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ORIGINAL ARTICLE

Phenotypic tests and Molecular detection of blaOXA-48 and blaNDM-1 genes among carbapenem-resistant *Enterobacteriaceae* isolated from allograft liver recipients

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ABSTRACT

Background: *Infections with carbapenem resistant Enterobacteriaceae (CRE) cause significant morbidity and mortality in Liver transplant recipients. Accurate detection of CRE is the first step in combating this problem.* ***Objectives:*** *to study the distribution of pathogens and CRE causing infections in liver allograft recipients with special reference to the impact of pre-operative intestinal colonization with CRE on post-operative infections, and to investigate the phenotypic tests and to detect the frequency of blaOXA-48 and blaNDM-1 genes by duplex PCR.* ***Methodology:*** *A total of 23 CRE isolates were investigated for carbapenemase production by phenotypic methods such as the modified Hodge test (MHT), douple disc synergy test (DDST), E test and Rabidec Carba NP (RCNP) test. Production of blaOXA 48 and blaNDM-1genes was investigated by duplex PCR.* ***Results:*** *Frequency of CRE that harbored blaOXA-48 and blaNDM-1 genes, as obtained by PCR, was 47.8% for blaOXA-48, 20.1% for blaNDM-1 and 8.7% of CRE isolates co-produced the two genes. RCNP test exhibited Sensitivity of 90.9% and 100% versus 81.1% and 66.6% sensitivities by MHT in detecting blaOXA-48 and blaNDM-1genes respectively. E-test had a much higher sensitivity in identifying NDM-1 carpabenemase compared to DDST (100% versus 66.6% respectively).****Conclusion:*** *RCNP test can be done instead of MHT in detecting CRE, as it is more accurate, rapid and having excellent sensitivity in detecting blaNDM-1 and blaOXA-48 producing isolates. E-test is more reliable than DDST and can be a valid alternative to PCR in detecting MBL especially NDM-1 type.*

INTRODUCTION

Carbapenem-resistant *Enterobacteriaceae* (CRE), especially carbapenem-resistant *Klebsiella pneumoniae* (CRKP), has been increasingly reported among liver transplantation (LT) recipients in the past decade and are responsible for poor outcomes [1]. Risk factors include poor functional status of the patients, frequent need for antimicrobial therapy, intensive care unit (ICU) admissions, mechanical ventilation, and prolonged hospitalizations [2].

LT recipients remain at high risk for both colonization and infection with these multidrug-resistant organisms due to prolonged wait times and unavoidable repeated exposures to both antibiotics and health care settings. Reported rates of CRKP in LT recipients range from 6% to 12.9% and reported associated mortality ranges from 18% to nearly 80% [1].

Enterobacteriaceae can become resistance to carbapenem by three mechanisms, either permeability loss; efflux pumps; and carbapenemase production. However, production of carbapenemases is the most common mechanism and represents a major concern, since they hydrolyze not only carbapenems but also other B-lactam antibiotics. Carbapenemases are classified into three classes, A, B or D B-lactamases, based on the molecular structure [3].

Klebisella pneumoniae carbapenemases (KPCs), a class A β- lactamase is the most common observed resistance mechanism among *Enterobacteriaceae* in the United States, South America, China and Mediterranean Europe. However, expression of metallo-b-lactamases (MBLs) (class B) and carbapenem-hydrolyzing class D carbapenemases (oxacillinase [OXA]) has been reported[4].

MBLs are other β-lactams, also hydrolyze carbapenems and are stable against the β-lactamase inhibitors[5]. The most common MBLs among *Enterobacteriaceae* were Verona integron–encoded (VIM) and IMP. However, New Delhi MBL (NDM), another plasmid-encoded enzyme, was identified in 2009 and quickly become the prevalent carbapenemase in India, the United Kingdom and Pakistan. Class D β-lactamases or OXA-type enzymes hydrolyze oxacillin,

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hence its name. OXA-48 type enzymes is a member of class D β-lactamases, which are predominant in North Africa, Turkey, and India [5].

