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Comparison Of Modified Hodge Test With Triton Hodge Test For Carbapenemase Detection In Gram Negative Bacteria

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Abstract

Background: Widespread use of carbapenem drugs has led to an increasing trend in the incidence of carbapenem resistant cases. The rapid dissemination of carbapenem resistance has necessitated early identification of carbapenem resistant gram negative bacteria from clinical samples. Modified Hodge test, a CLSI recommended screening test has limitations in detecting metallobetalactamases & OXA-type carbapenemases. Addition of Triton X 100 may increase the detection rate of these carbapenemases.

Materials and Methods: This cross sectional analytical study was carried out in the department of Microbiology,Yenepoya medical college,Mangalore,Karnataka, India. Gram negative isolates that were identified as carbapenem resistant by BD Phoenix were tested for carbapenemase production by both Modified Hodge test & Triton Hodge test. Results of both tests were analysed.

Results: Out of 96 gram negative bacteria isolated, 46 (47.9%) carbapenem resistant isolates were detected by BD Phoenix automated system. Carbapenem resistant isolates were recovered mainly in the age group of 50-70 years & majority of them were males (n=34 ,73.9%) . Wound swab (n=18, 39.1%) was the clinical specimen that isolated most of the resistant isolates. Ecoli & Klebsiella pneumoniae(both n= 14, 30.4%) were the predominantly isolated carbapenem resistant gram negative bacteria. Detection rate of Modified Hodge test & Triton Hodge test for carbapenemase production were 69.5% & 91.3% respectively with significant p- value (<0.001).

Conclusion: Early & rapid detection of carbapenem resistance among gram negative bacteria is necessary to curb the spread of these multidrug resistant organisms. Adding a non ionic surfactant like Triton X 100 to Modified Hodge test increases the detection rate of carbapenemases which could make it a better alternative to Modified Hodge test.

Keywords: Carbapenemases, Metallobetalactamases, Modified Hodge test, OXA-type carbapenemases, Triton Hodge test, Triton X 100

Introduction

Carbapenems are a class of beta lactam antibiotics that are most effective against gram negative bacteria. They have been preferred as the last resort drugs for treatment of gram negative infections especially multidrug resistant infections. Carbapenems could efficiently treat infections caused by ESBL & AmpC betalactamase producing gram negative bacteria.

However, widespread use of carbapenems has led to an increasing trend in the incidence of carbapenem resistant cases. This rise in carbapenem resistance among gram negative bacteria poses a global challenge due to the rapid spread of the drug resistant genes from one bacterium to another. The emerging

carbapenem resistance has limited the treatment options leading to significant morbidity & mortality. Various types of infections caused by carbapenem resistant strains can result in prolonged hospital stay, increased economic & social burden as well as emotional stress[1,2].

Gram negative bacteria exhibits various mechanisms of resistance to carbapenems. These mechanisms include synthesis of carbapenemase enzymes, overexpression of efflux pumps & loss of porin channels or mutation of porin channels. Out of these various mechanisms, carbapenemase enzyme production is the prominent mechanism of carbapenem resistance in gram negative bacteria. According to Ambler classification, carbapenemase enzymes can be classified into Class A, Class B & Class D carbapenemases.Class A carbapenemases includes KPC, IMI,GES, SME & NMC which are predominantly seen in all Enterobacteriaceae. Class B carbapenemases includes NDM, IMP,VIM, GIM, SPM that are mainly seen in Pseudomonas aeruginosa, Acinetobacter species & Enterobacteriaceae. Class D carbapenemases has OXA type carbapenemases, mainly seen in Acinetobacter species. Carbapenemase encoding genes can get easily transferred among pathogenic gram negative bacteria through mobile genetic elements like plasmids & transposons. This rapid dissemination of carbapenem resistance has necessitated the early identification of carbapenem resistant gram negative bacteria from clinical samples which helps in optimal management of patients thus improving the clinical outcome[3,4].

