MECHANISMS OF RESISTANCE TO CARBAPENEMS IN MEROPENEM-RESISTANT ACINETOBACTER ISOLATES FROM CLINICAL SAMPLES

*M Sinha, H Srinivasa

Abstract

Purpose: To analyze the resistance mechanisms in Acinetobacter species by phenotypic methods. Methods: Antibiotic susceptibility profile for 150 clinical isolates of Acinetobacter was determined by the standard disk diffusion method. Isolates detected to be meropenem resistant were tested further by broth microdilution minimum inhibitory concentration (MIC) for meropenem. The resistant isolates were also tested for metallo β-lactamase (MBL) production by the double-disk approximation test, for AmpC β-lactamase production and efflux pump detection by agar microdilution MIC with and without reserpine. Results: Twenty-one isolates were found resistant to meropenem by the standard disk diffusion method. Nine samples were from patients admitted in intensive care units (ICUs). Broth microdilution MICs of the isolates revealed low-level resistance to meropenem. MBL was not produced by any of these isolates. AmpC β-lactamases were produced by nine (43%) isolates. ‘Efflux pump’-mediated resistance to meropenem was detected in two out of nine random isolates tested for the same. Conclusions: Carbapenem resistance is not uncommon in Acinetobacter isolates. AmpC production may cause carbapenem resistance. MBL and efflux pump may not be important causes of carbapenem resistance.

Key words: Acinetobacter, carbapenem, meropenem, metallo β-lactamase.

Acinetobacter causes a wide variety of illnesses in debilitated and hospitalized patients, especially in the intensive care units (ICUs). These bacteria survive for a long time in the hospital environment, and thereby the opportunities for cross infection between patients are enhanced. Because of frequent resistance to aminoglycosides, fluoroquinolones, ursodoplicins and third-generation cephalosporins, carbapenems are important agents for managing Acinetobacter infections. In view of their resistance to hydrolysis by most β-lactamases (extended spectrum and AmpC β-lactamases), carbapenems are often used as a last resort in infections due to multidrug-resistant gram-negative bacilli. However, there has been an alarming increase in reports of carbapenem-resistant Acinetobacter spp. over the last decade. Carbapenem resistance is also being increasingly reported in Pseudomonas aeruginosa and Acinetobacter baumannii.

Carbapenem resistance in Acinetobacter is attributed to various causes such as reduced expression of outer membrane proteins (29 kDa, 33-36 kDa) and carbapenemases-β-lactamases. Some carbapenem-resistant isolates produce either metallo-β-lactamases (Ambler class B β-lactamases); or more commonly, OXA-type enzymes (Ambler class D β-lactamases or oxacillinases) having weak activity against carbapenems. Acinetobacters, especially A. baumannii, are known to produce the following metallo-β-lactamases (MBLs) – IMP-1, 2, 4 and 5 and VIM-1 and 2. Oxacillinases found in Acinetobacters are OXA-23, 24, 25, 26, 27, 40, 58. Since oxacillinases are chromosomally mediated, spread of ox genes to other microorganisms or Acinetobacters is difficult. AmpC β-lactamases have been found to be an important factor in carbapenem resistance in porin-deficient isolates, but the carbapenem hydrolytic activity is weak. Though efflux pumps causing resistance to certain antibiotics have been described in A. baumannii, these are not responsible for carbapenem resistance. Alterations in the penicillin-binding protein (PBP) with reduced affinity are not one of the known causes of carbapenem resistance.

The objective of this study was to identify mechanisms of resistance to carbapenems in meropenem-resistant Acinetobacter spp. obtained from clinical samples by phenotypic methods.

Materials and Methods

This prospective study was conducted at the Department of Microbiology at St. John’s Medical College and Hospital (SJMCH), Bangalore. One hundred fifty isolates of Acinetobacter spp. obtained from clinical samples from March 2003 to March 2004 were included in the study. All the samples were processed in the microbiology laboratory according to the standard procedures.

Preliminary identification of Acinetobacters was done by the Gram stain findings, testing for motility and the oxidase reaction in all the samples. Non-fermenting gram-negative bacilli that were oxidase-negative and non-motile were identified as Acinetobacter spp. Tests for speciating...
the isolates included glucose oxidation test by Hugh and Leifson’s OF glucose, growth at 37°C and 44°C, hemolysis on sheep blood agar, gelatin hydrolysis and arginine dihydrolase. Carbon assimilation tests were performed on isolates using a simplified panel of carbon sources (histamine, histidine, citrate, malonate, trans-aconitate, phenylalanine, DL-4 aminobutyrate).

