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Comparison of Various Phenotypic Methods in Detection of Carbapenemases and Metallo-Beta-Lactamases in Carbapenem Resistant Clinical Isolates of *Acinetobacter* Species at a Tertiary Care Centre in North India

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A B S T R A C T

Carbapenem resistance has become a worldwide issue and a major reason of concern for the treating physicians. Plasmid mediated metallo-beta-lactamases (MBLs) as well as oxacillinases (OXA) are responsible for carbapenem resistance. Various phenotypic as well as genotypic screening methods are used for detecting carbapenemases and MBLs. The strains were first and foremost screened for carbapenem resistance by Kirby-Bauer Disc Diffusion method. The resistant strains were detected for the production of carbapenemases by Modified Hodge Test (MHT) followed by MBL detection using Disc Potentiation Test (DPT) and EDTA Disc Synergy (EDS) test simultaneously. Our study emphasizes on the detection of carbapenem resistant *Acinetobacter sps.* emerging due to MBL production, and also for understanding the impact of MBL production in carbapenem resistant strains. Among the 100 clinical isolates of carbapenem resistant *Acinetobacter sps.* subjected to the phenotypic detection of carbapenemases and metallo-beta-lactamases, carbapenemases production was found to be 86 per cent by MHT, MBL production as 43 per cent and 86 per cent by DPT and EDS respectively. Thus, according to our study, EDS was considered to be more sensitive and reliable phenotypic test for MBL detection in our isolates.

Introduction

Acinetobacter sps. are Gram negative, aerobic, non-fermenting bacteria playing a vital role in hospital acquired infections. Due to various obstacles in the treatment and control of the pathogen, it continues to be a major threat (Giamarellou H *et al., 2008)*. Resistance for the β-lactam antibiotics has become widespread among *Acinetobacter* strains. There are several factors contributing for the carbapenem resistance such as hydrolysis by β-lactams (MBLs), lack of drug penetration due to porin mutation, loss of several outer membrane proteins and efflux mechanisms (Walsh TR *et al.,* 2002).

Resistance due to hydrolysis by β-lactamase enzymes is a major cause of carbapenem resistance worldwide. Many times, multi-resistant and PAN resistant *Acinetobacter* sps. have become a threat to any hospital in current

antibiotic era. Therefore, rapid, simple and cost effective screening tests are adopted for identification of MBL and carbapenemases. Various phenotypic tests like EDTA (EDS) test (Lee *et al.,* 2001), MBL E-test (Walsh TR *et al.,* 2002), EDTA based microbiological assay (Marchiaro *et al.,* 2005), DPT (Collee *et al.,* 2003) are used for identification of MBLs and Modified Hodge Test (MHT) for carbapenemases.

Hence, the present study was undertaken to isolate *Acinetobacter sps.* resistant to carbapenems and study the production of MBLs and carbapenemases by various phenotypic methods. We have also compared various methods in the present study.

Materials and Methods

The study was conducted in department of microbiology of a tertiary care centre of North India. A total of 100 consecutive, non-duplicate, clinical strains of *Acinetobacter sps.* were isolated from various samples such as endotracheal aspirate, blood and pus respectively. These isolates were subjected to various phenotypic methods for detection of MBL and carbapenemases.

MHT

The carbapenem resistant strains were at first subjected to MHT for detecting carbapenemases production. The quality strain used is *Escherichia coli*_ATCC (American Type Culture Collection) 25922. This *E.coli* ATCC 25922 strain is adjusted to 0.5 McFarland's standard and inoculated on the surface of Muller-Hinton Agar (MHA) using a sterile cotton swab. After drying, a 10 µg meropenem/imipenem disc was placed at the centre of the plate and the strains to be analyzed were streaked from the edge of the disc to the edge of the plate in four different directions. The plate was then incubated overnight at 37^0C. Presence of a clover leaf shaped zone of inhibition along the growth of the test strain was considered as positive for carbapenemases production (Noyal MJC *et al.,* 2009) (Fig. 1).

All strains were further subjected to Disc Potentiation Test (DPT) and EDTA Disc Synergy (EDS) test for the detection of MBLs.

