of carbapenem resistant isolates (66.7%) were collected from blood cultures. *Bla*VIM gene was the most detected gene among the carbapenem resistant isolates. It was found in 27 isolates (90%). *bla*NDM-1 genes were found in 7 isolates (23.3%) while *bla*KPC was found in 3 isolates only (10%). Carbapenem resistance among Enterobacteriaceae is widely spreading and mostly caused by *bla*VIM gene. More attention is needed to control the spread of carbapenem resistance among Enterobacteriaceae in Egypt.

INTRODUCTION

Carbapenems (imipenem, meropenem, doripenem and ertapenem) are classified as β-lactam antibiotics. These antibiotics have a broad spectrum activity, and are often considered 'drugs of last resort' for treatment of serious infections caused by Enterobacteriaceae (Riera et al., 2013). Carbapenem-resistant Enterobacteriaceae (CRE) is multidrug-resistant organisms exhibiting resistance to most, if not all, available antibiotics (CDC, 2013).

Resistance to carbapenems in Enterobacteriaceae is mediated by different mechanisms; one of them is mainly due to the production of hydrolyzing enzymes, carbapenemases (Meletis, 2016). Carbapenemases are members of the molecular class A, B, and D β-lactamases. The class A carbapenemases which are inhibited by clavulanic acid. Class B or metallo-β-lactamases (MβLs) which are inhibited by ethylene diamine tetra-acetic acid (EDTA). Lastly class D oxacillinases which are not affected by clavulanic acid or EDTA (Hammoudi et al., 2015).

The MβLs have emerged as one of the most feared resistance mechanisms because of their ability to hydrolyze virtually all β-lactam agents, including the carbapenems. Moreover, MβLs are not susceptible to therapeutic β-lactamase inhibitors and their genes also are carried on highly mobile elements (Cornaglia et al., 2011).

Several types of MβLs, such as IMP (imipenemase), VIM (Verona integron-encoded MβL), SPM (Sao Paulo MβL), GIM (German imipenemase), SIM (Seoul imipenemase), NDM-1 (New Delhi MβL), KHM-1 and DIM-1 (Dutch imipenemase) and their variants have been emerging as the most notable resistance determinants in Enterobacteriaceae (Pollini et al., 2013).

Since the beginning of the 2000s *Klebsiella pneumoniae* carbapenemase (KPC) enzymes have become increasingly prevalent among *K. pneumoniae* and other species of Enterobacteriaceae on the East Coast of the USA (Bratu et al., 2005).

The rapid dissemination of carbapenemase producing CRE that accounts for worldwide outbreaks could be attributable to the location of the carbapenemase encoding genes on mobile elements (Dupont et al., 2016). Infections with CRE are very difficult to treat, and they are also associated with high mortality rates. Thus, the rapid detection of CRE is of significant clinical and public health concern (Lutgring and Limbago, 2016). The implementation of a simple and accurate laboratory method to detect CRE is crucial. Polymerase chain reaction (PCR) analysis is the gold standard method for the detection of carbapenemase producing CRE, but it is not suitable for daily testing in clinical laboratories due to the cost and inconvenience.

Aim of the work

The aim of this study was to detect the frequency of positive (*bla*VIM, *bla*NDM-1 and *bla*KPC) CRE isolates isolated from Egypt Children's Hospital. Additionally, to evaluate phenotypic methods of CRE detection.

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MATERIALS AND METHODS

Patients and clinical isolates

A total of 100 Enterobacteriaceae isolates were isolated from different clinical samples from patients admitted to Egypt Children's Hospital, Cairo, Egypt during a period of 5 months from February 2017 to June 2017. The bacterial isolates were identified by conventional bacteriological methods according to Collee et al. (1996).