CRE spread can be controlled through the application of effective infection control measures and judge the use of antibiotics. Thus the introduction of fast and accurate tests for the detection of CRE is of urgent need[6].

For decades, Molecular assays (such as PCR) have been considered the gold standard technique for the carbapenemases detection. However, they are costly and need significant expertise; these factors limited their practical use in most laboratories. Nowadays, a variety of phenotypic methodologies are available for screening CRE in hospital settings, including growth-based assays (modified Hodge test [MHT]) [7] and detection of the enzymatic activity of carbapenemases [8].

This activity might be detected by biochemical assays such as Blue-Carba, CarbaNP and Rabidec Carba NP (RCNP) methodologies. [8] In which colour change of a pH indicator is visualized by hydrolysis of the B-lactam ring. Other methods include the use of mass spectroscopy, and most recently, the modified carbapenem inactivation method (mCIM) [3].

Thus we aimed to study the incidence of CRE post living donor liver transplantation and to investigate the phenotypic and genotypic characteristics of isolates, also to evaluate the accuracy of these phenotypic methods (MHT, RCNP, DDT, E-test) in relation to PCR results.

METHODIOLOGY

This study was carried out during the period from September 2017 to September 2018 and included 51 allograft LT recipients who admitted to transplant unit ,National Liver Institute, Menoufia University and had living donor liver transplantation during this period. Data were collected and recorded up to one month post transplantation. Episodes of infection with significant growth were reported. The institutional review board of the National Liver Institute approved this study and written informed consents were obtained from all patients before their enrollment in the study.

Different clinical specimens were obtained from LT recipients during episodes of infection (blood, urine, pus, swab, surgical site, stool, ascetic fluid and sputum). Identification of different organisms including *Enterobactericeae* species and antibiotic sensitivity testing were done using VITEK 2 compact system (bioMerieux, France). CRE was defined as resistance to at least one of carbapenems (Imipenem, Ertapenem or Meropenem) [3].

A total of 23 CRE isolates were obtained and stored at -80˚C in nutrient broth with supplemented with 16% glycerol until further use for phenotypic and genotypic evaluation. The 23 CRE isolates were investigated by phenotypic methods and duplex PCR for blaOXA-48 and blaNDM-1 carbapenemase production.

Phenotypic tests

Modified Hodge Test (MHT): We used the MHT test as per the CLSI recommendation [9]: a lawn of 1:10 dilution of 0.5 McFarland standard suspension of E. coli ATCC 25922 was streaked to a Muller Hinton agar plate and allowed to dry 3-5 mins. Then, a 10 µg Ertapenem disk was placed in the center of the plate. In a straight line, the test organism was streaked from the disk to the edge of the plate. The MHT test was considered positive if clover leaf-like indentation of E. coli 25922 growing along the growth streak of the organism within the disk diffusion zone was noted after overnight incubation at 37°C.

Imipenem-EDTA Double Disk Synergy Test (DDST): We used an Imipenem (10µg) disk and an Ethylene diamine tetra acetic acid (EDTA) disk, and placed them 20 mm apart center to center on Mueller Hinton agar plate inoculated with 0.5 McFarland suspension of the test isolate. An increase of 7 mm or more in the zone diameter of EDTA -imipenem disk in comparison to imipenem disk alone was interpreted as a positive result for MBL production. [10]

E–test MβL detection test (AB Biodisk, Solna, Sweden): E-test was performed according to the manufacturer's instructions. The test strips contained imipenem (4 to 256 µg/ ml) and imipenem (1 to 64 µg/ml)-EDTA combinations. A reduction in imipenem MIC (three-fold or greater) in the presence of EDTA was considered positive result for MBL production. Also the detection of a "phantom" zone between the two gradient sections or a deformation of the imipenem ellipses was interpreted as a positive result.[11]