Modified Hodge test (MHT) is a CLSI recommended test that have been used as a phenotypic screening test to detect carbapenemase production in gram negative bacteria.However, it has some limitations like test result interpretation is subjective which could lead to false positive & false negative results. Though it has high sensitivity for detecting Class A carbapenemases, it is not sensitive enough for detection of Class B carbapenemases ,metallobetalactamases & Class D carbapenemases , OXA type carbapenemases. Recent studies have concluded that many of the New Delhi metallobetalactamases (NDM) are membrane bound carbapenemases which could not be detected by MHT[5]. In such cases, addition of a non ionic surfactant like Triton X 100 could release the membrane bound carbapenemases thus increasing their detection rate[6].

So, in this particular study we have performed both Modified Hodge test (MHT) and Triton Hodge test (THT) & compared their performances to see the detection rate of carbapenemases in gram negative bacteria by both methods.

Materials And Methods

This was a cross sectional analytical study carried out over a period of 4 months from June 2022 to September 2022 in the department of Microbiology,Yenepoya medical college,Mangalore, a tertiary care centre in the Southern Indian state of Karnataka.The study was initiated after approval from the institutional ethics committee.

Inclusion Criteria:

1.Carbapenem resistant gram negative bacteria isolated from various clinical specimens that were sent for culture were included in the study.

2. If same patient sample gives 2 different isolates that were carbapenem resistant, it was considered as 2 different isolates & was included in the study.

Exclusion Criteria:

Isolates that were obtained in duplicates from clinical samples from same site of infection was considered as single isolate & excluded from the study.

Clinical specimens from various sites (sputum, ET aspirate,blood,urine,pus,wound swab) isolating gram negative bacteria were randomly collected in microbiology lab of Yenepoya medical college. Identification & antimicrobial susceptibility testing (AST) of isolated gram negative bacteria was performed using BD Phoenix automated system. AST pattern was studied to identify carbapenem resistant isolates. Characterization of the isolated carbapenem resistant isolates were done with respect to age & gender of patients from whom these samples were collected & also with respect to the type of specimen from which these carbapenem resistant bacteria were isolated. Modified Hodge test & Triton Hodge test was performed on isolates identified as carbapenem resistant by BD Phoenix & the results of both tests were compared.

Modified Hodge Test

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Modified Hodge test was performed according to the CLSI guidelines[7]. A 0.5 Mcfarland dilution of Ecoli ATCC 25922 in 5ml broth/saline was prepared. A 1:10 dilution was streaked on to a Mueller Hinton agar plate as lawn culture. A 10 mcg meropenem disk was placed in the centre of test area. Test organism was streaked in a straight line from the edge of disk to the edge of plate. Plates were incubated overnight at 35 ± 2^{o}C for 16-24hrs. After 24 hrs, MHT positive test showed a clover leaf like indentation of E.coli ATCC 25922 growing along the test organism growth streak within the disk diffusion. MHT negative test showed absence of growth of indicator strain towards carbapenem disk.

Triton Hodge Test

Triton Hodge test was performed by a slight modification of adding Triton X 100 to MHT[6]. For Triton Hodge test, 50 µl of pure Triton X 100 reagent (0.2% vol/vol) was dripped in the centre of plate. Using a swab, the detergent was uniformly distributed over the agar surface by streaking the swab over the entire agar surface 4 to 6 times, until the detergent was completely absorbed. Subsequent methods of Triton Hodge test was similar to Modified Hodge test. Triton Hodge test positive test showed a clover leaf like indentation of Ecoli ATCC 25922 growing along the test organism growth streak. Triton Hodge test negative test showed absence of growth of indicator strain towards carbapenem disk.

