Original article

Comparative analysis of various phenotypic methods for the detection of beta-lactamase in Acinetobacter baumannii isolates from a referral hospital in Sikkim, India

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ABSTRACT

Introduction: Several phenotypic methods are available for the detection of the resistance to β- lactam drugs in the bacteria. The present study was undertaken to compare the efficacy of various tests for the detection of AmpC β-lactamases (AmpC) and Metallo- β-lactamases (MBL) in clinical isolates of Acinetobacter baumannii by using different phenotypic methods. Methods: For the phenotypic detection of Metallo β- lactamase: modified hodge test (MHT), the imipenem/ceftazidime–EDTA combined disk method (CDM) and the imipenem-EDTA double disk synergy test (DDST) were performed and for the phenotypic detection of AmpC β-lactamase: AmpC disc test and boronic acid disc test were used. Result: Out of 87 samples, 72(82.7%) were positive with imipenem-EDTA combined disk test, 68(78.1%) were positive in ceftazidime-EDTA combined disk test, 41(47.1%) were positive in imipenem- EDTA double disk synergy test and 62(71.2%) were positive in the modified hodge test. In the boronic acid test 38(43.6%) and in the AmpC disk test 50(57.4%) were positive for AmpC beta lactamases. Acinetobacter baumannii showed 100% resistance to ampicillin, amoxicillin /clavulinic acid and trimethoprim/sulfamethaxole and 100% sensitivity to tigecycline and colistin. Conclusion: Our study showed that the combined disk test and AmpC disk test were effective for the detection of Metallo β- lactamase and AmpC β-lactamase respectively. These methods are recommended for the detection of drug resistance especially for nosocomial pathogens like Acinetobacter baumannii.

Keywords: Acinetobacter baumannii, AmpC β-lactamases, Metallo- β-lactamases.

INTRODUCTION

Acinetobacter species, especially Acinetobacter baumannii, has emerged in the last few years as a major etiological agent of nosocomial pathogen as they are known to be responsible for a number of hospital outbreaks like hospital-acquired pneumonia, respiratory-tract infection, urinary tract infections, surgical site infections, and bloodstream infections[(1)]. These outbreaks have primarily been observed in intensive care units (ICUs)[(2-3)] and also in surgical wards, burn units and general medical wards[(4-5)]. The resistant strains of A. baumannii have a knack to survive for prolonged periods in the hospital environment, contributing significantly to the growing antimicrobial resistance, thereby posing a difficult challenge for clinicians to treat, because of

the widespread resistance to major groups of antibiotics like aminoglycosides, fluoroquinolones and third generation cephalosporins. The most common cause of bacterial resistance to beta lactam antibiotics is the production of betalactamases[6]. Many of the second and third generation penicillin and cephalosporins were specifically designed to resist the hydrolytic action of major beta lactamases. However, new beta lactamases emerged against each of the new classes of beta-lactams that were introduced and caused resistance[7]. The chromosomally mediated beta-lactamase production is mainly through expression of Amp C gene which is either constitutive or inducible[8]. These enzymes have been described in many bacteria[9]. Metallo-β Lactamases (MBL) have recently emerged as one of the most worrisome resistance mechanisms owing to their capacity to hydrolyze with the exception of aztreonam, all beta lactams including carbapenems[10]. Management of multi-drug resistant Acinetobacter species infections is a great task for clinical microbiologist and physicians as therapeutic options have become very limited raising infection control concern worldwide. Drug resistance detection is highly specific and sensitive by molecular methods however limited in certain laboratories on the other hand, the phenotypic methods being simple, sensitive, economical and reliable can be routinely performed in microbiological laboratories. Several phenotypic methods are available for the detection of the resistance to β- lactam drugs in the bacteria. Most of these methods are based on the ability of the metal chelator (EDTA) and the thiol based compounds to inhibit the enzyme activities[11]. Hence, the present study was undertaken to compare the efficacy of various test for the detection of AmpC β-lactamases and MBL in clinical isolates of Acinetobacter spp. by using different phenotypic methods.