Detection of CRE by disc diffusion

All collected isolates were screened for carbapenem resistance with imipenem 10 µg (IPM) and meropenem 10 µg (MEM) discs by disc diffusion method (CLSI, 2017). The isolates that were resistant to both discs were tested for antibiotic susceptibility testing by the Kirby-Bauer method (CLSI, 2017). The following antibiotics were tested: amoxicillin/clavulanic acid (AMC, 20/10 µg), piperacillin/tazobactam (TPZ, 100/10 µg), ceftriaxone (CRO, 30 µg), ceftazidime (CAZ, 30 µg), cefotaxime (CTX, 30 µg), cefotaxime/clavulanic acid (30/10 µg), cefepime (FEP, 30 µg), imipenem (IPM, 10 µg), meropenem (MEM, 10 µg), gentamicin (CN, 10 µg), amikacin (AK, 30 µg), ciprofloxacin (CIP, 5 µg), and levofloxacin (LEV, 5 µg) (Oxoid, UK). *Escherichia coli* ATCC 25922 was used as a quality control strain. The isolate was considered multidrug resistant (MDR) if it was resistant to three or more classes of antimicrobial agents (Cohen et al., 2008).

Detection of carbapenemase producing isolates by Modified Hodge test (MHT)

All the carbapenem resistant strains were subjected to Modified Hodge test for detection of carbapenemases (CLSI, 2017). Firstly, A 0.5 McFarland dilution of the *E. coli* ATCC 25922 in 5 ml of saline was prepared. Then, A 1:10 dilution was streaked as a lawn onto a Mueller Hinton agar plate. A 10 µg meropenem susceptibility disc was placed in the center of the plate after streaking of diluted *E. coli* ATCC 25922 onto a Mueller Hinton agar plate. The tested organism was streaked from the edge of the disc to the edge of the plate. Four organisms were tested on the same plate with one disc. Finally, the plate was incubated overnight at 37°C. All in all, the tested strain was considered as positive for carbapenemases production if it forms a clover leaf shaped zone of inhibition (Figure 1).

Phenotypic detection of MβL by imipenem-EDTA combined disc diffusion method (IMP-EDTA CDT)

According to Yong et al. (2002), 0.5 McFarland of

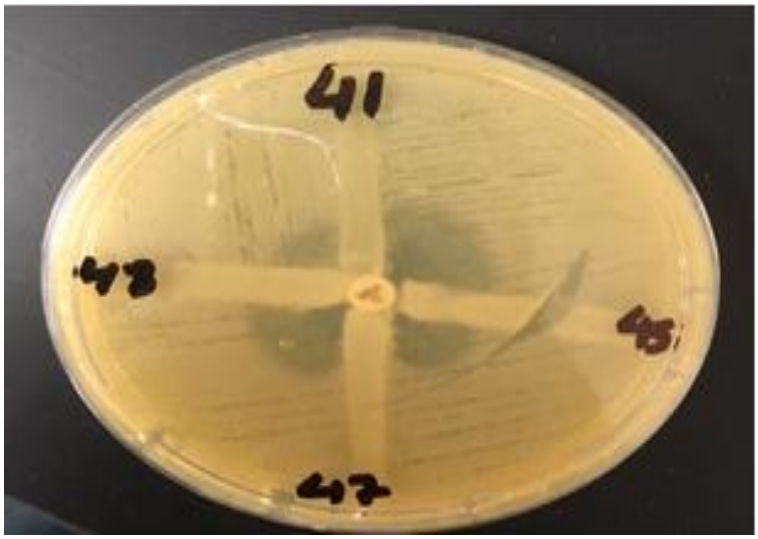

Figure 1. Positive MHT of samples numbers (45 and 47) and negative MHT sample No (48).

Figure 2. Positive IMP-EDTA CDT.

overnight culture of the tested isolate was streaked onto a Mueller Hinton agar plate. Two of 10 µg imipenem discs were used; one imipenem disc alone and one with 10 µl of 0.5 ml EDTA solution. Then, the two discs were placed on inoculated plates. The test was considered as positive if the zone of inhibition of imipenem + EDTA disc was>7 mm compared to imipenem alone, after overnight incubation (Figure 2).

