

Original Research Article

Carbapenem Resistant Mechanism in Carbapenem Resistant Gram Negative Bacilli Other Than Carbapenemase

Sibin P S¹, Anuranjini C², Jeeva Rani³

¹Microbiologist, Department of Medical Microbiology, VPS Lake Shore Hospital and Research Centre, Ernankulam, Cochin, Kerala.

²Assistant Professor, Department of Medical Microbiology, School of Health Sciences, Kannur University, Kerala.

³Microbiologist, Department of Medical Microbiology, Aster Malabar Institute of Medical Sciences, Calicut, Kerala.

Corresponding Author: Sibin P S

ABSTRACT

Introduction - Infections have been the major cause of disease throughout the history of human population. In the middle of the nineteenth century antibiotics are discovered as a powerful weapon in the battle against infectious diseases. Antibiotic resistance has been detected in many bacterial species. As each new class of antibiotic introduced into the clinical use, resistance starts to develop in the microorganism they are intended to kill. Both innate and acquired mechanisms contribute to the development of antibiotic resistance in bacteria.

Objectives - The present study was conducted to analyze the prevalence of Carbapenem resistance in gram negative bacilli and their carbapenem resistance mechanisms other than carbapenemase in tertiary care hospital.

Methods - The objectives of this study includes to analyze the carbapenemase producers and non carbapenemase producers by Modified Hodge Test (MHT) and to detect the presence of Efflux pump mechanism in MHT positive and negative strains by Ethidium Bromide (EtBR) agar cartwheel method.

Result - Klebsiella was found to be predominant form of carbapenem resistant gram negative bacilli. Among the 144 MHT negative strains all the strains showed either Efflux pump or Impermeability mechanisms. By Ethidium Bromide agar Cartwheel method only 10% strains were positive for efflux activity. Current study also indicates that the carbapenem resistant Pseudomonas was mainly due to Efflux activity. But in the case of carbapenem resistant Klebsiella was mainly due to impermeability mechanism. The overall prevalence of carbapenem resistant Gram Negative Bacilli was found to be 10%.

Key Words: Carbapenem resistance, Modified Hodge Test, Efflux pump, Gram Negative Bacilli, Klebsiella.

INTRODUCTION

Infections have been the major cause of disease throughout the history of human population. In the middle of the nineteenth century antibiotics are discovered as a powerful weapon in the battle against infectious diseases. Scientific antibiotic discovery started by Alexander Fleming,

who observed inhibition of growth on his agar plate on which he was growing Staphylococcus species. It was later found that a microorganism that was later to be called Penicillium notatum was the cause of inhibition of the Staphylococcus around it as a result of excreting some chemical into the media. That marked the beginning of the

discovery of penicillin which together with several other different antimicrobial agents was later to save millions of humans and animals from infectious disease causing organisms. However bacteria and other organisms are remarkably resistant and can develop ways to survive drugs meant to kill or weaken them. Such antibiotic resistance or drug resistance is largely due to the increasing use of antibiotics. Therefore antibiotic resistance is the major challenge in the 21st century.

Antibiotic resistance has been detected in many bacterial species. As each new class of antibiotic introduced into the clinical use, resistance starts to develop in the microorganism they are intended to kill. Both innate and acquired mechanisms contribute to the development of antibiotic resistance in bacteria.

Carbapenem play a critically important role in our antibiotic armamentarium. Of the many hundreds of different beta lactams, carbapenems possesses the broadest spectrum of activity and greatest potency against Gram positive and Gram negative bacteria. As a result, they are often used as “last line agents” or “antibiotic of last resort” when patients with infections become gravely ill or are suspected of harbouring resistant bacteria. [1-3] Unfortunately, the recent emergence multidrug resistance (MDR) pathogens seriously threaten this class of life saving drugs. [4] Several recent studies were available from various countries including India. [5-7] Enterobacteriaceae are the members of the intestinal flora. They are most common cause of infections such as cystitis and pyelonephritis with fever, septicemia, pneumonia, peritonitis, meningitis, and catheter associated infections.