MATERIALS & METHODS

The present prospective study was conducted in the Department of Microbiology, Central Referral Hospital (CRH), Sikkim, during a period of one year (September 2015 to August 2016). Samples like blood, lower respiratory tract (LRT) samples (endotracheal aspirates, bronchoalveolar lavage, sputum), urine, pus, throat swabs, CSF and other body fluids sent from the CRH was processed in the Department of Microbiology for the diagnosis of Acinetobacter baumannii infection. All the **c**linical samples were cultured on the various culture media like MacConkey agar, blood agar and HICROME Acinetobacter agar base media (HiMedia, Mumbai). The HICROME agar were prepared according to the manufacturer recommendations and incubated at 37°C for 18-24 hours; a positive growth of A.baumanii was identified by light purple colour colonies on the media. All the isolates were further processed by using the VITEK-2 automated system, specially designed for the microbial identification and antibiotic susceptibility testing as per Clinical and Laboratory Standard Institute (CLSI) criteria.

Phenotypic detection of Metallo β- lactamase (MBL)

Modified Hodge Test (MHT)

It is a simple phenotypic test for detection of carbapenemase activity in bacteria. A 0.5 McFarland dilution of the Escherichia coli ATCC 25922 in 5 ml of broth or saline was prepared. A 1:10 dilution was streaked as lawn on to a Mueller Hinton agar (MHA) plate. A 10 µg imipenem susceptibility disk was placed in the center of the test area. Test organism was streaked in a straight line from the edge of the disk to the edge of the plate and then incubated at

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35°C ± 2°C in a bacteriological incubator for 24-48 hrs. Carbapenemases production was detected by the appearance of the enhanced ATCC E. coli 25922 growth along the test organism that revealed a clover-leaf-like indentation which indicated a positive test[12].

EDTA combined disk /disk synergy (EDS) test

The imipenem –EDTA combined disk method (CDM) – A 10µg imipenem disk was placed on a Mueller Hinton agar plate at a distance of 20mm from an imipenem-EDTA disk, on a lawn culture of an imipenem resistant isolate. The plates were incubated overnight at 37^{o} C and the zone of inhibition of the imipenem and the imipenem -EDTA disks were compared on the next day. If the increase in the inhibition zone with the imipenem-EDTA disk was ≥ 7mm than the imipenem disk alone, it was considered to be MBL positive[13].

The ceftazidime - EDTA combined disk method (CDM) - Two 30µg ceftazidime disks were placed on a Muller Hinton agar plate, on which the lawn culture of an imipenem resistant isolate was made. 10µl of EDTA solution was added to one of them, to obtain the desired concentration of 750µg. The plates were incubated overnight at 37^{o}C and the zone of inhibition of the ceftazidime and the ceftazidime EDTA disks were compared on the next day. If the increase in the inhibition zone with the ceftazidime-EDTA disk was ≥ 7 mm than the ceftazidime disk alone, it was considered to be MBL positive[13].

The imipenem - EDTA double disk synergy test (DDST) – A 10 µg imipenem disk was placed at 20 mm centre to centre from a blank disk which contained 10ul of 0.5 molar EDTA (750 µg). After an overnight incubation at 37^{o}C, the enhancement of the zone of inhibition around the imipenem EDTA disk in comparison with the zone of inhibition on the far side of the drug was interpreted as positive for MBL production[12].

Phenotypic detection of AmpC β-lactamase

AmpC disc test

On a MHA plate, lawn culture of E.coli ATCC 25922 was made from an overnight culture suspension adjusted to 0.5 McFarland standard. A 3 µg cefoxitin disk was kept on the surface of the agar. A blank disk (6 mm in diameter, Whatmann filter paper no.1) was moistened with sterile saline and inoculated with a few colonies of the test strain. The inoculated disk was then placed beside the cefoxitin disk almost touching it. The plate was incubated overnight at 37^{0}C. A flattening or indentation of the cefoxitin inhibition zone in the vicinity of the disk with test strain was interpreted as positive for the production of AmpC β-lactamase. An undistorted zone was considered as negative[14].

Boronic acid disc test method[15]

Boronic acid test was performed by disc containing boronic acid, The stock solution was prepared as previously recommended[16]. From this solution, 20 µl (containing 400 µg of boronic acid) was dispensed onto commercially available antibiotic disks. The disks were then dried and used within 60 min. The tests were performed by inoculating Mueller-Hinton agar by the standard diffusion method[17] and placing disks containing eight different β-lactams (imipenem, meropenem, ertapenem, cefepime, cefoxitin, cefotetan, cefotaxime, and ceftazidime) with and without boronic acid onto two separate agar. The agar plates were incubated at 37°C overnight. The diameter of the growth-inhibitory zone around a β-lactam disk with boronic acid was compared with that around the corresponding β-lactam disk without boronic acid. The test was considered positive for the detection of KPC enzyme production when the

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diameter of the growth-inhibitory zone around a β-lactam disk with boronic acid was ≥5 mm larger than that around a disk containing the β-lactam substrate alone.