Molecular detection of carbapenems resistance genes (*bla*VIM, *bla*KPC and *bla*NDM-1)

All carbapenem resistant isolates were tested by conventional PCR for detection of carbapenemase genes (*bla*VIM, *bla*KPC and *bla*NDM-1). Firstly, DNA extraction was done as per manufacturer's instructions using PCR Kits; (QuiagenMini kit), for purification of DNA from the bacterial isolates. Then, amplification of class A carbapenemase (*bla*KPC) and class B MβLs (*bla*VIM and *bla*NDM-1) were carried out on the isolates by using primers supplied from Invitrogen as shown in Table 1. Finally, PCR products were run on 1.5% agarose gel, stained with ethidium bromide and visualized under UV light and photographed.

Statistical analysis

All data were analyzed by using SPSS for Windows version 22.0 (IBM Corp. 2013). Chi-square test was used

Table 1. Primers sequences used in this study.

Target	Primer name	Sequence (5' → 3')	Amplicon size (bp)
*Bla*VIM	VIM, forward primer	TCTCCACGCACTTTCATGAC	390 (Poirel et al., 2011)
	VIM, reverse primer	GTGGGAATCTCGTTCCCCTC	
*Bla*KPC	KPC, forward primer	GGC CGC CGT GCA ATA C	890 (Rasheed et al., 2013)
	KPC, reverse primer	GCC GCC CAA CTC CTT CA	
*Bla*NDM-1	NDM, forward primer	GAC CGC CCA GAT CCT CAA	1013 (Rasheed et al., 2013)
	NDM, Reverse primer	CGC GAC CGG CAG GTT	

Table 2. Modified Hodge test results in comparison to the PCR.

		Modified Hodge test		Sens.	Spec.	PPV	NPV	Accuracy
		Positive	Negative					
KPC gene	Negative	13	14	12.50	92.86	66.67	48.15	50.00
	Positive	1	2					
VIM gene	Negative	0	3	81.25	0.00	48.15	0.00	43.33
	Positive	14	13					
NDM-1 gene	Negative	11	12	25.00	78.57	57.14	47.83	50.00
	Positive	3	4					

to correlate genes with MHTs and IMP-EDTA CDT results. Screening test results were also calculated. Descriptive statistics, frequencies and percentages were given. PCR was considered as a gold standard method. Sensitivity and specificity, negative predictive values (NPV), and positive predictive values (PPV) were also calculated. The statistical significance level (P-value) was set to 0.05.

RESULTS

CRE Isolates

Out of 100 bacterial isolates tested, 30 isolates were initially identified as carbapenem resistant based on IPM 10 µg and MEM 10 µg resistance by disc diffusion method. *K. pneumoniae* were the most common isolated species, 22 (73.33%) followed by 8 isolates of *E. coli* (26.67%). The majority of carbapenem resistant isolates, 20 isolates (66.7%) were collected from blood, 8 isolates were isolated from endo-tracheal tube (ETT) (26.7%) and 2 isolates were isolated from wound swab (6.6%).

Antimicrobial susceptibility testing

All the CRE isolates (100%) were resistant to all beta-lactam/beta-lactamase inhibitor combinations, 3rd generation cephalosporine and cefepime. 28 isolates (93.3%) were resistant to gentamicin, 24 isolates (80%) were resistant to levofloxacin and 33 isolates (76.7%) were resistant to ciprofloxacin and amikacin.

Detection of carbapenemase producing isolates by MHT

Out of 30 CRE isolates, 16 isolates (53.33%) were positive for carbapenemase by MHT (Figure 1). Among these isolates, 8 isolates were *bla*VIM positive, three isolates were coharboring *bla*VIM and *bla*NDM-1, 1 isolate was coharboring *bla*VIM and *bla*KPC, 1 isolate was coharboring the three genes *bla*VIM, *bla*KPC and *bla*NDM-1 and 3 isolates (10%) were negative for the three genes.