Rabidec Carba NP Test (RCNP): This test was performed according to the manufacturer's instructions. 100 ul of API suspension medium was pipetted into wells a, b, c. on the test strip and left for 4-10 minutes at room temperature. Well c was inoculated with the colonies to be tested and left for 30 minutes at room temperature. Then 25 ul was transferred from well c to wells d and e and 25 uL from well a to wells d and e. If the color of the strip changed from red to yellow, orange, or dark orange in well e after incubation for 30 minutes at 33-38ºC, the result was interpreted positive and the test is complete. [12]

Duplex PCR for blaOXA-48 and blaNDM-1 genes detection

Bacterial DNA was extracted from cell pellets using Gene Jet Genomic DNA purification kit (Qiagen, Hilden, Germany) following manufacturer's instructions. DNA concentration was measured by Nano drop 2000 Thermoscientific spectrophotometer. Target genes were amplified using the following primers:
OXA-F: GCGTGGTTAAGGATGAACAC and
OXA-R: CATCAAGTTCAACCCAACCG and
NDM-F: GGTTTGGCGATCTGGTTTTC,

NDM-R: CGGAATGGCTCATCACGATC[13].

DNA amplification was carried out in a thermal cycler (Veriti Applied Biosystem USA) in a total volume of 40 µl. Each reaction contained 20 µl of 2x PCR master mix (Hot start DNA polymerase, buffer, dNTP mix, Thermoscientific vilinus, Lithuania), 10 µmol/L of each primer, 6µl of template DNA (about 20ng according to DNA concentration). PCR cycling conditions were set up as follows: initial denaturation at 95˚C for 10 min, followed by 35 cycles of 95˚C for 30 seconds, annealing at 62.5˚C for 10 min. Duplex PCR was carried out by the simultaneous addition of the two primers in the same reaction mixture. [13]

Amplified products were separated by agarose (1.5%) gel electrophoresis in 1xTBE, stained with ethidum bromide and visualized with UV transillumination and were photographed by a Bio-Rad-Gel Doc device.

Statistical analysis

The data were collected, tabulated, statistically analyzed using the IBM personal computer with Statistical Package of Social Science (SPSS) version 22 (SPSS, Inc, Chicago, Illinois, USA).

RESULTS

We prospectively followed 51 patients who underwent liver transplantation at National Liver Institute, Menoufia University. Episodes of infection with significant growth were reported. Out of 51 allograft liver recipients, 25 (49.1%) patients had 34 infection episodes (30 bacterial and 4 fungal episodes) with no reported viral infections in the studied patients.

Thirty four isolates caused episodes of infection with significant symptoms and signs. *Klebsiella Pneumoniae* caused the highest rate of infection (44.11%); associated with pneumonia, UTI, blood stream infection, peritonitis and device associated infection. *E.coli* represented 11.76% of the isolates, associated with UTI, peritonitis. However the least incidence of infection caused by *Enterococci*, *strept pneumoniae* and *candida* (Table 1).

Table 1: Distribution of isolated organisms associated with post- operative infections according to the type of infection.

Isolated organisms	Type of infection						% to total no of isolates (no=34)
	Pneumonia	UTI	Blood stream infection	Peritonitis	Device associated infection	SSI	
Klebsiella Pneumoniae	5	4	1	2	3	0	15(44.11%)
E.coli	2	0	0	2	0	0	4(11.76%)
Proteus spp	0	0	0	1	0	1	2(5.88%)
Pseudomonas spp	0	0	0	1	0	0	1(2.94%)
Acinetobacter spp	0	0	0	1	0	0	1(2.94%)
Staph aureus	2	0	1	0	1	1	5(14.7%)
St.pneumoniae	0	1	0	0	0	0	1(2.9%)
Enterococci	0	0	1	0	0	0	1(2.9%)
Candida albicans	0	0	1	0	0	0	1(2.9%)
Non albicans candida	0	2	0	0	0	1	3(8.82%)
Total No	9	7	4	7	4	3	34(100%)

Twenty three CRE were isolated from the studied patients, six of them were associated with pre-operative intestinal colonization, and 17 CRE isolates were associated with post-transplantation infections. The most common infection caused by CRE isolates was pneumonia followed by UTI and peritonitis and least common infection was blood stream infection (Table 2).