Antimicrobial susceptibility of all isolates was determined by the standard Kirby Bauer disk diffusion method.12 Antibiotics included were amikacin (10 µg), cefotaxime (30 µg), ceftazidime (30 µg), ciprofloxacin (5 µg), meropenem (10 µg), gentamicin (10 µg), piperacillin (100 µg) and cefoperazone-sulbactam. Susceptibility to cefoxitin (30 µg) was also tested to screen for AmpC β-lactamase production.

**MIC of meropenem by broth microdilution method**

Strains found resistant to meropenem by the disk diffusion test were tested by NCCLS broth microdilution method using Mueller-Hinton broth. Doubling dilutions for meropenem ranging from 0.25 µg/mL through 64 µg/mL were tested. Meropenem powder for injection (Astra Zeneca, U.K. Ltd.) was used for the test. ATCC Pseudomonas aeruginosa 27853 was used as the control strain. MIC of 16 µg/mL or above was interpreted as resistant to meropenem.13

**Metallo β-lactamase (MBL) detection**

This was performed by the procedure recommended by Lee et al.13 EDTA disks were prepared using 0.5 molar solution of EDTA (Sigma Chemicals) in distilled water. The pH was adjusted to 8, and the EDTA powder was completely dissolved. It was sterilized by autoclaving. Ten microliters of the EDTA solution was added to a 6-mm disk (Whatman filter paper 4) and allowed to dry. This disk contained approximately 1,900 µg of EDTA. MBL detection was performed by the disk diffusion method. A 0.5 McFarland adjusted suspension of the organism was inoculated on the Mueller-Hinton agar (MHA) plate. A 10 mg meropenem disk was placed in the center of the plate. An EDTA disk (1,900 µg) was placed at a distance of 15 mm, center to center, from the meropenem disk. The plate was incubated at 37°C overnight. The zone around the meropenem disk would be extended on the side nearest the EDTA disk for a MBL producer.

**AmpC detection**

Cefoxitin (30 µg) resistant isolates were tested for AmpC activity by a three-dimensional extract method.14 A 0.5 McFarland (50 µL) bacterial suspension was inoculated into 12 mL of trypticase soy broth and was incubated at 37°C for four hours. The cells were centrifuged, and crude enzyme preparations were made by freeze-thawing the pellets. The surface of a MHA plate was inoculated with Escherichia coli ATCC 25922 (sensitive to cefoxitin), as described for standard disk diffusion method. A 30 µg cefoxitin disk was placed in the center of the inoculated plate. With a sterile scalpel blade, a slit beginning 5 mm from the edge of the disk was cut in the agar in an outward radial direction. Using a pipette, 30 µL of the enzyme preparation was dispensed into the slit, beginning near the disk and moving outward. Slit overfill was avoided. This medium was incubated overnight at 37°C. Enhanced growth of the surface organism (E. coli) at the point where the slit intersected the zone of inhibition was considered a positive result and was interpreted as evidence for presence of AmpC β-lactamase.

**Detection of efflux mechanism of resistance**

Minimum inhibitory concentration (MIC) assays by agar dilution method for meropenem were performed with MHA plates with and without reserpine (25 and 50 µg/mL) for nine strains. Difference in the MICs of the strains (decrease in MIC in the control plates with reserpine) suggests a putative efflux mechanism.

**Results**

Among 150 isolates of Acinetobacter spp. obtained from clinical samples, 21 isolates from 19 patients were resistant to meropenem by the standard disk diffusion method. The clinical profiles of these 19 patients are depicted in Table 1. The antibiotic susceptibility patterns of these 21 resistant isolates are depicted in Fig. 1. Six isolates were found resistant to all antibiotics tested, including cefoperazone-sulbactam. Most of these resistant isolates were from patients admitted in intensive care units (Fig. 2).

MIC determined by the broth microdilution method for the 21 isolates showed low-level resistance or sensitivity to meropenem. Taking an MIC of 16 µg/mL or above as resistant to meropenem, all but three isolates were found to have MICs to meropenem in the sensitive zone. The MIC of the three highly resistant isolates, obtained from two patients, was 64 µg/mL. Two isolates showed MICs of 4 µg/mL; and two other isolates, 2 µg/mL. The remaining 14 isolates showed MICs of 0.25-0.5 µg/mL.

Of the nine isolates tested for efflux mechanism of resistance, seven did not show any differences in the MICs when reserpine was added, thus suggesting that a putative efflux mechanism was not responsible for carbapenem resistance in these strains. However, two isolates obtained from one patient showed 1-2 dilution decrease in the MICs of meropenem with reserpine, suggesting that an efflux mechanism may be responsible for the resistance to carbapenems in these two isolates.