This method showed low sensitivity compared to PCR especially for *bla*KPC detection. The sensitivity, specificity, PPV and NPV of the MHT were listed in Table 2.

Phenotypic detection of MβL

IMP-EDTA CDT reveled 22 isolates (73.3%) were positive for MβL production (Figure 2). These isolates include; 15 positive *bla*VIM isolates, 3 isolates were coharboring *bla*VIM and *bla*NDM-1, 2 isolates were coharboring *bla*VIM and *bla*KPC, 1 isolate was coharboring the three genes *bla*VIM, *bla*KPC and

Table 3. IMP-EDTA CDT results in comparison to the PCR.

		IMP-EDTA CDT		Sens.	Spec.	PPV	NPV	Accuracy
		Negative	Positive					
***Bla*KPC gene**	Negative	8	19	13.64	100.00	100.00	29.63	36.67
	Positive	0	3					
***Bla*VIM gene**	Negative	2	1	95.45	25.00	77.78	66.67	76.67
	Positive	6	21					
***Bla*NDM-1 gene**	Negative	5	18	18.18	62.50	57.14	21.74	30.00
	Positive	3	4					

*bla*NDM-1 and 1 isolate was negative for the three genes. IMP-EDTA CDT showed low sensitivity compared to PCR especially for detection of *bla*KPC and *bla*NDM-1. The sensitivity, specificity, PPV and NPV of the IMP-EDTA CDT were listed in Table 3.

Molecular detection of *bla*KPC gene, *bla*VIM gene and *bla*NDM-1gene

*bla*VIM gene was the most detected gene among the CRE isolates. It was found in (27/30 isolates, 90%). *bla*NDM-1gene were found in (7/30 isolates, 23.3%) while *bla*KPC was found in (3/30 isolates, 10%). Among the CRE isolates, 6 isolates were coharboring *bla*VIM and *bla*NDM-1, 2 isolates were coharboring *bla*VIM and *bla*KPC, and 1 *klebsiella* isolate was coharboring *bla*VIM, *bla*NDM-1 and *bla*KPC (Figure 3 and 4). In 3 isolates (2 *E. coli and* 1 *Klebsiella*), none of carbapenemase genes were detected

DISCUSSION

CRE are an important health concern because of plasmid-encoded and easily transferable carbapenemases. Infections with these organisms are highly mortal due to narrow treatment options (Djahmi et al., 2014). It is crucial to rapidly identify CRE to prevent their spread in hospitals. In this study IMP EDTA CDT and MHT were used as phenotypic methods for detection of carbapemenases production among Enterobacteriaceae. Conventional PCR was the gold standard method used for detection of *bla*KPC, *bla*VIM, and *bla*NDM-1 among the Enterobacteriaceae isolates.

In this study, *K. pneumoniae* was the most common isolated species (73.33%) followed by *E. coli* (26.67%). This result come in accordance with Zhang et al. (2018) who found *K. pneumoniae* (73.9%), followed by *E. coli* (16.6%). Also, this result was in agreement with a study conducted in Egypt by El-Ghazzawy et al. (2016) who reported that the most common organism was *K. pneumoniae* (93.75%) followed by *E. coli* (3.75%). On the other hand, Mende et al. (2017) found *K. pneumoniae* by (37%) and *E. coli* (19%) in their study.

The majority of carbapenem resistant isolates in this study were isolated from blood. This was similar to the results of Baran and Aksu (2016) and El-Ghazzawy et al. (2016). On the contrary Dahab et al. (2017) reported that the most of carbapenem resistant isolates were isolated from urine.