Table 2: Number and percent of CRE isolated from allograft liver recipients.

Type of infection	CRE isolates		% of total no of CRE isolates(no=23)
	Klebsellia pneumoniae	*E.coli*	
Pre-operative intestinal colonization	4	2	6(26.08%)
Post-transplantation infection:			
Pneumonia	3	2	5(21.73%)
UTI	4	0	4(17.4%)
Blood stream infection	1	0	1(4.34%)
Device associated infections	3`	0	3(13.04%)
Peritonitis	2	2	4(17.04%)
Total No	17	6	23(100%)

There was a highly statistical significant correlation between preoperative intestinal colonization and post-transplantation infection with CRE (P-value <0.01). (Table 3). Antimicrobial susceptibility testing to 23 CRE isolates was done using VITEK 2 compact system. We found that 100 % of CRE was resistant to Ampicillin, Ampicillin-Clavulinc, Cefoxitin, Cefepime, Ceftazidine, Ceftriaxone, Cefotaxime, Gentamycin and Meropenem. While 30.4% were sensitive to Tigecycline and Levofloxacin. CRE isolates showed different degrees of resistance to Carbapenems as resistance was 65.2% to Ertapenem and 100% to Imipenem and Meropenem. In our study 100% of isolates were sensitive to Colistin.

Table 3: Correlation between pre-operative intestinal colonization with CRE and post-transplantation infections in the studied allograft Liver recipients.

Pre-operative intestinal colonization of CRK (number of patients)	Post- transplantation infection with CRK		P-value
	Yes (13)	No (38)	
Yes (n=6)	4	2	<0.01
No (n=45)	9	36	

RCNP test detected the presence of carbapenamase enzyme in 87% of total number of CRE, while MHT was positive in only 56.6% of CRE (figure 1 & 2). E-test detected MBL enzyme production in 43.5% of CRE isolates, while DDST was positive in only 30.4% of CRE isolates**.**

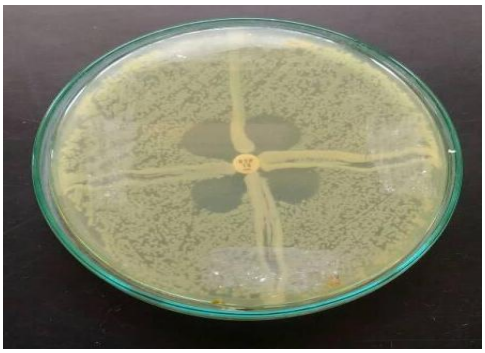

Fig. 1: Modified Hodge Test (MHT) positive result with clover leaf-like indentation of E. coli 25922 growing along the growth streak of the tested organisms within the Ertapenem diffusion zone

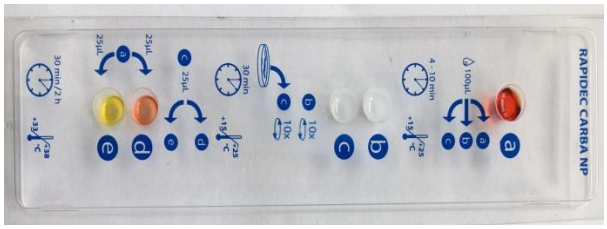

Fig. 2: Positive *Klebsellia pneumonia* isolate for carbapenemase production by RCNP test.

Frequency of CRE isolates that harbored blaOXA48 and blaNDM-1 genes, as obtained by PCR, was 47.8% for blaOXA48, 20.1% for blaNDM-1 gene and 8.7% of CRE isolates co-produced the two genes **(**table 4 & figure 3).