RESULT

Out of 87 Acinetobacter baumannii isolates, the most common sites of isolation included endotracheal secretions 35(40.2%), followed by pus 19(21.8%), sputum 15(17.2%), catheter tip 11(12.6%) and blood 7 (8%). About 29(33%) samples came from medical intensive care unit (MICU) 23(26.4%) from surgical ward, 13(14.9%) from surgery intensive care unit (SICU) and the rest 22(25.2%) samples included the cardio-care unit, emergency and neurology department. Out of 87 samples, only 63(72%) showed resistance to imipenem antibiotic by VITEK-2 automated system and when the various phenotypic confirmatory test was performed. It was observed that 72(82.7%) were positive with imipenem-EDTA combined disk test, 68(78.1%) were positive in ceftazidime-EDTA combined disk test, whereas 41(47.1%) were positive in imipenem- EDTA double disk synergy test. However in the modified hodge test, among 87 isolates 62(71.2%) were positive for metallo beta lactamas.

In the boronic acid test, the number of samples positive for the AmpC beta lactamases were 38(43.6%) and in the Amp c disk test only 50(57.4%) were positive for AmpC beta lactamases. Among isolates of Acinetobacter baumannii, showed 100% resistant to ampicillin, amoxicillin /clavulinic acid and trimethoprim/sulfamethaxole whereas resistance to other antibiotics were variable from 72% to 95.4% (imipenem, ceftriaxone, ciprofloxacin, gentamicin, amikacin, and cefuroxime) on the other hand it showed 100% sensitivity to tigecycline and colistin (Table 1). The treatment outcome status of the patients in the following study showed that about 52 (59.7%) of the patients were treated well and normally discharged, 17(19.4%) had requested for discharge, 16(18.3%) was still under medication and 2 (2.2%) of the patients had expired.

Table 1: Antibiotic susceptibility pattern of Acinetobacter baumannii

Antibiotic	**Sensitive n (%)**	**Intermediate n(%)**	**Resistant n (%)**
Ampicillin	-	-	87 (100%)
Amoxicillin/clavulinic acid	-	-	87 (100%)
Trimethoprim/sulfamethaxole	-	-	87 (100%)
Cefoperazon/sulbactam	39(44.8%)	11(12.6%)	37 (42.5%)
Ceftriaxone	11(12.6%)	-	76 (87%)
Cefuroxime axetil	-	7(8%)	80(91.9%)
Ciprofloxacin	11(12.6%)	-	76 (87%)
Amikacin	10 (11%)	-	77(88.5%)
Gentamicin	11(12.6%)	-	76 (87%)
Imipenem	20(22.9%)	4(4.5%)	63 (72%)
Meropenem	18(20.6%)	4(4.5%)	65(74.7%)
Nalidixic acid	39(44.8%)	4(4.5)	44 (50.5%)
Tigecycline	87 (100%)	-	-
Colistin	87 (100%)	-	-

