

Study to detect incidence of carbapenemase among Gram negative clinical isolates from tertiary care hospital

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Abstract: Purpose: To study incidence of carbapenemases in tertiary care hospital among gram negative clinical isolates of both Enterobacteriaceae and nonenterobacteriaceae (nonfermenters).

Materials and Methods: A total of 200 gram negative isolates were subjected to various phenotypic methods for carbapenemase detection along with minimum inhibitory concentration (MIC) for Imipenem and Meropenem by agar dilution technique. The phenotypic methods tested like Double disc synergy test (DDST), Combined disc test (CDT), Modified Hodge test (MHT).

Results: Of 200 isolates, 114 were members of family enterobacteriaceae and 86 were nonfermenters. Out of 114, 71 were *E. coli*, 32 *Klebsiella* spp, 08 enterobacter and 03 citrobacter. Out of 86 nonfermenters 55 were *Pseudomonas* spp and 31 were *Acinetobacter* spp. Of the 200 isolates tested by DDST, 06 showed distortion of the one towards the EDTA disc. In CDT test 30 isolates showed an increase of >7mm zone of inhibition around the imipenem+EDTA disc in comparison to imipenem alone. Similarly 18 isolates had an increase of >7mm around the ceftazidime+EDTA in comparison to ceftazidime alone. Only one *Klebsiella* isolate was found to be positive with MHT, where a clover leaf shaped zone of inhibition was noted. Of 200 isolates, 167 were found to have an MIC of < 2 µg/ml, 25 had an MIC value of 2 µg/ml, 07 had an MIC of 4 µg/ml and 01 had an MIC of 8 µg/ml against imipenem. For meropenem, 170 isolates had an MIC of < 2 µg/ml, 22 had an MIC of 2 µg/ml, 07 had an MIC of 4 µg/ml and 01 had an MIC of 8 µg/ml. The strain, *Klebsiella* spp, showed higher MIC 8 µg/ml for both imipenem and meropenem.

Conclusions: Different phenotypic methods for detection of these carbapenemases are available, but controversies exist regarding the choice of optimal laboratory method. Molecular methods though expensive are the confirmatory tests

I. Introduction

Resistant bacteria are emerging worldwide both in community and hospital settings. There are many reports of increase in the incidence of nosocomial infections by multidrug resistant organisms.⁽¹⁾

Bacteria have developed several mechanisms of resistance against various antibiotics. Synthesis of drug inactivating enzymes like β lactamases, which hydrolyse the β lactam antibiotics, decreased target susceptibility by target alteration, development of efflux system and modification of diffusion barrier, altered metabolic activity are the main mechanisms of drug resistance. Many studies were done on β lactam group of antibiotics and β lactamases.⁽²⁾

In the past, multidrug resistant gram negative bacterial infections were successfully treated with penicillin group of antibiotics like carbenicillin, ticarcillin, piperacillin etc. Among all β lactams, carbapenem group of drugs have maximum antimicrobial spectrum. This spectrum is due to their high affinity for penicillin binding protein 2, good stability against most serine based β lactamases and excellent outer membrane permeability. Carbapenems are among the few backup agents for multidrug resistant gram negative pathogens. However, increasing usage of carbapenems has resulted in development of resistance to this group of drugs as well.⁽³⁾

II. Materials and methods

Two hundred gram negative isolates from various clinical specimens like pus 129, urine 52, pleural fluid 03, sputum 07, endotracheal secretions 07 and blood 02 were taken for the study. Of these 200 samples, 147 were from in patients from wards and 53 were from ICU (Table 1) Of the 200 isolates, 114 were members of the family enterobacteriaceae and 86 isolates were nonfermenters. Out of these 114, 71 were *E. coli*, 32 *Klebsiella* spp, 08 enterobacter spp and 03 citrobacter spp. Out of the 86 nonfermenters, 55

were *Pseudomonas* spp and 31 *Acinetobacter* spp. These isolates were subjected to various phenotypic methods of carbapenemase detection along with MIC determination for imipenem and meropenem. The phenotypic methods tested were double disc synergy test, combined disc test and Modified Hodge test. The MIC was determined by agar dilution technique.