Table 4: Phenotypic and Genotypic characteristics of 23 CRE isolated from allograft Liver recipients.

Test	Positive % (N)	Negative % (N)
MHT	56.5% (13)	43.5% (10)
DDST	30.4% (7)	69.6% (16)
E-Test	43.5% (10)	56.5% (13)
RCNP	87% (20)	13% (3)
blaOXA-48 gene production	47.8% (11)	56.5% (13)
blaNDM-1 gene production	20.1% (6)	73.9% (17)
blaOXA-48 and blaNDM-1 co-production	8.7% (2)	91.3% (21)

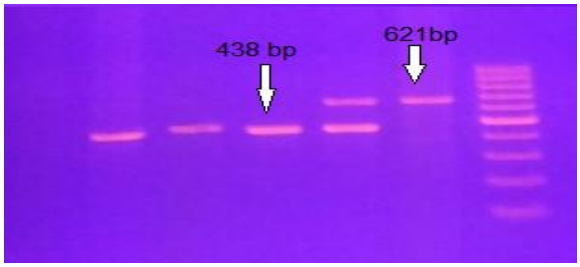

Fig. 3: Agarose gel electrophoresis of CRE isolates contained NDM-1(621bp), OXA-48 (438bp) and combined NDM-1, OXA-48 genes using ladder 100.

Table (5) shows that, among 10 CRE isolates considered negative for carbapenemase production by MHT, 7 isolates were positive by RCNP test (P value <0.01).

Table 5: Comparison between MHT and RCNP test for detection of carbapenamase production among CRE isolates.

MHT	Positive RCNP	Negative RCNP	P-value
Positive (13)	13	0	**<0.01**
Negative (10)	7	3	
Total (23)	20	3	

This table shows a significant difference between E-test and DDST in detection of MBL production by CRE isolates. E-test detected MβL production in 52.9% of CRKP, while DDST detected MβL production in 35.3% CRKP only (P value < 0.05). Also E-test and DDST detected MβL production in 16.6% of carbapenem resistant *E.coli*.

Table 6: Comparison of DDST and E-test for detection of MβL among CRE isolates.

MβL		DDST	E-test
***Klebsiella pneumoniae* (n=17)**	**MβL +ve**	**6 (35.3%)**	**9 (52.9%)**
	MβL –ve	**11 (64.7 %)**	**8 (47.1 %)**
	P-value	**<0.05**	
***E.coli* (n=6)**	**MβL +ve**	**1 (16.6%)**	**1 (16.6%)**
	MβL –ve	**5 (83.3%)**	**5 (83.3 %)**
	P-value	**0.061**	

MHT exhibited 81.1% and 66.6% sensitivities in detecting blaOXA-48 gene and blaNDM-1carbapenemases respectively, while failed to recognize 19.9% and 34.6% blaOXA-48 and blaNDM-1producing isolates respectively. Sensitivity of 90.9% and 100% was shown by RCNP test in detecting blaOXA-48 and blaNDM-1carbapenemases respectively. E-test had a much higher sensitivity in identifying NDM-1 carpabenemase compared to DDST (100% versus 66.6% respectively) (Table 7).

Table 7: Performance characteristics of the four phenotypic methods in the detection of blaOXA-48 and blaNDM-1 genes

carbapenemase gene	%positive (no. of positive tests/no. of isolates) producing specific carbapenemase gene			
	MHT	**RCNP test**	**E-test**	**DDST**
blaOXA-48 (n =11)	**81.1%(9/11)**	**90.9%(10/11)**	**-**	**-**
blaNDM-1 (n =6)	**66.6%(4/6)**	**100%(6/6)**	**100%(6/6)**	**66.6(4/6)**
blaOXA-48 & blaNDM-1 (n = 2)	**100%(2/2)**	**100%(2/2)**	**100%(2/2)**	**50%(1/2)**
Total (n = 19)	**78.9%(15/19)**	**94.7%(18/19)**	**100%(8/8)**	**60.25(5/8)**