In both Modified Hodge test & Triton Hodge test, length of enhanced growth of indicator strain (Ecoli ATCC 25922) was measured using a ruler in mm. When the length of growth of indicator strain L=0 mm, then the test isolate was interpreted as negative for carbapenemase production in both Modified Hodge test & Triton Hodge test. When the length of growth of indicator strain was more than 0mm but less than 3 mm (0 <L< 3 mm), the test isolate was considered to be weakly positive for carbapenemase production. If the length of growth of indicator strain exceeds 3mm or is equal to 3mm (L≥3mm), the test isolate is considered as positive for carbapenemase production[6]. Results of both Modified Hodge test & Triton Hodge test were compared & analysed.

Statistical analysis

Data was recorded in MS Excel sheet & data analysis was done using ISM SPSS 20 program running on windows. Enhanced growth of Ecoli indicator strain was compared in both Modified Hodge test & Triton Hodge test using paired t test. Prevalence ratio was calculated. Resistance was calculated in percentages.

Results

A total of 96 gram negative bacteria were isolated from various clinical specimens. Out of these, 46 (47.9%) non duplicate carbapenem resistant clinical isolates were detected by BD Phoenix automated system.

Age, gender & samplewise distribution of carbapenem resistant clinical isolates

Majority of carbapenem resistant isolates (65.2 %) were recovered in the age group of 50-70 years. 34 (73.9%) carbapenem resistant isolates were recovered from males & 12 (26%) from females. Wound swab (n=18, 39.1%) was the predominant clinical specimen isolating carbapenem resistant isolates followed by pus (n=8, 17.3%), urine (n=7, 15.2%), sputum (n=6, 13 %), ET aspirate (n=5, 10.86 %) & blood (n=2, 4.3 %)[Table 1].

Carbapenem resistant isolates

Ecoli & Klebsiella pneumoniae(both n= 14, 30.4%) were the most commonly isolated carbapenem resistant gram negative bacteria from various clinical specimens. Total 11 (23.9%) isolates of Acinetobacter baumanii, 3 (6.5%) isolates of Pseudomonas aeruginosa & 1 (2.1%) isolate each of Proteus mirabilis, Enterobacter aerogenes, Acinetobacter haemolyticus & Acinetobacter calcoaceticus were recovered from the clinical specimens [Figure 1]. Majority of wound swab & urine samples isolated Ecoli as the carbapenem resistant isolate . However Klebsiella pneumoniae predominated the sputum & pus samples. ET aspirate predominantly isolated Acinetobacter baumanii as the carbapenem resistant isolate. The details are shown in Table 1.

Modified Hodge test & Triton Hodge test results

Out of the total 46 carbapenem resistant isolates, 14(30.4%) isolates were Modified Hodge test negative (L=0mm). Among the 32 (69.5%) positive isolates in Modified Hodge test, there were 21 (65.6%) weak positive (0<L<3mm) & 11 (34.3%)

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positive isolates (L ≥3mm). 4 carbapenem resistant isolates each of Acinetobacter baumanii, Ecoli & 3 isolates of Pseudomonas aeruginosa were Modified Hodge test negative. Majority of the weak positive isolates in Modified Hodge test were Ecoli & Klebsiella pneumoniae (each n=7, 33.3%).

4 (8.6%) carbapenem resistant isolates which were Triton Hodge test negative were also negative by Modified Hodge test. There were total 42 (91.3%) positive isolates in Triton Hodge test out of which 17(40.4%) isolates were weak positive & 25 (59.5%) isolates were positive. Carbapenem resistant isolates of Ecoli (n=1), Acinetobacter baumanii (n=2) & Pseudomonas aeruginosa (n=2) which were MHT negative became positive for carbapenemase production in Triton Hodge test. Some of the weak positive isolates in MHT like Acinetobacter baumanii (n=3),Ecoli (n=3) & Klebsiella pneumoniae (n=1) became positive after addition of Triton X reagent to MHT. Details are summarised in Table 2.