According to the PCR results, 27 out of 30 (90%) CRE isolates were *bla*VIM MβL producers. The *bla*VIM gene is extensively distributed worldwide (Nordmann et al., 2011). Endemicity of *bla*VIM enzymes has been reported in Greece, Taiwan and Japan (Queenan and Bush, 2007). Also, *K. pneumoniae* isolates with *bla*VIM-MβLs have been found as causes of country wide epidemics in the USA, several Latin American countries, China and Europe (Nordmann et al., 2011). *bla*VIM gene was reported in many Egyptian studies (Mohamed and Raafat, 2011; Fouad et al., 2013). It was detected in five isolates (20%) of *Pseudomonas aeruginosa* (*P. aeruginosa*) in a study conducted in Egypt by Abbas et al. (2018). Also, Melake et al. (2016) found 27.5% of *K. pneumoniae* isolates were positive for the *bla*VIM gene alone in thier study that conducted in Egypt. Zafer et al. (2014) demonstrated that 58.3% of *P. aeruginosa* isolates were positive for *bla*VIM gene. Another study conducted by Diab et al. (2013) revealed that 70% of *P. aeruginosa* isolates were positive for *bla*VIM.

*Bla*NDM-1 was the second most frequently detected gene in this study. It was detected in 7 isolates (23.3%). Higher prevalence of *bla*NDM-1 gene was detected by El-Ghazzawy et al. (2016) and Sultan and Irfan (2014) who detected *bla*NDM-1 enzymes in 96.4 and 94% of their clinical isolates, respectively. The Middle East region might be a secondary reservoir for the spread of *bla*NDM-1 isolates after India (Nordmann et al., 2011). Kaase et al. (2011) showed the first identification of a *bla*NDM-2 gene in a clinical isolate arising from Egypt, without obvious link with the Indian subcontinent. Also, Abdelaziz et al. (2013) reported the first flare-up of *bla*NDM-1-producing pneumonia that was isolated from patient

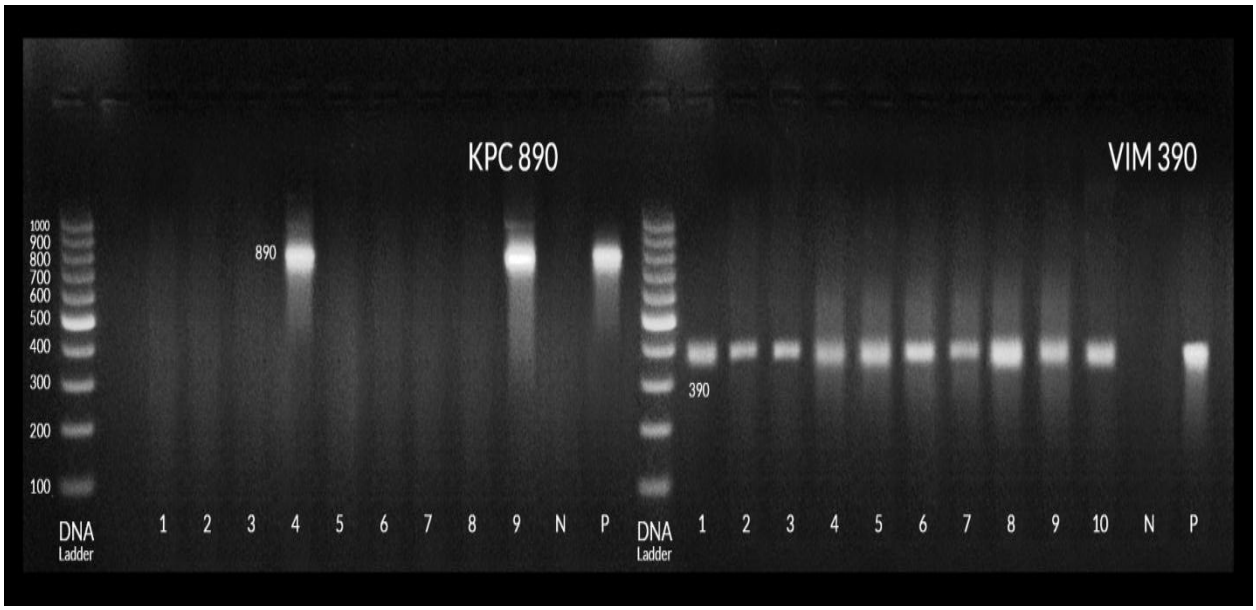

Figure 3. Positive detection of *bla*KPC gene at 890 bp and *bla*VIM gene at 390bp.