III. Phenotypic Methods

1. DOUBLE DISC SYNERGY TEST (DDST) ⁽⁴⁾

After purity check, isolated colonies were inoculated into peptone water and kept in incubator at 37°C for 2 hours to get a turbidity to match 0.5 Mc Farland tube. The test organism inoculum was spread as a lawn onto Muller Hinton agar (MHA) plate using a sterile cotton swab. A 6 mm in diameter Whatman No 1 filter paper disc with 10 µl of EDTA (≈ 1900 µg of disodium salt dehydrate) solution was placed at center of the plate. On either sides of this EDTA disc, at a distance of 10 mm from the edge to edge, an imipenem (10µg) and a ceftazidime (30 µg) discs were placed. The inoculated plates were incubated at 35°C in the incubator for 18-24 hours. Presence of any distortion in the zone of inhibition or any indentation towards the EDTA disc was interpreted as positive.

2. COMBINED DISC TEST (CDT) ⁽⁴⁾

In this test after spreading the test inoculum (0.5 Mc Farland turbidity) on to MHA plate, two imipenem (10 µg) discs and two ceftazidime (30 µg) discs were placed onto one of the imipenem and ceftazidime discs, 10 µl of EDTA solution was added. The plates were incubated at 35°C for 18-24 hours. The zone of inhibition between the imipenem and imipenem + EDTA and ceftazidime and ceftazidime + EDTA discs were compared. Difference in the zone diameter of ≥7mm was interpreted as positive for MBL production.

3. MODIFIED HODGE TEST (MHT) ⁽⁵⁾

The ATCC *E.coli* 25922 at a turbidity equivalent to that of 0.5 Mc Farland was inoculated onto the MHA plate. After brief drying for five minutes, a meropenem (10 µg) disc was placed at the centre of the plate. The test strain was heavily streaked from the edge of the meropenem disc to the periphery of the plate. Four isolates were inoculated in one plated at 90° to each other and the plates were incubated at 35°C for 18-24 hours. Presence of a clover leaf type of zone of inhibition near the test organism was interpreted as positive for carbapenem hydrolyzing enzymes, as per the CLSI guidelines.

MINIMUM INHIBITORY CONCENTRATION (MIC)

MIC to imipenem and meropenem was done by agar dilution technique. Commercial preparation of imipenem (500 mg) (Manufacturers: M/S. Zeiss pharma (P) Ltd) and meropenem (500 mg) (Astra Zeneca pharma India Ltd) were taken for the study. ATCC *Pseudomonas aeruginosa* 27853 was used as control strain MIC values for both imipenem and meropenem were interpreted as follows: ≤ 1 µg/ml as sensitive, 2 µg/ml as intermediate and ≥ 4 µg/ml as resistant as per CLSI guidelines. ⁽⁶⁾

IV. Results

Of these 200 samples, 114 yielded organisms belonging to enterobacteriaceae and 86 yielded growth of nonfermenters. Out of 114 members of enterobacteriaceae family, 71 were *E.coli*, 32 klebsiella spp, 08 enterobacter spp and 03 citrobacter spp. Out of 86 nonfermenters, 55 were pseudomonas spp and 31 were acinetobacter spp.

Table 1: Shows the breakup of organisms isolated from patient in to various wards

Organism	ICU	Surgery	Ortho	Medicine	Gynae	Paed	Total
<i>E.coli</i>	16	24	1	15	13	01	71
<i>Pseudomonas</i> spp	17	25	05	05	03	-	55
<i>Klebsiella</i> spp	12	08	03	07	01	01	32
<i>Acinetobacter</i> spp	08	18	-	03	02	-	31
<i>Enterobacter</i> spp	-	04	02	02	-	-	08
<i>Citrobacter</i> spp	-	01	-	02	-	-	03
Total	53	80	11	94	19	02	200

Of the 200 isolates tested by DDST, 06 showed distortion of the zone towards the EDTA disc. Of these 06 isolates 02 were *E.coli*, 02 acinetobacter spp, 01 klebsiella spp and 01 pseudomonas spp.