Positive isolates of Ecoli showed maximum length of indentation of indicator strain (Ecoli ATCC 25922) upto 3mm in Modified Hodge test but exhibited the length of indentation of indicator strain upto 5 mm in Triton Hodge test. Similar results were observed in Klebsiella pneumoniae & Acinetobacter baumanii with maximum length of indentation of indicator strain upto 6 mm in Triton Hodge test. 2 isolates of Pseudomonas aeruginosa which were MHT negative came out as positive in Triton Hodge test with maximum length of indentation of indicator strain being 7 mm [figure 2].

Detection rate of both Modified Hodge test & Triton Hodge test for carbapenemase production were 69.5% & 91.3% respectively. Mean length of indentation of indicator strain was 1.3mm in Modified Hodge test & 3.4mm in Triton Hodge test. P value was calculated to be <0.001 indicating a significant difference in indentation of indicator strain by both methods.

Table 1. Carbapenem resistant gram negative bacteria isolated from clinical specimens

Carbapenem resistant gram negative bacteria isolated	Wound swab (n=18,39.1)	Pus (n=8,17.3%)	Urine (n=7,15.2%)	Sputum (n=6,13%)	ET aspirate (n=5,10.8%)	Blood (n=2,4.3%)
Ecoli (n=14, 30.4 %)	8	2	4	0	0	0
Klebsiella pneumoniae (n=14,30.4 %)	4	4	0	4	1	1
Acinetobacter baumanii (n=11,23.9%)	4	0	2	2	3	0
Pseudomonas aeruginosa (n=3, 6.5%)	1	1	0	0	1	0

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Acinetobacter haemolyticus (n=1, 2.1%)	0	0	0	0	0	1
Acinetobacter calcoaceticus (n=1, 2.1%)	0	0	1	0	0	0
Enterobacter aerogenes (n=1,2.1%)	0	1	0	0	0	0
Proteus mirabilis (n=1, 2.1%)	1	0	0	0	0	0

Table 2. Comparison of Modified Hodge test & Triton Hodge test results

	Modified Hodge Test			Triton Hodge Test		
Total no.of carbapenem resistant isolates (n=46)	Negative (L= 0 mm)	Weak positive (L>0mm but L<3mm)	Positive L ≥3mm)	Negative (L= 0 mm)	Weak positive (L > 0 mm but L<3mm)	Positive (L ≥ 3 mm)
Ecoli (n=14)	4	7	3	2	5	7
Klebsiella pneumoniae (n=14)	1	7	6	0	7	7
Acinetobacter baumanii (n=11)	4	5	2	0	4	7
Pseudomonas aeruginosa (n=3)	3	0	0	1	0	2
Acinetobacter haemolyticus (n=1)	0	1	0	0	0	1
Acinetobacter calcoaceticus						

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(n=1)	1	0	0	0	0	1
Enterobacter aerogenes (n=1)	1	0	0	1	0	0
Proteus mirabilis (n=1)	0	1	0	0	1	0

Figure 1. Distribution of carbapenem resistant gram negative bacteria in various clinical specimens