DISCUSSION

LT recipients are vulnerable to infections with multidrug-resistant pathogens especially those caused by CRE. Infections with CRE prove to be more serious and are responsible for high rates of both morbidity and mortality in solid organ transplant recipients accounting for 29% survival rate compared to 86% to those with no CRE infections. [1]

CRE can spread clonally from person to other and carbapenamase genes can spread horizontally between bacteria**.** The most important carbapenamase are OXA-48, NDM, VIM and KPC**.** Rapid identification of carbapenamase producing strains is the first step for limiting their spread and preventing hospital infections**.** [3] Thus we aimed to study the incidence of CRE post living donor liver transplantation and to investigate the phenotypic and genotypic characteristics of CRE isolates.

In the current study, out of 51 allograft liver recipients, 25 (49.1%) patients had 34 infection episodes (30 bacterial and 4 fungal episodes) with no reported viral infections in the studied patients. As regard the incidence of isolated organisms in our study, *Klebsiella pneumoniae* was the commonest organism that caused 44.2% of infections mainly pneumonia, followed by *Staph aureus* (14.7%) and *E.coli* (11.8%). These results were in accordance with Pereira et al., [14] and Singh et al.[15] who found that *Klebsiella pneumoniae* was the commonest organisms isolated from LT with post-operative infection. There was a clear shift that the gram-negative bacteria had become the dominant pathogen, with the emerging of CRKP infections as a serious complication early post-transplantation, due to the lack of effective antibiotics prophylaxis. [15]

Twenty three CRE were isolated from the studied patients, six of them were associated with pre-operative intestinal colonization, and 17 CRE isolates were associated with post-transplantation infections. The most common infection caused by CRE isolates was pneumonia followed peritonitis and UTI and least common infection was blood stream infection. Our findings were in accordance with Kalpoe et al., [16] Clancy et al., [17] and Lubbert et al.,[18] who found that Pneumonia was the most common infection caused by CRE.

There was a highly statistical significance correlation between preoperative intestinal colonization and post-transplantation infection with CRE (P-value <0.01). As 66.7% of patients with pre-operative CRE colonization had post-operative infection with CRE during the first month after transplantation. Thus the preoperative intestinal colonization with CRE can be a significant risk factor for post-operative infection with CRE in LT receipents. Similar findings reported by Lubbert et al., [18] and Rasilainen et al., [19] who found that the LT recipients colonized prior to LT were at higher risk of LT infection than those not colonized.

In the current study we did antimicrobial susceptibility testing to 23 CRE isolates using VITEK 2 compact system. We found that 100 % of CRE was resistant to Ampicillin, Ampicillin-Clavulinc, Cefoxitin, Cefepime, Ceftazidine, Ceftriaxone, Cefotaxime, Gentamycin and Meropenem. While 30.4% were sensitive to Tigecycline and Levofloxacin. CRE isolates showed different degrees of resistance to Carbapenems as resistance was 65.2% to Ertapenem and 100% to Imipenem and Meropenem. In our study 100% of isolates were sensitive to Colistin. These results agreed with Foldes et al.,[20] and Baran and Asku,. [21] who reported nearly similar results.

Hence the accurate and rapid detection of carbapenemase producers is crucial to implementing immediate contact isolation and antibiotic treatment decisions, as CRE can lead to devastating consequences in health care settings. [3] Thus we investigated four phenotypic tests, including the recently RCNP test, to detect carbapenemase production among well characterized CRE isolates.

The first recognized carbapenemase screening test was the MHT, recommended by the CLSI in 2009[22]. It is a simple and inexpensive method that detects production of carbapenemase enzyme. In the current study, it gave positive results for 56.5% of CRE isolates. These results were nearly similar to Foldes et al., [20] and Baran and Asku,[21] who reported that 60.8% and 65.2% of CRE isolates were MHT positive respectively. However Ranjan et al., [23] and Tamma et al., [24] reported that MHT positive in 87.5% and 95% of isolates respectively (these results were slightly higher than results reported by our study).

