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RESEARCH ARTICLE

PHENOTYPIC EVALUATION OF DIFFERENT METHODS FOR THE DETECTION OF METALLO-BETA-LACTAMASES IN *PSEUDOMONAS AERUGINOSA*

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ABSTRACT

Pseudomonas aeruginosa is known to cause wide range of infections. It exhibits resistance to several antimicrobial agents and produces ß-lactamases, which are responsible for the widespread ß-lactam resistance. In the present study, 41 isolates of *Pseudomonas aeruginosa* were obtained from various clinical specimens like pus, urine, stool and wounds. 13/41 (32%) *Pseudomonas aeruginosa* isolates were found to be positive for the production of ESBL by disc diffusion method. 9/41 (22%) *Pseudomonas aeruginosa* isolates were found to be positive for the production of metallo-β-lactamases. All the 9 isolates were subjected for the detection of metallo-β-lactamases by different methods. Imipenem (IMP)- EDTA combined disc test detected 4(45%) compared to EDTA disc potentiation using Ceftazidime, Ceftizoxime and Cefotaxime which detected 3(33%), and Imipenem (IMP)- EDTA double disc synergy test detected 2(22%) of MBL producing *P.aeruginosa* and hence IMP-EDTA CDT was a better method compared to other methods used in the study to detect MBL producers.

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INTRODUCTION

Pseudomonas aeruginosa is one of the commonest causes of infection in burn patients and an important agent for hospital acquired infections and death in immunocompromised such as cystic fibrosis and cancer patients (Neu, 1983). This bacterium is often resistant to many antimicrobial agents. The cause of resistance can be efflux pumps, decreased outer membrane permeability and secretion of beta-lactamase enzymes (Arakawa *et al.,* 2000). *Pseudomonas aeruginosa* producing metallo-β-lactamases (MBLs) was first reported from Japan in 1991 (Watanabe *et al*., 1991) and since then has been described from various parts of the world, including Asia (Yan *et al*., 2001), Europe (Libisch *et al*., 2004), Australia (Peleg *et al*., 2004), South America (Gales *et al*., 2003), and North America (Toleman *et al*., 2004). MBLs are class B enzymes which hydrolyze carbapenems and are encoded by genes like IMP, VIM, etc (Jaykumar *et al*., 2007). They have been described as the enzymes which require divalent cations, usually zinc, as metal co-factors for their enzymatic activity. Metallo-β-lactamase-producing *P.aeruginosa* isolates have been responsible for several nosocomial outbreaks in tertiary centers in different parts of the world, illustrating the need for proper infection control practices. These isolates have also been responsible for serious infections, such as septicaemia and pneumonia (Cornaglia *et al*., 2000), and have been associated with failure of therapy with carbapenems (Nordmann & Poirel, 2002).

Several phenotypic methods are available for the detection of MBL-producing bacteria. All these methods are based on the ability of metal chelators, such as EDTA and thiol-based compounds, to inhibit the activity of MBLs. This study was taken up to evaluate three different methods for the detection of Metallo-β-lactamase production among *P.aeruginosa.*

MATERIALS AND METHODS

Collection and processing of samples

Pus and wound samples were collected with the help of sterile cotton swabs and the stool and urine samples were collected in sterile containers under aseptic conditions by standard procedures and were processed according to standard guidelines. Samples were inoculated onto blood agar, MacConkey agar, cetrimide agar and nutrient agar for primary isolation. Identification of Pseudomonas was done by using standard biochemical methods.

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ESBL screening test

All the isolates of *P.aeruginosa* which showed resistance to ceftazidime were evaluated for ESBL production. 0.5 Mc Farland's suspension of each isolate was spread on a Muller Hinton agar (MHA) plate and ceftazidime (30 µg) and ceftazidime / clavulanic acid (30 µg/ 10 µg) discs were placed aseptically on the agar plate. A distance of about 15mm was kept between the two discs (edge to edge) and the plates were incubated at 37°C for 24 hrs. An observation of a ≥ 5mm increase in the zone diameter for the antimicrobial agent which was tested in combination with clavulanic acid, versus its zone diameter when tested alone, confirmed the presence of ESBL production by the organism. The increase in the zone diameter was due to the inhibition of the β lactamase by clavulanic acid. (CLSI 2006)

MBL screening tests

a) Imipenem (IMP)-EDTA combined disc test (IMP-EDTA CDT): (Yong *et al.,* 2002)

MHA plate was inoculated with standard inoculum (0.5 McFarland) of the test organism to form a lawn culture. Two 10mcg Imipenem discs were placed on the plate. 10µl of 0.5M EDTA solution was added to one of the Imipenem discs to obtain a concentration of 750mcg and was incubated overnight at 37°C. If there was an increase in inhibition zone with IMP and EDTA disc was >7mm than IMP disc alone, it was considered as MBL producer.