In the combined disc test, 30 isolates showed an increase of > 7mm zone of inhibition around the imipenem+ EDTA disc in comparison to imipenem alone. Similarly 18 isolates had an increase of > 7mm around the ceftazidime+ EDTA disc in comparison to ceftazidime alone. Eleven isolates were positive (showed > 7mm difference in zone size) with both imipenem+EDTA and ceftazidime+ EDTA in comparison to imipenem and ceftazidime alone. Only one klebsiella isolate was found to be positive with modified hodge test, where a clover leaf shaped zone of inhibition was noted around the isolate

Table 2: Comparison of various phenotypic tests for detection of carbapenemases

Methods	No of strains	Percentage (%)
DDST	06	03
CDT		
a. Imipenem	30	15
b. Ceftazidime	18	09
MHT	01	0.5

MIC determination for imipenem and meropenem was done by agar dilution technique; where in a range of 2µg/ml to 16µg/ml of the drug concentrations were as covered. Of these 200 isolates, 167 isolates were found to have an MIC of < 2µg/ml, 25 had an MIC value of 2µg/ml, 07 had an MIC of 4µg/ml and 01 had an MIC of 8µg/ml against imipenem. For meropenem, 170 isolates had an MIC of < 2µg/ml, 22 had an MIC of 2µg/ml, 07 had an MIC of 4µg/ml and 01 had an MIC of 8µg/ml. The strain, Klebsiella spp, showed higher MIC 8µg/ml for both imipenem and meropenem.

Table 3: Shows minimum inhibitory concentration of imipenem and meropenem by agar dilution technique.

Drugs tested	Minimum Inhibitory Concentration in µg/ml			
	< 2	02	04	08
Imipenem	167	25	07	01
Meropenem	170	22	07	01

V. Discussion

The discovery and development of antibiotics is one of the greatest advances in modern medicine. Carbapenems are β lactam antibiotics presently considered as potent agents to target multidrug resistant gram negative infections, due to the stability of these agents against many β lactamases.

The prevalence of carbapenemase among gram negative bacilli varies greatly from country to country and among different institutions within the country. Carbapenemase production is only one mechanism of resistance and other mechanisms also exist for carbapenem resistance like porin loss, efflux of drug and target alteration. However, it is well known that multiple mechanisms of resistance and enzyme production can co-exist in a single organism.

Carbapenem hydrolyzing enzymes are most commonly seen in nonfermenter gram negative organisms (non enterobacteriaceae) ie. Pseudomonas and acinetobacter. However, in the recent years there is an increasing incidence of these enzymes in enterobacteriaceae family as well. In Enterobacteriaceae family carbapenemases are more common encountered in lactose-fermenters (e.g. *K.pneumoniae* and *E.coli*) than in non-lactose fermenters (e.g. *Serratia marcescens* and some Enterobacter spp).

The present study was under taken to compare the different phenotypic methods for carbapenemase detection in nonfermenters and enterobacteriaceae family from various clinical isolates in our hospital.

Though CLSI does not advocate the use of the modified Hodge test for the detection of carbapenemase production in nonfermenting Gram-negative bacilli, several authors have found the modified Hodge test using imipenem as a useful screening test for carbapenemase production. In the present study we screened all the isolates with three different phenotypic methods to detect both MBLs and other carbapenemase.

Initially carbapenem resistant strain were observed in *P.aeruginosa* and in no time they disseminated to different parts of the world.⁽⁷⁾ Two studies by Marchiaro *et al*⁽⁸⁾ and Toleman *et al*⁽⁹⁾ reported the prevalence of MBL to be 43.9% and 39.1% in *P.aeruginosa*. In the year 2010, in India, a study done by Manoharan *et al*⁽¹⁰⁾ quoted the prevalence of MBLs in *P.aeruginosa* to be as high as 65 %. In acinetobacter spp, in a study by Manikal *et al*,⁽¹¹⁾ in the year 2000 in New York hospitals, the MBLs prevalence was found to be 50%.

Later in one Indian study by Taneja *et al*, in 2003 the prevalence of MBL in acinetobacter was found to be 20%. There are very few studies for carbapenemase detection in acinetobacters when compared to *P.aeruginosa*.⁽⁷⁾ In the present study the prevalence of MBL in *P.aeruginosa* is 15% which correlates with two studies by Hemalatha *et al*⁽¹²⁾ and Navaneeth *et al*⁽¹³⁾ where the prevalence was 16% and 12%. Prevalence of

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MBL in acinetobacter spp in present study is 10.5% while a study by Glandstone *et al*⁽¹⁴⁾ in 2005 was 14.2% because in the Indian scenario production of MBL may not play a major role in carbapenem resistance among acinetobacters spp.⁽¹⁵⁾

In DDST 6 isolates (3%) were positive of which 02 were *E.coli*, 02 acinetobacters spp, 01 Klebsiella spp and 01 pseudomonas spp. In the present study the distance between the EDTA and the other two antibiotic discs was taken as 10mm. In literature many authors have chosen 10 mm distance between the discs as it was found to be more sensitive compare to other distance. The study by Galani *et al*⁽⁴⁾ observed that 10mm distance between imipenem and EDTA disc shows sensitivity (100 %) and specificity (91.9%). There are very few studies in literature to compare the sensitivity of imipenem and ceftazidime in detecting MBLs by DDST, two such studies are by Mendiratta *et al*⁽⁴⁾ and Hemalatha *et al*⁽¹²⁾ where they found ceftazidime to be a better disc in comparison to imipenem in detecting the MBLs. In the present study however, study we did not find any difference between imipenem and ceftazidime.