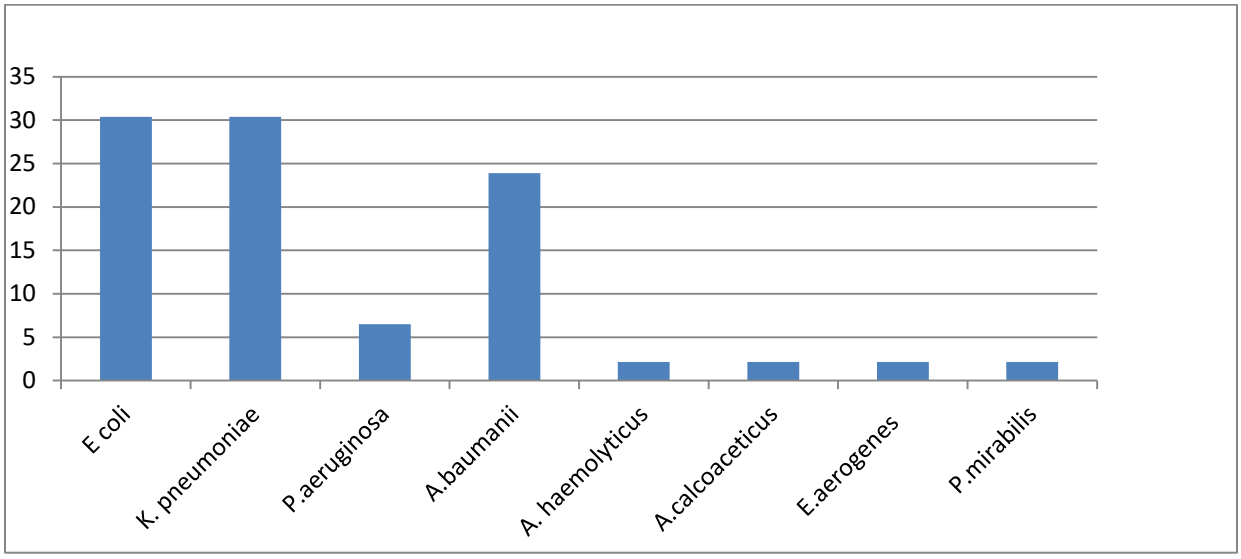
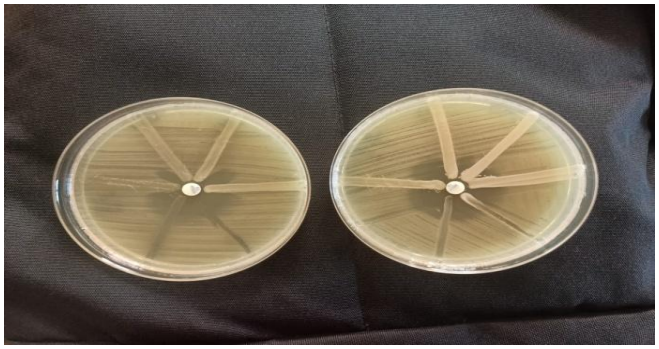


Figure 2. Triton Hodge test & Modified Hodge test results



Left side : Triton Hodge test, Right side: Modified Hodge test

Discussion

In the present study, 47.9% (n=46) of gram negative bacteria were identified as carbapenem resistant by BD Phoenix automated system. Diwakar J et al has reported 43.4% of gram negative bacteria as carbapenem resistant in his study. Much higher rate of carbapenem resistance, about 76.2% was reported among Enterobacteriaceae in a study conducted by Pooja et al in 2020. These findings suggest that knowledge regarding the prevalence of carbapenem resistant isolates is essential for judicious management of patients suffering from infections caused by carbapenem resistant strains[8,9].

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Authors have observed that majority of carbapenem resistant clinical isolates were recovered from the elderly age group, with the mean age being 58 years. This could be due to more hospital admissions among elderly, use of invasive devices,overuse of antimicrobials & inappropriate prescription of antimicrobials to the elderly age group (prescription not based on antimicrobial susceptibility testing reports) & acquiring of multidrug resistant isolates from the hospital environment during the prolonged hospital stay[10]. Carbapenem resistant isolates were predominantly recovered from males (n= 34 , 73.9%). Maximum number of carbapenem resistant isolates were recovered from wound swab (n= 18 , 39.1 %) followed by pus (n=8, 17.3 %) & urine (n= 7,15.2 %) in the present study.However, urine isolated the highest number of carbapenem resistant isolates in recent studies conducted by Akshaya R et al, Pooja et al & Namrata et al in 2016, 2020 & 2021 respectively[8,11,12]Ecoli & Klebsiella pneumoniae (n = 14 , 30.4 %) were the commonest carbapenem resistant isolates in our study. Previous studies also have concluded with similar findings.