b) Imipenem (IMP)-EDTA double disc synergy test(IMP-EDTA DDST): (Lee *et al*., 2003)

MHA plate was inoculated with standard inoculum (0.5 McFarland) of the test organism to form a lawn culture. An IMP (10mcg) disc was placed 20mm center to center (10mm edge to edge) from a blank disc(6mm diameter, Whatmann No.2) containing 10µl of 0.5 M EDTA(750mcg) and was incubated overnight at 37°C. Enhancement of zone of inhibition in area between IMP and EDTA disc in comparison with zone of inhibition on far side of the drug was considered as MBL producer.

a) EDTA disc potentiation using ceftazidime, ceftizoxime and cefotaxime

MHA plate was inoculated with standard inoculum (0.5 McFarland) of the test organism to form a lawn culture. A filter paper (6mm diameter, Whatmann No.2) was placed and the following discs such as ceftazidime (30 µg), ceftizoxime (30 µg), cefotaxime (30 µg), cefepime (30 µg) were placed 25mm center to center from blank disc.10µl of 0.5 M EDTA (750mcg) solution was added to the blank disc and was incubated overnight at 37°C. Enhancement of zone of inhibition in area between EDTA disc and any one of the four cephalosporin disc in comparison with zone of inhibition on far side of the drug was interpreted as an MBL producer. (Arakawa, 2000)

RESULTS

In the present study 41 isolates of *Pseudomonas aeruginosa* were obtained from various clinical specimens like pus, urine, stool and wounds. Isolation rate of *Pseudomonas aeruginosa* was found to be high in pus samples followed by wound samples (Table 1).

Screening of ESBL production among *P.aeruginosa*

Of the 41 isolates of *Pseudomonas aeruginosa*, 13 (32%) were found to be positive for the production of ESBL by disc diffusion method using ceftazidime and with ceftazidime/ clavulanic acid. All the 13 isolates were found to be resistant towards ceftazidime (Table 2).

Screening of MBL production among *P.aeruginosa*

Of the 41 isolates of *Pseudomonas aeruginosa*, 9 (22%) were found to be positive for the production of metallo-β-lactamases and 32 (78%) were non-metallo-β-lactamase producers (Table 3).

Evaluation of different phenotypic methods for the detection of Metallo-Beta-Lactamases

In the present study, 9 *P.aeruginosa* isolates which were resistant to Imipenem were screened for MBL detection.

Table 1. Isolation of *Pseudomonas aeruginosa* from clinical samples

S.No.	Source of sample	Total number of samples collected	Percentage of *Pseudomonas aeruginosa* from various samples
1	Pus	21	18 (86%)
2	Urine	15	7 (47%)
3	Wound Swabs	17	13 (76%)
4	Stool Samples	9	3 (33%)

Table 2. Ceftazidime antibiotic sensitivity pattern of *P.aeruginosa*

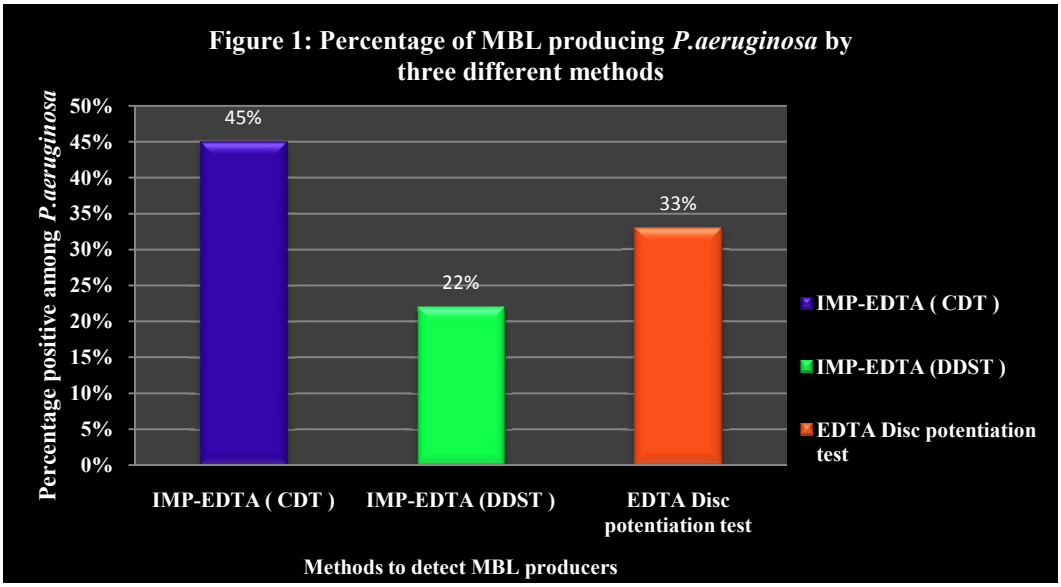
Total number of *P.aeruginosa*	Ceftazidime sensitive isolates	Ceftazidime resistant isolates
41	28 (68%)	13 (32%)