Mechanisms associated with carbapenem resistance are complex and involve various genes. Multidrug efflux system, Impermeability and production of Carbapenemases (Enzyme that inactivate carbapenems) are the most important molecular mechanisms that mediate

carbapenem resistance in Enterobacteriaceae. [8-12] Antibiotic efflux in bacteria was first reported in the late 1970s for tetracyclines in *Escherichia coli* and attributed to the plasmid encoded Tet protein. Impermeability due to the loss of OprD porin is the most common mechanism described in *Pseudomonas aeruginosa*. Carbapenem resistant Enterobacteriaceae have been reported worldwide as a consequence of large acquisition of Carbapenemase genes. The first Carbapenemase producer in Enterobacteriaceae (NmcA) was identified in 1993. Since then, a large variety of carbapenemase has been identified in Enterobacteriaceae belonging to 3 classes of beta lactamases: the Ambler class A, B and D beta lactamases. CLSI published a recommendation that carbapenems susceptible Enterobacteriaceae with elevated MICs or reduced disc diffusion zone sizes be tested for the presence of carbapenemases using the Modified Hodge Test (MHT). Ethyidium Bromide (EtBr) agar cartwheel method is easy to perform, there by facilitating the rapid identification of isolates to detect Multidrug resistance mediated by efflux and EDTA is the agent that increase the membrane permeability, therefore resistance due to impermeability can be detected by invitro susceptibility testing by adding this agent.

In the view of above, the present study was conducted to analyze the prevalence of Carbapenem resistance in gram negative bacilli and their carbapenem resistance mechanisms other than carbapenemase in tertiary care hospital. The carbapenems are β -lactum type of antibiotics with an exceptionally broad spectrum of activity coverage of Gram positive and Gram negative aerobes and are stable almost all bacteria. The first carbapenem to be identified in the mid 1970s was thienamycin, a compound produced by the soil organism *Streptomyces cattleya*. [13-14]

Carbapenems inhibit cell wall synthesis by binding to most high molecular

weight PBPs. They traverse the outer membrane of gram negative bacteria through specific outer membrane protein (OMPs) to each the periplasmic space. The most significant OMP is Opr D in *Pseudomonas aeruginosa*. Although variations exist depending on the specific agent, they preferentially bind to PBPs 1a, 1b, 2 and 4 and to a lesser extent PBP3, which is the primary target of amino – penicillins and cephalosporins. This low affinity with PBP3 is thought to be responsible for the formation of sphere forms without the production of long filaments of bacterial lysis. The affinity of carbapenems to multiple PBPs of various bacteria contributes to the broad spectrum of activity of these agents.^[15] The introduction of carbapenems into clinical practice represented a great advance for the treatment of serious bacterial infections caused by beta lactam resistant bacteria.^[13] Carbapenem antibiotics are the last treatment option for severe, life threatening infections caused by gram negative enteric bacilli with multiple drug resistance. Therefore, an understanding of carbapenem resistance is important. Carbapenem resistance among members of the family Enterobacteriaceae is mediated by the 1. Production of beta lactamases (Carbapenemases) that hydrolysis the carbapenems 2. Active pumping of the antibiotics out of the cell using complex “Efflux pumps” 3. Changes in outer membrane porins that block the entry of these antibiotics (Impermeability).^[15] Evidence of increasing incidence of infections due to carbapenem resistant enterobacteriaceae is reported from various countries. Guh Ay et al.,^[16] reported that 7 US communities (Colorado, Georgia, Maryland, Minnesota, New Mexico, New York and Oregon) the high prevalence of CRE during the period of 2012- 2013. Multidrug resistant organisms such as CRE have been labelled as a “serious threat to public health” by the Centres for Disease Control and prevention and “one of the greatest threats to human health”.

The present study was intended to analyze the mechanisms of carbapenem resistance and other than carbapenemase production in gram negative bacilli isolated from clinical samples in tertiary care hospital. The objectives of this study includes to analyze the Carbapenemase producers and non carbapenemase producers by Modified Hodge Test (MHT) and to detect the presence of Efflux pump mechanism in MHT positive and negative strains by Ethidium Bromide (EtBR) agar cartwheel method. Another objective was to detect the Impermeability in MHT positive and negative strains by augmented in vitro susceptibility test by adding EDTA.

MATERIALS AND METHODS

The study was carried out in Malabar Institute of Medical Sciences, Calicut over the period of January to March 2016. Clinical samples such as urine, pus, blood, body fluids catheterized specimens, sputum, respiratory specimens and tissue specimens were collected aseptically in a sterile container. Immediately after collection, the samples were transported to microbiology laboratory and processed. Whenever there was delay in processing the samples were transferred to appropriate transport media depending on the specimen and preserved under appropriate condition as per the standard protocols. All the samples were processed in appropriate media within 2-4 hours of collection. The bacterial isolates obtained were identified based on their morphological, cultural and biochemical characters. The unidentified organisms are identified by using automated culture system VITEK 2 compact. Modified Hodge Test were performed for the detection of Carbapenemase and non Carbapenemase, positive results were later confirmed by (for impermeability) Meropenem – EDTA disc synergy and detection of Efflux pump mechanism by using Ethidium bromide.