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DISCUSSION

In the present study an attempt has been made to compare the modified hodge test (MHT), EDTA double disk synergy (EDS),EDTA combined disk test for the MBL detection and AmpC disc test and boronic acid disc test method for detection of AmpC β-lactamase. In our study, only 62(71.2%) isolates showed the MBL production by the Modified Hodge test, since there are no standard detection methods available, many studies have used many different methods to detect and confirm the MBL production[18-19] by MHT. Some have used an inhibition assay to test the hydrolysis of carbapenem by an isolate of KPC-1-producing Klebsiella pneumoniae[20] while other have used zinc sulfate to improve the test[21]. Lee et al. (2003) have reported that the Hodge test can be used to screen carbapenemase-producing gram-negative bacilli and that the imipenem (IPM)-EDTA double-disk synergy test (DDST) can distinguish MBL-producing from MBL-nonproducing gram-negative bacilli[22]. The three different methods used in our test (CAZ-EDTA-CDM; I-EDTA-CDM; I-EDTA-DDST) have shown variable results. The ceftazidime-EDTA combined disk test detected 68(78.1%) out of 87 isolates as MBL phenotypic confirmed but on using imipenem-EDTA combined disk test detected 72(82.7%), also imipenem- EDTA double disk synergy test was only 41(47.1%) for MBL. This result is in agreement with that of studies carried out by Bashi et al[23](2011), Prakash et al[13](2012) and Arakawa et al[24](2000) suggesting that the combined disk test was far better in performance compared to other test. However, test carried by Prakash et al[13](2012) for double disk synergy test and modified hodge test varied. Arakawa et al[24] (2000) have reported a DDST using a ceftazidime (CAZ) and a 2-mercaptopropionic acid (MPA) disk. It has been suggested that the selection of the optimal MBL screening method be based not only on the bacterial species, but also on the strains which are collected and the local prevalence of the MBL producers[13].We recommend carbapenem to treat multidrug-resistant gram-negative bacilli as these drugs are quite stable and effective against extended-spectrum and AmpC β-lactamases. Further, in the present study, AmpC β-lactamases were detected in 57.4% of the Acinetobacter isolates by AmpC disc method and only 43.6% by boronic acid method. Parveen et al[25](2010) also detected AmpC β-lactamases in 50% isolates of the Acinetobacter species. However, some studies have described a lower rate of AmpC β-lactamase production in Acinetobacter sp., which ranged from 20% to 45%[26-28]. Black et al[29] (2005) study reported that AmpC disk test based on filter paper disks impregnated with EDTA demonstrated a highly sensitive, specific, and convenient means of detection of plasmid-mediated AmpC beta-lactamases in organisms lacking a chromosomally mediated AmpC beta-lactamase. AmpC beta-lactamases are largely unknown due to difficulties in the phenotypic detection and are often misidentified as ESBLs. Detection of AmpC isolates is important not only because of their broader cephalosporin resistance but also because of carbapenem resistance by mutations resulting in reduced porin expression[28]. There are various phenotypic methods for AmpC detection and none of them are standardized as they are time-consuming when screening large number of isolates. In addition to chromosomal AmpC, the production of plasmid-mediated AmpC presents a new threat in the treatment of infection[30]. Though there is limited data on the coexistence of MBL and AmpC resistance, in our study such coexistence was found to be 20%

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however, other studies have a much higher rate of coexistence 68.5%[31]. Another study showed the coproduction of metallo-β-lactamases and AmpC β-lactamases in 54% of the Acinetobacter sp[32]. The emergence of MDR isolates of A. baumannii has become a serious problem that has made it difficult to select an empirical antimicrobial for its treatment. Therefore, monitoring of the antibiotic resistance patterns of this organism over time may provide us useful information regarding its treatment policy. Currently, the antibiotics of choice for treatment of A. baumannii infections include the aminoglycosides, fluoroquinolones, and carbapenems[33]. Thus, it is concerning that our data demonstrate 100% resistant to ampicillin, amoxicillin /clavulinic acid and trimethoprim/sulfamethaxole whereas resistance to other antibiotics was variable from 72% to 95.4% (imipenem, meropenem, ceftriaxone, ciprofloxacin, gentamicin, amikacin, and cefuroxime). This is an alarming finding that strongly suggests the possibility of treatment failures in life-threatening A. baumannii infections due to carbapenem-resistance strains. The increase in resistance may be associated with its frequency of use in health care units. The antibiotic choice for the treatment of MDR A. baumannii infections is also limited and includes the lipopeptides[34-35] which the results of this study suggest is the best class of antibiotics to use for MDR because of its 100% sensitivity to tigecycline and colistin. Our findings indicate that the prevalence of antibiotic-resistant A. baumannii is highly common in Sikkim. Our study demands a need for effective surveillance of antimicrobial resistance, which is an emerging concern to public health, particularly in the clinical management of patients infected with life-threatening A. baumannii infections.

CONCLUSION

The results of our study showed that the combined disk test and AmpC disk test was effective for the detection of β-lactamases drug resistance compared to the other tests, however since molecular analysis and characterization of MBL and AmpC resistance was not carried out due to certain limitations, this study do help in early detection of β-lactamase especially for nosocomial pathogens. Also the antibiotic resistance pattern of A. baumannii in our study does warn us of further dissemination of these multi drug resistant strains, which points towards the implementation of a stronger antibiotic policy and infection control measures to limit the spread of these resistant microorganisms.

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