The rising trend of antimicrobial resistance has become a global issue of concern stressing on health care facilities early detection of carbapenem resistance. Various phenotypic methods are available to detect the production of carbapenemases like Modified Hodge test (MHT), Combined disc test (CDT), modified carbapenem inactivation method (mCIM) , Carba NP test & Epsilometer test (E test). Modified Hodge test or clover leaf test is the method recommended by CLSI in 2009 for early screening of carbapenemase activity. This screening method is easy to perform, cost effective & doesn't require any expensive reagents for the test to be done. In spite of these advantages, MHT has so many limitations like giving indeterminate results ,longer turn around time, interpretation is subjective which may vary from observer to observer. Though it has excellent sensitivity for detecting KPC type carbapenemases produced by gram negative bacteria, it is less sensitive for detecting OXA-type carbapenemases & metallobetalactamase (MBL) enzymes especially New Delhi metallobetalactamases (NDM)[13].In 2016, Fernando Pasteran et al came up with an improved version of Modified Hodge test by using Triton X 100 reagent to overcome the above limitations[6].

In the present study,we have compared carbapenemase detection performance of Triton Hodge test with Modified Hodge test. Authors have observed that 69.5% (n=32) of carbapenem resistant isolates came out as positive for carbapenemase production in Modified Hodge test. At the same time 91.3 % (n=42) of carbapenem resistant isolates turned out to be positive in Triton Hodge test. This concludes that Triton Hodge test has more detection performance than Modified Hodge test for detection of carbapenemases in gram negative bacteria. Our results are in agreement with the results obtained by Pasteran et al & Fan et al in their 2016 & 2020 studies respectively.Pasteran et al in his 2016 study has concluded with a sensitivity of 67% & 97 % for Modified Hodge test & Triton Hodge test respectively. A similar study conducted by Fan et al in 2020 for detection of Acinetobacter baumanii carbapenemases displayed a sensitivity of 59 % for Modified Hodge test & 100 % for Triton Hodge test. The low sensitivity of Modified Hodge test could be attributed to its reduced ability to detect OXA- type carbapenemases & metallobetalactamases[6,14].

A recently conducted study reported false negative MHT results among 1IMP-4 producer & 11 NDM producers. Other related studies also have demonstrated that MHT is least sensitive for detection of metallobetalactamases. Detection rate of MHT for NDM, VIM & IMP were 0 %, 30 % & 50% respectively in a 2017 study[15]. Kumudunie et al has reported a low positive predictive value of 56 % for MHT due to its poor sensitivity for identification of NDM producers[16]. Many related studies have observed that MHT is also not sensitive enough for detection of OXA-type carbapenemases like OXA-48 type carbapenemases.[14]

NDM-1, one of the most dominant carbapenemase is a cell membrane bound carbapenemase & it remains anchored to the cell membranes in gram negative bacteria. It is difficult to detect these membrane bound carbapenemases by MHT which could lead to false negative reporting of carbapenemase. We have observed that a slight modification of MHT by adding a very inexpensive reagent like Triton X 100 could cause a marked increase in the sensitivity of carbapenemase detection. Triton X 100 is one of the most widely used non ionic surfactant for cell lysis & extraction of proteins & nucleic acids. These

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detergents helps in membrane permeabilization & solubilization of membrane proteins. Addition of 50µl of pure Triton X 100 reagent to the Mueller Hinton agar plate helps to permeabilize the cell membranes of carbapenem resistant strains thus releasing the membrane bound carbapenemases[6]. Triton Hodge test has already proved its high detection performance in previous studies. Sensitivity improved from 59 % in MHT to 100 % in THT for detection of OXA type carbapenemases in a 2020 study[14]. Similarly, the sensitivity for NDM detection increased from 20 % in MHT to 92.5 % in THT in the study conducted by Pasteran et al[6].