MODIFIED HODGE TEST – FOR THE DETECTION OF CARBAPENAMSASE ACTIVITY:

The test was performed as suggested by Lee et al., [17] The Modified Hodge Test (MHT) detects Carbapenemase production in isolates of Enterobacteriaceae. Modified Hodge (clover leaf) Test involving distorted carbapenem inhibition zones. Carbapenemase production is detected by the MHT when the test isolate produces the enzyme and allows growth of a carbapenem susceptible strain (E. Coli ATCC 25922) towards a carbapenem disc where the concentration of the antibiotic is low. The result is a characteristic clover leaf like indentation. MHT positive test has a clover leaf like indentation of E. coli 25922 growing along the test organism growth streak within the disc diffusion zone. MHT negative test has no growth of the E. coli 25922 along the test organism growth streak within the disc diffusion.

MEROPENEM – EDTA DISC SYNERGY TEST:

The test strains were lawn cultured and 10 microgram meropenem disc and meropenem with EDTA were placed 20 mm apart. Following incubation, carbapenem resistant strains doesn't give sensitive zone. Metallo-beta-lactamase enzymes require zinc for their action. Since EDTA is a strong chelator of zinc, it chelates the zinc present in the medium making the zinc unavailable to the enzyme and thus suppresses the MBL activity and strains shows increased zone of diameter. Increased zone diameters of 6mm than the meropenem alone were considered to be positive. The detection of impermeability indicated by the formation of inhibitory zone greater than normal discs by adding permeabilizing agent like EDTA.

DETECTION OF EFFLUX AND NON EFFLUX ACTIVITY – BY USING ETHEDIUM BROMIDE CARTWHEEL METHOD:

The principle of this test is flurometric assay, the passage of the

common substrates of Efflux pumps EtBr across the cytoplasmic membrane and its subsequent intracellular accumulation inside the bacterial cell. EtBR traverses the bacterial cell wall and once inside, it can be concentrated to a point where it fluoresces when excited by Ultra Violet light (UV). Efflux pump of MDR bacteria recognize this substance and are able to extrude out in to the medium. [18]

Prepared the MHA plates containing EtBR concentration 20µL. MHA plates was prepared fresh on the previous or same day of the experiment and kept protected from the light. MHA plates were then divided into as many as 12 sectors by radial lines, forming a cartwheel pattern. Prepared 0.5 % McFarlands dilutions of the overnight cultures of the bacterial isolates. In a straight line streak the test cultures or swabbed on the EtBr – MHA plates. The MHA plates were incubated at 37⁰C for 16 hours. After this period MHA plates examined under a gel imaging system, the presence of fluorescent growth indicates absence of efflux activity where as absence of fluorescence growth indicates presence of efflux activity.

RESULT

During this period of study total of 5324 various clinical specimens were subjected to isolation of bacterial pathogens and 2510 gram negative organisms were isolated and identified in hospital. Based on morphological, biochemical characters and antibiotic susceptibility patterns of each gram negative isolates were analyzed and those isolates which showed meropenem and imipenem resistance were selected for screening carbapenem resistance other than carbapenemase. Among 2510 gram negative isolates 10% (250) are carbapenem resistant gram negative bacilli. These isolates include Klebsiella, E. coli, Enterobacter, Chromobacter, Acinetobacter, and Pseudomonas species.

Klebsiella was found to be predominant form of carbapenem resistant gram negative bacilli. From the total of 250

sample 138 Klebsiella were isolated. Followed by E.coli (87), Enterobacter (20), Pseudomonas (3), Acinetobacter and Chromobacter (1) each. Among these samples 143 (57.6%) Carbapenem resistant Gram negative bacilli were isolated from the urine sample. Apart from the urine sample Klebsiella and Enterobacter species, the most of the carbapenem resistant Gram Negative Bacilli isolated from the sputum samples 36 (14%). 27 (11%) carbapenem resistant Gram Negative Bacilli were isolated from the blood samples, and remaining 44 Carbapenem resistant Gram negative bacilli were isolated from the pus, swabs and exudates respectively

Modified Hodge Test (MHT) was the easy and simple test to be performed to detect carbapenemase producing bacteria. From this test classify the organisms as carbapenemase producers and non producers (may be efflux pump or impermeability mechanism).

Table 1 shows carbapenemase positive and negative strains by MHT.