DPT

For detecting MBLs in carbapenem resistant isolates using DPT, the test organism was first adjusted to 0.5 McFarland's opacity standards and inoculated on MHA plate. Two 10 µg imipenem discs, one containing 750 µg EDTA, obtained from Hi-Media Mumbai, were placed on the inoculated plate and incubated for 24 hours at 37^0C. The zones of inhibition around imipenem disc alone and imipenem-EDTA disc were recorded. An increase in the zone of inhibition of at least 7mm around the imipenem-EDTA disc as compared to imipenem alone was considered as positive for MBL production (Krista L *et al.,* 2006) (Fig. 2).

EDS

MBL detection by EDS is done by simultaneous testing of two different β-lactams imipenem and ceftazidime in carbapenem resistant strains (Lee *et al.,* 2001). A 0.5 M EDTA solution was prepared by dissolving 186.1 gms of Disodium EDTA.$2H_2O$ (obtained from SRL) in 1000 ml of distilled water. The pH is adjusted to 8.0 using NaOH (obtained from MERK) and was sterilized by autoclaving (Yong D *et al.,* 2002). An overnight culture of the test isolate is adjusted to a turbidity of 0.5 McFarland standard (Krista *et al.,* 2006) and spread on the surface of the MHA plate. A 10 µg imipenem disc and 30 µg ceftazidime disc is placed on the agar. A blank disc (6mm in diameter,

Whatmann filter paper no. 1) was kept on the inner surface of the lid of the MHA plate and 10 µl of 0.5 M EDTA was added to it. This EDTA disc was then transferred to the surface of the agar and kept 10 mm edge to edge apart from the imipenem or ceftazidime disc. After incubating overnight at 37^0C, the presence of an expanded growth inhibition zone between the two discs was interpreted as positive for MBL production (Fig. 3).

Results and Discussion

Among the total 100 carbapenem resistant clinical isolates of *Acinetobacter sps.,* 96 per cent were isolated from the endotracheal aspirate, 2 per cent from pus and 2 per cent from blood respectively.

In our study, 100 per cent strains were identified as enzyme producers, either as carbapenemases or as metallo-beta-lactamases.

Out of the total carbapenem resistant clinical isolates, 86 per cent (86/100) of the strains were found to be positive for carbapenemases production by MHT. Whereas, 14 per cent of the strains not detected by MHT for carbapenemases production, were all detected as MBL producers by EDS and 29 per cent (4/14) of these MHT negative strains were reported as MBL producers by DPT as well.

In the present study, EDS reported 86 per cent (86/100) of the strains positive for MBL production. However, total 14 MBL negative strains were all positive for carbapenemase production by MHT. We used two drugs ceftazidime and imipenem to see MBL production by EDS method. On comparing individual drugs, 9 per cent of the strains showed synergetic effect only by ceftazidme and in 67 per cent of the strains, synergetic effect is shown by only imipenem. However, 24 per cent of the isolates showed synergetic effect by both ceftazidme and imipenem. Intrestingly, 85 per cent (12/14) carbapenemase negative strains by MHT were found as MBL producers by imipenem in EDS.

DPT using imipenem detected only 48 per cent (48/100) of the isolates as MBL producers. Out of 52 per cent undetected by DPT, 76.9% were detected as MBL producers by EDS and 80% as carbapenemases producers by MHT. An important observation that authors feel, is that despite using imipenem and imipenem EDTA in DPT, those strains which were negative by DPT, most of these strains 73% (38/52 strains) showed MBL production by EDS method using imipenem and freshly prepared EDTA discs. Therefore, we can assume that commercially available imipenem-EDTA disc may not contain right amount or right potency of EDTA solution in the disc. Although the sensitivity of DPT was the least, but interestingly, DPT was able to detect 14% (2/14) isolates as MBL producers that were reported negative by EDS.

Carbapenems have a wide spectrum of antibacterial activity, and are resistant to hydrolysis by most of the β-lactams including ESBL and Amp C β-lactamases (Payne DJ *et al.,* 1997).