None of the 21 isolates were found to produce MBL by the disk approximation test using meropenem and EDTA disks. Among 21 resistant isolates, nine isolates (42.85%) were AmpC β-lactamase producers.
Discussion

Carbapenem resistance in Acinetobacter spp. is an emerging problem and is a cause of concern as many nosocomial Acinetobacters are detected to be resistant to most other antibiotics. Several phenotypic and molecular typing methods are used to investigate the origin of infection, route of spread and prevalence of isolates in a bacterial population. However, certain simple tests may be performed to determine a few common mechanisms of resistance, and these can be performed in most laboratories.

In the present study involving 150 clinical isolates of Acinetobacters, 21 (14%) isolates were detected to have resistant zone sizes for meropenem when tested by disk diffusion method. Another Indian study by Taneja et al. in 2003 reported a high incidence of more than 20% carbapenem resistance among Acinetobacters.\textsuperscript{15} Corbella and co-workers found carbapenem resistance among the Acinetobacter spp. from patients in ICU to be as high as 36%.\textsuperscript{5} Manikal et al. observed a high rate of 50% carbapenem resistance among Acinetobacters in a New York hospital.\textsuperscript{2} Sub-analysis from the present study revealed that like some of the above studies,
majority (42.8%) of the meropenem-resistant isolates were also from the patients admitted in medical ICUs.

Low-level resistance to carbapenems as determined by MIC has been reported in several studies. In a study by Weinbren et al., isolates were detected to be resistant to carbapenem by disk diffusion method and revealed MICs of 0.5-2 µg/mL, which is below the recommended MIC breakpoint for resistant isolates. In this study, majority of the isolates detected resistant by disk diffusion method were found to have MICs in the sensitive range. All the 21 meropenem-resistant isolates were also resistant to most of the other antimicrobials tested. One of the major concerns emerging from this study is the fact that six meropenem-resistant isolates were resistant to cefoperazone sulbactam also. When such resistant isolates are responsible for infection in debilitated patients, therapeutic options are narrowed. It may pose a threat if disseminated to other patients and will be a major reason of concern in the near future.

The present study found that none of the meropenem-resistant Acinetobacter isolates produced metallo beta-lactamases (MBLs), as detected by the double disk approximation test. Other studies have detected MBL production rates in carbapenem-resistant Acinetobacters ranging from very occasional to as high as 50%. Lee et al. had reported MBL production in resistant Acinetobacters to be 15.1% (range: 0-34%). Studies from the Indian subcontinent on occurrence of MBL production by resistant Acinetobacters are lacking. Clinical microbiology laboratories should therefore be extremely vigilant for the imminent detection of plasmid-mediated metalloenzymes in resistant Acinetobacters.

Occurrence of OXA-type β lactamases (class D) could not be ruled out in this study, as these enzymes are difficult to detect by simple procedures. Oxacillinases are less efficient in hydrolyzing carbapenems than the metalloenzymes but are known to occur commonly in carbapenem-resistant Acinetobacters.

Reduced outer-membrane permeability and increased AmpC beta-lactamase production are known important factors leading to carbapenem resistance in Acinetobacters. Reduced outer membrane permeability occurs due to loss of particular outer-membrane proteins (OMP) and can be easily detected by sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) of the OMPs extracted from the bacterial cells but was not performed in this study.

AmpC β-lactamases were found in 42.85% of the resistant isolates and may contribute to the carbapenem resistance in these isolates. The role of efflux pumps in carbapenem resistance among Acinetobacters is unknown. But in the present study, two isolates obtained from one patient revealed efflux pump as a possible cause for the carbapenem resistance. However, more isolates need to be tested before one concludes about the role of efflux pumps in pumping out carbapenems from the bacterial cells.

This study demonstrated that multidrug-resistant Acinetobacters are common in hospitals, especially in the ICUs. Unwarranted and unrestricted usage of antibiotics is associated with emergence of resistance in common nosocomial pathogens like Acinetobacter spp. Use of third-generation cephalosporins has been shown to increase carbapenem resistance in Acinetobacters. Also, increased use of Imipenem against cephalosporin-resistant Klebsiella pneumoniae had encouraged development of Imipenem-resistant A. baumannii.

The present study has sampled a very small number of meropenem-resistant Acinetobacters, and lack of MBL production cannot be generalized without further studies for the same. Multiple mechanisms account for carbapenem resistance in Acinetobacters, and understanding its mechanisms might be crucial for the development of novel therapeutic strategies. A coordinated effort to limit inappropriate use of broad-spectrum antibiotics, efficient hospital antibiotic policies, vigilant detection of resistant Acinetobacters, rigorous surveillance and infection-control protocols are needed to control the increasing incidence of highly resistant Acinetobacters.

References

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Source of Support: Nil, Conflict of Interest: None declared.


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