Total number of strains	No. of strains showing positive in MHT	No. of strains showing negative MHT
Klebsiella	63 (45.7%)	75 (54.3%)
E. coli	30 (34.4%)	57 (65.5%)
Enterobacter	13	7
Chromobacter	0	1 (100%)
Acinetobacter	0	1
Pseudomonas	0	3
Total	106(42.4%)	144(57.6%)

Present study it was observed that 54.3% (75) of the Klebsiella and 65.5% (57) of the E.coli were the MHT negative strains, therefore it indicates that these Klebsiella and E.coli is having other than carbapenemase mechanisms. But in the case of Enterobacter species carbapenemase mechanism was predominant. Only the isolate of Pseudomonas, Acinetobacter and Chromobacter species were obtained which are MHT negative. In our study it revealed that high prevalence of other than carbapenemase mechanism is predominant such as impermeability and efflux pump. Out of 250 strains collected, total 42.4% (106) shows carbapenemase producers and remain 57.6% (144) are non carbapenemase producers.

Table 2 reveals the Efflux pump, Impermeability mechanism and carbapenemase activity in MHT positive strains.

Number of MHT positive strains	Efflux pump by EtBr Method	Impermeability by adding EDTA
Klebsiella N=63	0	63
E. coli N=30	10	20
Enterobacter N= 13	13	0
Chromobacter N=0	0	0
Acinetobacter N= 0	0	0
Pseudomonas N= 0	0	0
Total N=106	23	83

In order to identify the efflux pump mediated carbapenem resistance in MHT positive strains Ethidium Bromide agar Cartwheel method was used. Since only 22% strains shows the Efflux pump mechanism. In our study only E. coli and Enterobacter species showed Efflux pumps mediated carbapenem resistance. Whereas Klebsiella strains does not shows efflux mechanism. To identify the Impermeability mechanisms we added EDTA, an agent that increases the permeability. Out of 106 strains 78% shows the impermeability. 100% of Klebsiella species and 19% E. coli were exhibited this mechanism. In this current study it indicates carbapenemase positive strains are also shown the impermeability and efflux mechanisms. In carbapenem resistant Gram Negative Bacilli a single mechanism may not be sufficient to cause a clinically relevant degree of resistance.

Table 3 Depicts the Efflux pump and Impermeability mechanism in MHT negative strains.

Number of MHT negative strains	Efflux pump by EtBr Method	Impermeability by adding EDTA
Klebsiella N=75	6	69
E. coli N=57	5	52
Enterobacter N= 7	0	7
Chromobacter N=1	0	1
Acinetobacter N= 1	0	1
Pseudomonas N= 3	3	0
Total N=144	14	130

Among the 144 MHT negative strains all the strains showed either the Efflux pump or Impermeability mechanisms. By the Ethidium Bromide agar Cartwheel method only 10% strains were positive for efflux activity. Current study also indicates that the carbapenem resistant Pseudomonas was mainly due to Efflux activity. But in the case of carbapenem resistant Klebsiella was mainly due to

impermeability mechanism and all the Acinetobacter, Enterobacter, Chromobacter and majority of E.coli strains were having impermeable mechanism. In Chromobacter, Acinetobacter and Enterobacter species, efflux pump mediated resistance mechanism were absent in our study, where as 90% strains were showed carbapenem resistance due to impermeability by augmented in vitro susceptibility assay.

DISCUSSION

A CRE prevalence rate of rate of 12.26% have been obtained in tertiary care hospital in Mumbai, India over a period of 12 months, from which majority of isolates were detected in urine sample. [19] In this present study the prevalence of Carbapenem resistant gram negative bacilli rate was 10%. Among the samples analyzed in the laboratory 57% carbapenem resistant Gram Negative Bacilli are isolated from urine sample followed by sputum (14.4%) and blood (11%) samples. Klebsiella was the most prevalent carbapenem resistant gram negative bacilli from the total samples followed by E.coli and Enterobacter species. CRE that can cause different types of health care associated infections, wound or surgical site infections and meningitis. They are normally found in human intestine. In health care settings CRE infections commonly occurs among sick patient who are received treatment for other conditions such as diabetes or patients undergo any transplantation procedure or an stem cell implantation and long term stay in hospital along with exposure to antimicrobial therapy. Patients whose care requires devices like ventilators, and patient who are taking long course of certain antibiotics are the most risk for Klebsiella infections. [20-22] Organisms isolated from the blood samples showed Klebsiella was the most prevalent carbapenem resistant Enterobacteriaceae followed by E.coli which may be indicate the blood stream infections. European authorities report local outbreak in numerous countries across the continent, which a high proportion of CRKP