However, due to over and irrational use of carbapenems, an alarming increase in carbapenem resistance has been reported in many gram negative bacteria including *Acinetobacter sps.* (Walsh *et al.,* 2005). *Acinetobacter sps.* has become a vital nosocomial pathogen in the last few years especially in ICU setting.

Fig.1 Modified Hodge Test (MHT). Positive strain shows a 'cloverleaf shaped' zone of inhibition due to carbapenemase production

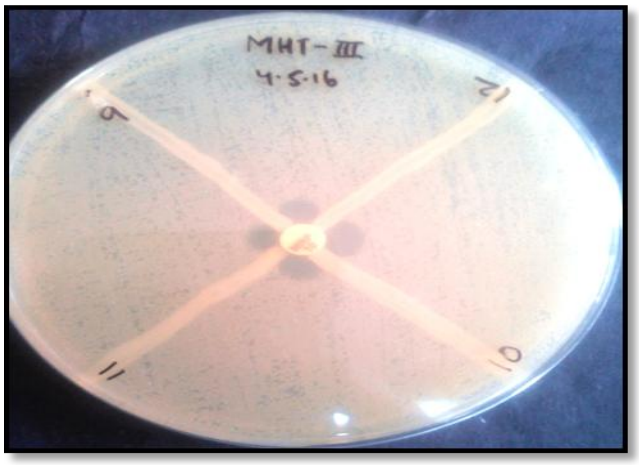

Fig.2 Disc Potentiation Test (DPT). An increase in the zone of inhibition of >7mm around the imipenem-EDTA disc as compared to imipenem alone is positive for MBL production

Fig.3 EDTA Disc Synergy Test (EDS). Positive strain shows a synergistic zone of inhibition between ceftazidime or imipenem disc and EDTA disc

In the present study, we focused on carbapenem resistant *Acinetobacter sps.* for carbapenemases and MBL production in our set-up. Being a tertiary care centre, we also encountered high prevalence of carbapenem resistant bugs, especially *Acinetobacter sps.* We took 100 non- consecutive *Acinetobacter sp.* isolates in the present study. We found that 96% of our isolates were from the endotracheal secretions, which was in concordance to a study conducted by Muthuswamy *et al.,* in Coimbatore (2012) where most of isolates were from respiratory secretions.

Many other studies (Duygu Aksoy *et al.,* 2015, Aparna Shivaprasad *et al.,* 2012, Jaggi *et al.,* 2014) also reported high prevalence of *Acinetobacter sp.* in respiratory secretions. Therefore, we conclude that respiratory tract is a preferred site for *Acinetobacter sp.* infection. Most of our isolates were from ICU settings. A very similar observation was given by other authors like Sinha *et al.,* (2007) and Richa Hans *et al.,* (2015). They felt that a lot of risk factors associated with *Acinetobacter sp.* infections exist in ICU like opportunity for cross transmission many environment reservoirs, immuno-compromised status of the patients, multiple indwelling devices, etc,.

A study conducted in Karnataka by Aparna Shivaprasad *et al.,* (2014) has documented 100% strains positive for carbapenemases production by MHT. Similarly, we observed a very high prevalence (100%) of enzyme production in our study i.e. the isolates were either carbapenemase or MBL producers. Phenotypically, Modified Hodge Test (MHT) for carbapenemases production confirmed a high prevalence of 86% (86/100) in our isolates, which is quite similar to a study by Duygu Aksoy *et al.,* where 50 strains (96%) were detected as carbapenemase positive by the modified Hodge test.

In another study by Anil V. Kumar *et al.,* (2011), 71% of *Acinetobacter sps.* were found to be carbapenemases producers. This was also in concordance with the results which were obtained by Lee *et al.,* in Korea (2001), where 73% (59/81) of the isolates were found to be carbapenemase positive by the MHT (Lee *et al.,* 2001). This suggests that carbapenamase producing *Acinetobacter sp* might be on a rise worldwide which could be due to indiscriminate and overuse of carbapenems in our hospitals. On a contrary although, Noyal *et al.,* in 2009 reported only 1 strain (14.3%) positive for carbapenemases production by Modified Hodge test (Noyal *et al.,* 2009). Likewise, another study of same geographical area, by Richa Hans *et al.,* in 2015 documented 26.4% carbapenemase production and a very low prevalence of 2.96% was also reported by Patwardhan *et al.,* 2013. A study by Uma *et al.,* (2009) reported 70.9 % MBL production. On a contrary, another study conducted by Noyal *et al.,* in 2008, only 3 (6.5%) were MBL producers.