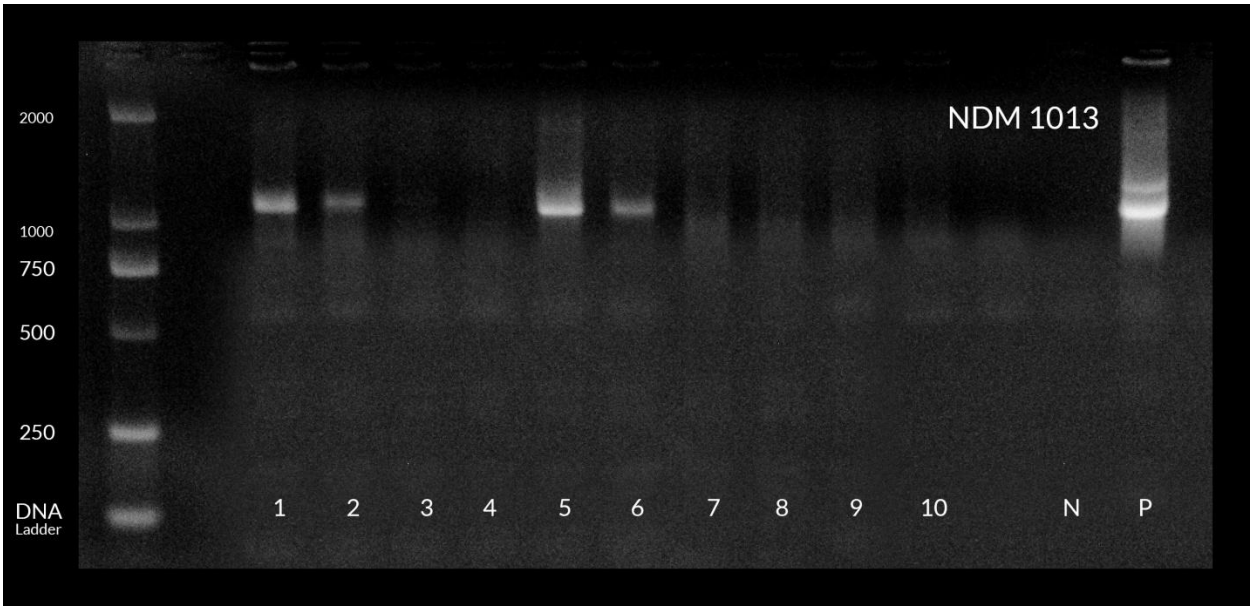

Figure 4. Positive detection of *bla*NDM-1 gene by PCR at 1013 bp.

with cancer from Cairo. Additionally, El-Ghazzawy et al. (2016) reported the first *bla*NDM-1 carrying *K. pneumoniae* in Alexandria. However, the data on the prevalence of *bla*NDM-1 in Egypt is still limited.

*Bla*KPC was the least detected gene. It was found in 3 isolates (10%). However higher percentage was detected by Metwally et al. (2013) who found *bla*KPC in 14 out 20 (70%) of their isolates. In a study done by Melake et al. (2016), *bla*KPC was founded in 17.5% of IPM-resistant isolates. Also, Amer et al. (2016) found *bla*KPC in 46.8% of their isolates which collected from Tanta University Hospital.

MHT is simple, inexpensive phenotypic confirmation test for carbapenemases that is currently proposed by the

CLSI (2017). In this study 16 isolates (53.33%) of CRE isolates were MHT positive. This result comes in accordance with the study done by Dahab et al. (2017) who reported that 50.6% of the total carbapenem resistant isolates were MHT positive. Lower percentages were reported by Pandurangan et al. (2015) and Mahmoud et al. (2016) who found 30.5 and 46.28% of isolates were MHT positive respectively.