(Carbapenem resistant Klebsiella pneumoniae) in blood stream isolates in Greece, Italy and Cyprus. Multiple countries in the Middle East and southern Mediterranean basis, South America and Asia are affected. CRKP has become endemic in the Indian subcontinent. CRKP blood stream (BSI) is associated with an immense case fatality rate of 40% to 70%. [23-29]

From our study it is revealed that 144 carbapenem resistant Gram Negative Bacilli strains (57.6%) are non carbapenemase producers and remaining 106 (42.4%) shows the carbapenemase production. Which indicates the high prevalence of other than carbapenemase mechanisms such as efflux and impermeability are common. Active Efflux pumps transport drug through the bacterial envelope and limit the intracellular accumulation of toxic components. It was well established the multidrug resistance efflux pumps encoded by bacteria can confer clinically relevant resistance to antibiotics. [29]

The MHT negative strains are subjected to efflux activity, among this 14 out of 144 non carbapenemase producers shows efflux activity. Klebsiella (59 out of 75), E. Coli (52 out of 57) and Enterobacter, Chromobacter and Acinetobacter were the most prevalent organism shows carbapenem resistance through the impermeability mechanism. Enterobacter causing nosocomial infection involving the urinary tract, lower respiratory tract, skin and soft tissue, biliary tract, wounds, intravenous catheters and central nervous system. [30] Fuchen Yang et. al., [31] reported that the Ertapenem resistant Enterobacter cloacae in the Taiwanese university hospital mainly due to the active efflux pump mechanism. Athul Khajuria et al., [32] conducted a study in tertiary care hospitals in Central India and the study states that MBL production contribute carbapenem resistance in Enterobacter species.

When analyzed MHT positive strains by EtBr method, it showed that 33%

of *E. coli* strains were carbapenem resistance which was mediated by efflux pumps. The outer membrane of gram negative bacteria performs the crucial role of providing an extra layer of protection to the organism. By combining a highly hydrophobic lipid bilayer with pore forming proteins of specific size exclusion properties, the OM acts as selective barrier. The permeability properties of this barrier, therefore, have a major impact on the susceptibility of organisms to major antibiotics. Porin protein play an important role in the permeability of the outer membrane of gram negative bacteria to small hydrophilic molecules, such as beta lactam antibiotics.

In MHT negative strains 90% shows the impermeability mechanism. Among the 92% of *Klebsiella*, 91% *E. coli* and 100% *Enterobacter* species showed carbapenem resistance which was mediated by impermeability mechanism. EDTA was the excellent agents that increase the permeability of bacterial cellwall. In MHT positive strains the present data proves that not only carbapenemase mechanism but also other mechanisms like Efflux and Impermeability have provide major resistant mechanism. Current study strongly supported the only 22% Enterobacteriaceae were the Efflux pump mechanism but 78% shows the Impermeability. The study strongly supported the Yohei Doi et al., 2006 ^[15] that gram negative bacteria although a single mechanism may not be sufficient to cause a clinically relevant degree of resistance, it occurs through an interplay involving carbapenemase production, impaired Impermeability, and enhanced Efflux.

Among this most of the Carbapenem resistant bacteria were isolated from the urine samples (57%) followed by sputum (14%) and blood (11%) samples. Carbapenem resistant Gram Negative Bacilli were the major nosocomial pathogen causing significant morbidity and mortality. Carbapenem resistant *Klebsiella* species poses a major problem for clinical

therapeutics. Modified Hodge Test (MHT) can be used to detect carbapenem resistance. The present study identified that other than carbapenemase were the major cause of resistance in Gram negative bacteria and 57.6% were the non carbapenemase mediated mechanism. The outer membrane barrier can produce significant levels of resistance in carbapenem resistant Gram Negative Bacilli.

CONCLUSION

In Conclusion the overall prevalence of carbapenem resistant Gram Negative Bacilli were found to be 10% (250/2510). Carbapenem resistance *Klebsiella* and *E. coli* were mostly due to the impermeability mechanisms. In carbapenem resistant Gram Negative Bacilli although a single mechanism may not be sufficient cause a clinically relevant degree of resistance, frank resistance occurs through an interplay involving carbapenemase production, impaired permeability and enhanced efflux.

In future, the design of new antimicrobial preparations might aim to ensure the accumulation of active agent within the cell, relaying on several principle strategies to do so :(A) Increasing uptake of the antimicrobial compounds by (1) Enhancing self promoted uptake mechanisms (2) Combining the active agents with a permeabilizer (3) A synergistic physical process, and or (B) increasing accumulation of the antimicrobials by reducing the activity of multidrug efflux system.

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