Table 3. Imipenem antibiotic sensitivity pattern of *P.aeruginosa*

Total number of *P.aeruginosa*	Imipenem sensitive isolates	Imipenem resistant isolates
41	32 (78%)	9(22%)

Table 4. Phenotypic evaluation of Metallo-Beta-Lactamases

Total number of *P.aeruginosa* resistant to imipenem	IMP-EDTA CDT	IMP-EDTA DDST	EDTA disc potentiation test
9	4 (45%)	2 (22%)	3 (33%)



Imipenem (IMP)-EDTA combined disc test detected 4(45%) compared to EDTA disc potentiation using Ceftazidime, Ceftizoxime and Cefotaxime which detected 3(33%), and Imipenem (IMP) - EDTA double disc synergy test detected 2(22%) of MBL producing *P.aeruginosa* and hence IMP-EDTA CDT was a better method compared to other methods used in the study to detect MBL producers (Table 4 & Figure 1).

DISCUSSION

MBL producing isolates are associated with a higher morbidity and mortality. Moreover given the fact that MBLs will hydrolyze all classes of ß-lactams. The occurrence of an MBL positive isolate poses not only a therapeutic problem but is also a serious concern for infection control management. As a result of being difficult to detect, such organisms pose significant risks particularly due to their role in unnoticed spread within institutions and their ability to participate in horizontal MBL gene transfer, with other pathogens in the hospital (Agarwal *et al*., 2006). In the present study 41 isolates of *Pseudomonas aeruginosa* were obtained from different clinical specimens like pus, urine, stool and wounds. Maximum number of *P.aeruginosa* were obtained from pus samples giving a percentage isolation of 86% (18) followed by wound swabs 76% (13), urine samples 47% (7) and stool samples 33% (3). 32% (13/41) *P.aeruginosa* were resistant to ceftazidime which was similar to the study done by Agarwal *et al*., (2008) at Haryana showing 20.27% of cefazidime resistant *P.aeruginosa*. Another study done by Singh *et al*., (2012) at Mysore also showed 27.2% *P.aeruginosa* resistant to cefazidime and 20.27% by a study by Wayne, 2010. Studies in some places like in Nagpur, the figures of ESBL producers were 50%.

A study in 2005, from New Delhi, showed 68.78 % of the strains of gram negative bacteria to be ESBL producers which is high compared to our study. Studies in few other places like in Varanasi, Upadhyay *et al., (*2010) showed the prevalence of ESBL producing *P.aeruginosa* was 3.3% and Rodrigues *et al.*, (2004) in their study showed 5.9% of *P.aeruginosa* isolates harbored ESBLs in Mumbai which is less in comparison to our study.

In our study 22% (9/41) *P.aeruginosa* were resistant to Imipenem which was similar to the study done by Shobha *et al.*, 2009 showing 30% of Imipenem resistant *P.aeruginosa*. Another study done by Varaiya *et al.* (2008) showed 20.8% *P.aeruginosa* resistant to Imipenem. A study by Irfan *et al*., (2008) showed 25% and Kumar *et al*., 2012 showed 32.4% Imipenem resistant *P.aeruginosa*. In the present study, 9 *P.aeruginosa* isolates resistant to Imipenem were screened for MBL detection. Imipenem (IMP)-EDTA combined disc test detected 4(45%) compared to EDTA disc potentiation using Ceftazidime, Ceftizoxime and Cefotaxime which detected 3(33%), and Imipenem (IMP)- EDTA double disc synergy test detected 2(22%) of MBL producing *P.aeruginosa* and hence IMP-EDTA CDT was a better method compared to other methods used in the study. This is in accordance with a study by Behera *et al.,* 2008 who demonstrated CDT method to be superior to DDST and DPT for detection on MBL producing *P.aeruginosa*. Another study by Yan *et al.,* 2004 also showed that CDT as a better method to detect MBL producing *P.aeruginosa*.

Conclusion

Combined Disc method was found to be a good method to detect ESBL producing *P.aeruginosa* in our study. Detection of ESBL and MBL in *Pseudomonas aeruginosa* should be routinely done in laboratories for an early and accurate detection of ESBL and MBL producers as this will help to start the treatment at the earliest and thus reduces mortality and morbidity due to indiscriminate antibiotic usage.

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