MHT is known to be reliable for detection of KPCs and OXA-48-like enzymes (Doyle et al., 2012). In the present study, the MHT had a low sensitivity of detecting KPC producers as only 2 isolates were MHT positive and harbor *bla*KPC (12.5%). This result was against results of Doyle et al. (2012) and AlTamimi et al. (2017) who reported higher sensitivity of MHT 98% and 89.65% respectively. MHT perform poorly for MβL (NDMs, VIMs and IMPs) (Doyle et al., 2012). However, we found that 13 isolates (43.3%) *bla*VIM positive and 4 isolates (13.3%) *bla*NDM-1 positive were MHT positive. This result was in agreement with Girlich et al. (2012) who found 7 of 14 *bla*NDM-1 isolates were MHT positive. MHT sensitivity for detection *bla*NDM-1 and *bla*VIM among all isolates were 25% and 81.25% respectively. These results were higher than result reported by Doyle et al. (2012). Many studies reported the MHT sensitivity to detect *bla*NDM-1 was below 50% and this was in agreement with our results (Girlich et al., 2012; Bonnin et al., 2012; Saito et al., 2015).

In our study MHT gave false-positive results as 14 isolates were MHT positive and *bla*KPC negative, 12 isolates were MHT positive and *bla*NDM-1 negative and 3 isolates were MHT positive and *bla*VIM negative. This false positive result may be due to the production of AmpC enzymes combined with porin mutations (Carvalhaes et al., 2010). However, MHT remains an important part of many testing algorithms because it is simple to perform and uses reagents readily available in most microbiology laboratories.

MβL screening can be done by different methods but the CLSI does not meet the standard guidelines for detection of MβLs. PCR is considered the gold standard for carbapenemase gene detection, but it is not available in routine microbiology laboratory (Bartolini et al., 2014).

In the present study, 22 isolates (73.3%) were positive for MβL production by IMP-EDTA CDT. This come in concordance with a study done in Menoufia University by Mahmoud et al. (2016) who reported that (75.34%) of isolates were MβL producing. Also, this result was in agreement with a study done by Alshara et al. (2014), in which (77.8%) of the isolates were MβL producers. However, Pandya et al. (2011) reported higher percentage of MβL producing strains (96.3%) using the same phenotypic test.

When comparing the sensitivity of the IMP-EDTA CDT to detect *bla*VIM to the PCR, it was 95.4%. This result was in agreement with Pandya et al. (2011) and Arunagiri et al. (2012) who found that the sensitivity of IMP-EDTA CDT was 96.3 and 94% respectively. lower sensitivity of IMP-EDTA CDT was reported by Picão et al. (2008) and El-Ghazzawy et al. (2016).

In our study IMP-EDTA CDT failed to detect MβL production among 3 isolates that carry both *bla*NDM and *bla*VIM. This result was in agreement with Bartolini et al. (2014) who reported that phenotypic tests failed to identify the presence of MβL in isolates harboring more than one carbapenemase gene. This false negative result might arise from carbapenem hydrolysis or inactivation caused by EDTA (Picão et al., 2008).

Also, IMP-EDTA CDT gave false negative results as it was negative in 14 isolates harbor *bla*VIM. However, using the IMP-EDTA CDT test for detecting MβL production was recommended since this test is simple to perform, can be easily introduced into the workflow of a clinical laboratory, and is less expensive than the other methods (Farajzadeh et al., 2014).

Conclusion

Based on our results MHT and IMP-EDTA CDT can give false positive results but still more available and simple screening methods for carbapenemases production. The high prevalence of carbapenmases production by these methods may be due the presence of other unstudied genes. *bla*VIM gene was the most prevalent detected MβL production encoded gene.

Limitations of the study

This study conducted on small number of isolates due to lack of resources so we could not perform PCR for large number of isolates and detect others related genes.

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