Many authors have conducted studies on addition of Triton X 100 reagent to other methods of carbapenemase detection like carbapenem inactivation method (CIM) & Carba NP test (CNPt). All these studies exhibited higher sensitivity for carbapenemase detection with the addition of Triton[14,16,17] .CNPt- direct test is a modification of Carba NP assay where the bacterial lysis solution is replaced with Triton X 100. CNPt – direct assay has shown a higher performance for detection of carbapenemases with reduction in cost compared to CNPt[16,18].

Authors of the present study have found that the number of positive isolates (n=25, 59.5%) were more compared to weak positive isolates (n=17, 40.4 %) in Triton Hodge test. All 10 isolates of gram negative bacteria which were MHT negative (L=0 mm) became positive (L≥ 3 mm) by THT with the maximum length of indentation of indicator strain upto 7mm. Carbapenem resistant isolates (n=11) which were MHT positive (L ≥ 3mm) were also positive by THT (L ≥ 3mm) but with enhanced growth of indicator strain upto a maximum of 6mm length in THT. For few gram negative isolates that were weak positive in MHT, length of indentation of indicator strain measured 0.5mm , 0.8 mm & 1mm. This could lead to indeterminate results as interpretation of small length of indentation is very difficult. The same isolates became positive (L ≥ 3mm) for carbapenemase production inTHT . This helped in better visualization & measurement of enhanced growth of indicator strain, thus avoiding indeterminate results .

Out of the 46 carbapenem resistant isolates, 4 (8.7%) gram negative isolates were both MHT & THT negative. Carbapenem resistance in these isolates could be probably due to resistance mechanisms other than carbapenemase production like loss of porin channels, overexpression of efflux pumps[3].

Porins are outer membrane proteins which helps in uptake of amino acids, metabolites & antibiotics in gram negative bacteria. Previous studies have concluded that decrease in the number of porin channels or in the size of channels or even electrostatic modification of porin channels can reduce the cellular permeability. This can result in reduced uptake of drug leading to carbapenem resistance. Main porin channels related to carbapenem resistance are OprD in P.aeruginosa, Omp C & Omp F in Ecoli & E.aerogenes, OmpK35 & OmpK36 in K.pneumoniae & CarO in A.baumanii.[19,20,21]

Similarly, antimicrobial agents can be easily expelled to the external environment through efflux pumps. The reduced intracellular accumulation of antibiotics can result in carbapenem resistance. Over expression of efflux pumps are seen more frequently when meropenem & ertapenem are used. There are 5 super families of efflux pumps out of which RND family (Resistance Nodulation Division family) constitutes the major efflux pump in gram negative bacteria. Upregulation of MexAB-OprM efflux pump, a class of RND family as well as loss of OprD porins are the major mechanisms of carbapenem resistance in P.aeruginosa. Non carbapenemase mechanisms like porin loss coupled with efflux pump production has been a cause of carbapenem resistance in various other gram negative bacteria including Acinetobacter baumanii.[22,23]

Authors have observed that in the present study, 2 isolates of Ecoli, 1 isolate of P.aeruginosa & 1 isolate of E.aerogenes were both MHT & THT negative. The results indicates that these bacteria would be exhibiting non carbapenemase mechanisms of resistance like porin loss, efflux pump production or a combination of both mechanisms[23].

The present study has few limitations.

1. Only those isolates which were identified as carbapenem resistant by BD Phoenix automated system were included in the study. Carbapenem sensitive isolates were not tested for carbapenemase production by both MHT & THT.

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2. PCR , which is considered to be the gold standard reference method for carbapenemase detection was not performed in the present study.

Conclusion

Early & rapid detection of carbapenem resistance among gram negative bacteria is necessary to curb the spread of these multidrug resistant organisms around the globe. Modified Hodge test is a CLSI recommended screening method for carbapenemase detection but it has limitations in detecting metallobetalactamases & OXA type carbapenemases. Adding a non ionic surfactant like Triton X 100 helps to overcome the above limitations. Detection rate of MHT & THT were 69.5% & 91.3% respectively in the present study. Improved performance of THT could make it a better alternative to MHT for detection of carbapenemases.

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