RCNP, a novel phenotypic assay, gives reliable results within two hours making it fast and easy test to control carbapenamase producers. RCNP test was found to be positive in 20 isoaltes from 23 CRE. These results were in agreement with Poirel et al.[12] and Tamma et al.,[24] who reported nearly similar findings.

Among 10 CRE isolates considered negative for carbapenemase production by MHT, 7 isolates were positive by RCNP test (P value <0.01). Thus RCNP is more accurate and sensitive than MHT in detecting carbapenemase production among CRE isolates. [12]

In the present study we reported that E-test was positive in 43.5% of CRE isolates. This result was in agreement with Ranjan et al., [23] and Tamma et al., [24] who reported that E-test was positive in 33.4% and 43% of CRE isolates respectively. The results of our study were much smaller than the results reported by Baran and Asku. [21] Also Sioud et al.,[25] stated that EDTA are more reliable than E-test in detecting MBL as E-test are expensive and not usually available.

As regard the genotypic characteristics of CRE isolates, blaOXA-48 gene was observed in 47.8% of isolates, and blaNDM-1 gene was observed in 20.1% of isolates. 8.7 of isolated CRE were observed to co-produce the two genes. These results were consistent with the previous report that stated that OXA-48 is commonly distributed in the Mediterranean region of Africa and Europe and NDM is endemic in northern Africa. [26] Our results were in accordance with other study in Egypt that reported that the most prevalent carbapenemase gene was blaOXA-48-like (49.2% of CRE isolates,) followed by blaNDM-1. [27] Our results were also agreed with a Saudi Arabia study [28], where OXA-48 and NDM-1 were the most common among *Enterobacteriaceae*, particularly *E. coli* and *K. pneumoniae*.

The performance characteristics of the various assays in detecting blaOXA-48 and blaNDM-1 genes was investigated in the current study. The MHT had lower sensitivity (81.1%) for the detection of blaOXA-48 producers and had limited sensitivity (66.6%) in detecting blaNDM-1 producers. RCNP test exhibited 100% sensitivity in detecting blaNDM-1 type carbapenemases, and had sensitivity of 90.9% in detecting blaOXA-48 producers.

Our results nearly similar to Pancotto et al., [3] who found sensitivities of 100% and 93% of RCNP in detecting blaNDM-1 and blaOXA-48- carbapenemases in *Enterobacteraecea* respectively, while MHT exhibited less sensitivity.

These results were also in consistent with the previous studies that found that MHT had much lower sensitivity in identifying NDM-1 carbapenemase compared to colorimetric assays such as CarbNP and RCNP [3, 24]

The present study also found that E-test had much higher sensitivity than DDST in detecting blaNDM-1 gene (100% versus 66.6% respectively). Our result agreed with Sakanashi et al., [30] who found that sensitivity of E-test was 100% while DDST was inadequate for NDM-1 producing isolates.

CONCLUSION

In conclusion, the preoperative intestinal colonization with CRE is a significant risk factor for post-transplantation infection with CRE, thus pre-operative intestinal decolonization of LT recipients may

be considered. Identifying the carbapenem resistance mechanisms and the local molecular epidemiology of carbapenemases in a health care setting is essential to consider the required phenotypic tests. MHT exhibited low diagnostic sensitivity especially in detecting MBL, thus, it is no longer necessary for routine testing. So, we recommend to use RCNP test instead, as it is more accurate, rapid and having excellent sensitivity in detecting blaNDM-1 and blaOXA-48 producing isolates. E-test is more reliable than DDST and can be a valid alternative to PCR in detecting MBL especially NDM-1 type.

The authors declare that they have no financial or non-financial conflicts of interest related to the work done in the manuscript.

- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

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