Although there are no established phenotypic methods available for the detection of specific serine carbapenemases. However, for zinc based carbapenemases (MBLs) various methods like EDTA-disc potentiation test, MBL E-test and EDTA based microbiological assay are available (Richa Hans *et al*, 2015).

All our isolates were thus, further subjected to DPT and EDS simultaneously to detect MBL production. Overall MBL production was found quite significant 88% (88/100) among our isolates close to a study by Uma *et al.,* (2009) reporting 70.9 % MBL production. Another Indian study by Shanthi *et al.,* (2009) observed 80% MBL production in non-fermenters. Whereas, on a contrary, in a study conducted in 2007 by Sinha *et al*, none of the isolates were reported as MBL producers. Dheepa Muthusamy *et al.,* (2012) detected 10% of the strains to be MBL producers. In a

similar study conducted by Noyal *et al.*, (2009) in Pondicherry, South India, in the year 2009, 6.5% MBL producers were identified among *Acinetobacter sps.*

According to Yong *et al., (2002)*, the imipenem (IMP) 10 µg-EDTA 750 µg combined disc test has 95.7% sensitivity and 91.0% specificity for detection of metallo-β-lactamases in MBL-producing *Ps. aeruginosa* and *Acinetobacter spp*. (Yong D et al, 2002).

In our study EDS detected 86% (86/100) of the strains as MBL producers. This was in concordance to various studies reporting EDS as one of the convenient methods for the detection of Ambler class B MBL production. In 2005, a similar study by Jesudasan *et al.*, reported 72 per cent of the strains to be MBL producers. In 2014, a study by Aparna S *et al.*, documented 67.05 per cent MBL production. By the same method although, in 2011 John S *et al.*, reported only 14.8 per cent MBL production among *Acinetobacter sps*.

In our study, DPT confirmed only 48% (48/100) of the isolates as MBL producers. This was contrary to certain studies which revealed 81.18% and 96.6% of the strains positive by DPT (Aparna *et al.*, 2014 and Irfan *et al*, 2008)

Therefore, probably the low potency of commercially available imipenem-EDTA combination discs used in DPT may be a reason of low sensitivity of DPT in the present study. Therefore, according to our study, EDS is found more sensitive 86% (86/100) than DPT 48% (48/100).

Significance of the results

Modified Hodge Test (MHT) is found to be a reliable phenotypic method for the detection of carbapenemases production. However for the detection of MBL production, EDTA Disc Synergy (EDS) test is found to be more sensitive and reliable in comparison to Disc Potentiation Test (DPT).

Both ceftazidime and imipenem should be used simultaneously to conduct EDS for avoiding false negative results. Moreover, there is a need of two or more tests to identify all the carbapenemases producing isolates. MBL production is an important mechanism of carbapenem resistance among *Acinetobacter sps.* in the present study.

Thus, it is recommended to perform these simple phenotypic tests like Modified Hodge Test (MHT) for carbapenemases production and Disc Potentiation test (DPT) and EDTA Disc Synergy (EDS) test for keeping a check on MBL production in small tertiary care centers or in resource limited set-ups to determine resistance mechanisms and prevent the indiscriminate usage of antibiotics. These tests are comparatively easily applicable and economical for screening of enzyme production in *Acinetobacter species*. and can be incorporated into the routine testing of any busy microbiology laboratory.

Our study concludes that high prevalence of carbapenem resistant *Acinetobacter species* with MBL production is an alarming situation which needs strict infection control measures.

Abbreviations

MHT: Modified Hodge Test
EDS: EDTA Disc Synergy (EDS)
DPT: Disc Potentiation Test (DPT)

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