Many chelating agents were used in testing MBLs, however most commonly used is EDTA, but it has a direct bactericidal effect on test strain which can give rise to false positive.⁽¹⁵⁾ A study by Galani *et al*,⁽¹⁷⁾ shows the sensitivity of DDST varies from 44 to 67 %. However EDTA disc were more sensitive than SMA disc for detection of MBL production in pseudomonas, whereas SMA was better for acinetobacter spp. In the present study we used only EDTA as a chelating agent.

In CDT, 30 isolates (15%) were found to be positive with imipenem + EDTA and 18 isolates (09%) with ceftazidime + EDTA, in detecting MBLs. Similar to our study, Sakshi *et al*⁽¹⁸⁾ in 2009 found a sensitivity of 95.2% using IPM+EDTA, in comparison to 38.1% using ceftazidime +EDTA. In the same year Manoharan *et al*⁽¹⁰⁾ also found a better sensitivity of 93.3% with imipenem +EDTA in detecting MBL as compared to EDTA+ceftazidime. In contrast to our study, studies by Hemalatha *et al*⁽¹²⁾ and Arakawa *et al*⁽¹⁹⁾ found ceftazidime to be a better agent in detecting MBL than imipenem, however, in our study imipenem was found to be a better choice for detection of MBL production by CDT.

So CDT is more sensitive screening method in detecting MBL.⁽⁷⁾ However, though different phenotypic methods have been described for a long time there are no standard guidelines for screening of carbapenemase.⁽¹⁵⁾ CLSI has recommended MHT for detection of carbapenemase activity in enterobacteriaceae, especially for KPC in klebsiella spp, but not in nonfermenters. However, we performed this test on all isolates to see for any co-existence of different types of enzymes. In our study, only one out (2.8%) of 35 klebsiella isolates was found to be positive with MDT, where a clover shaped zone of inhibition was noted around the isolate.

In the present study, 167 (83.5%) out of 200 the isolates were sensitive to imipenem, where the MIC was <2 µg/ml, 25 isolates (12.5%) were intermediate sensitive with an MIC of 2 µg/ml and 08 isolates (04%) were found to be resistant with 07 having an MIC of 4 µg/ml and one with an MIC of 8 µg/ml.

In 2008 Agarwal *et al*⁽²⁰⁾ observed that 77.01% of strains for imipenem were having MIC as low as ≤2 µg/ml, while 1.15% of strains were having MIC as high as 1024 µg/ml. Such a high MIC was not observed in present study.

MICs against meropenem was as follows 85% strains had MIC of <2 µg/ml, 11% show MIC of 2 µg/ml, 3.5% showed 4 µg/ml and 0.5% showed MIC of 8 µg/ml. A study done by Hemalatha *et al* observed that MIC ranges from 8-128 µg/ml but in our study only one isolate showed high MIC of 8 µg/ml. Four isolates which shows high MIC of 4µg/ml did not exhibit zone size potentiation with EDTA impregnated antibiotics discs which suggests other mechanism of resistance like impaired permeability or active efflux pump associated with overproduction of endogenous β lactamase being responsible for this high MIC.

Of the 200 strains, eight isolates showed an MIC of >4 µg/ml for both imipenem and meropenem. When these isolates were compared to various phenotypic tests (DDST, CDT, MHT), seven strains were positive for CDT, two strains were found to be positive for DDST and one isolate which is klebsiella spp. positive for all three test like DDST, CDT and MHT. This shows CDT can detect more number of isolates than DDST.

VI. Conclusion

Penems are β lactam antibiotics that are the last line of drugs for treating gram negative infections. However various mechanisms of resistance have evolved for these antibiotics too due to their extensive use throughout the world. Different phenotypic methods for detection of these carbapenemases are available, but controversies exist regarding the choice of optimal laboratory method. Molecular methods though expensive are the confirmatory tests.

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