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*RESEARCH ARTICLE*

# COMPARISON OF PHENOTYPIC AND GENOTYPIC METHODS FOR DETECTION OF CARBAPENEM RESISTANCE IN AN INDIAN TERTIARY CARE HOSPITAL.

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*Abstract*



## Introduction:-

Carbapenem-resistant *Enterobacteriaceae* (CRE), an emerging threat to public health, belong to a family of microorganisms that are difficult to treat because they are highly resistant to antibiotics.These bacteria can cause serious hospital- and community-acquired infections, such as bloodstream infections, wound infections, urinary tract infections and pneumonia.[1]

Unlike Methicillin Resistant *Staphylococcus aureus* (MRSA) resistance, which is mediated by a single mechanism in a single bacterial species, the mechanisms of carbapenem resistance are complex because they involve a broad range of organisms and are mediated by different mechanisms, such as the production of β-lactamases,efflux pump and porin mutations. [2]

Carbapenemases are β-lactamases with versatile hydrolytic capacities. They hydrolyze penicillins, cephalosporins, monobactams, and carbapenems. Bacteria producing these β-lactamases may cause serious infections in which the carbapenemase activity renders many β-lactams ineffective. Carbapenemases are members of the Ambler class A, B, and D β-lactamases. The class A carbapenemase group includes members of the SME, IMI, NMC, GES, and KPC families. Of these, the KPC carbapenemases are the most prevalent,found mostly on plasmids in *Klebsiella pneumoniae*.[3] The first member of the KPC family was discovered through the ICARE surveillance project in a *K. pneumoniae* clinical isolate from North Carolina in 1996.[4]

The gene encoding the KPC enzyme is usually flanked by transposon-related sequences and has been identified on conjugative plasmids,therefore,potential for dissemination is significant.[5,6,7] Isolates that acquire this enzyme are usually resistant to several other classes of antimicrobial agents used as treatment options. Laboratory identification of KPC-producing clinical isolates will be critical for limiting the spread of this resistance mechanism.[8]

The most commonly used method for detection of CRE is the measurement of minimum inhibitory concentration (MIC). MICs are important in diagnostic laboratories to confirm resistance of microorganisms to an antimicrobial agent . It is a quantitative measurement of antibiotic activity, and it is defined as the minimum concentration of an antibiotic that can inhibit visible microbial growth under normal conditions.[9,10] In 2009, CLSI published a recommendation that carbapenem susceptible *Enterobacteriaceae* with susceptible, but elevated MIC, be tested for the presence of the carbapenemase enzyme using the Modified Hodge Test (MHT). [11] In 2010, the CLSI changed

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the carbapenem resistance criteria to ensure that KPC-producing organisms were not misclassified. This change eliminated the need for secondary testing by MHT. So,a lower MIC for antibiotic resistance was established for CREs and these criteria were again revised in 2012.[12] Polymerase chain reaction confirms the detection of a particular gene.So the present study was conducted to compare MIC with blakpc gene detection in meropenem resistant Klebsiella pneumoniae isolates.

## Material and methods:-

**Study design:-**

This study was conducted in the Department of Microbiology, Sher-i-Kashmir Institute of Medical Sciences, Srinagar; over a period of one year and three months.

**Methodology:-**

The study included all isolates of *Klebsiella pneumoniae* recovered from blood culture of the patients. The identification and antimicrobial susceptibility of the isolates was done on Vitek 2. Isolates that are resistant to Meropenem were included for phenotypic(MIC) and genotypic(PCR) testing of KPC. MIC was determined by broth microdilution for meropenem.[13] These isolates were then tested for *bla*KPC gene by conventional polymerase chain reaction.[14]

**MIC was done by Broth Microdilution method as under:-**

**Preparation of antibiotic stock solution for Meropenem:-**

Stock solution was prepared using the formula**:-**

$$1000/P \times V \times C = W$$

Where, P= potency given by manufacturer (µg/mg),V = volume required (ml),C = final concentration of solution (mg/ L) and W = weight of antibiotic (mg) to be dissolved in volume V (ml).

The stock solution was prepared in such a way that its concentration was 1mg/ml or greater .Meropenem stock solution was prepared by dissolving 55.43 mg of the antibiotic powder in 1ml of distilled water.

**Preparation of working antibiotic solution:-**

Working **s**olution was prepared as per the formula V1C1=V2C2(V1=volume of starting solution needed,C1-concentration of starting solution needed,C2=final concentration of new solution,V2=final volume of new solution).The working solution was prepared one concentration higher than the highest concentration of the drug being tested.Thus for meropenem,256µg/ml of working solution was prepared by dissolving 51.2µl of stock solution in Muller-Hinton broth.

**Microbroth dilution method:-**

Using a micropipette 50 µl of Muller Hinton broth was dispensed into all wells of a microtitre plate leaving the first column unfilled.After this 100µl of working antibiotic solution (concentration 256µg/ml) was added to the wells of the first column.From the first well 50µl of the working antibiotic solution was pipetted out and added to the second well,already containing 50 µl of MH broth .From the second well 50 µl of solution was added into the next well and so on and so forth till the well well number 10 was reached from which 50 µl of solution was discarded .The final concentration in the wells ranged from 256-0.5µg/ml.The last two columns served as growth control and sterility control respectively.

The turbidity of the bacterial inoculum was adjusted to 0.5 McFarland standards and 50 µl of it was dispensed into all the wells of microtitre plate.Finally the plates were incubated at 37ºC overnight and read the other day.

Results were recorded by visual inspection of the microtitre plates after overnight incubation at 37ºC as per CLSI guidelines. The test was considered valid when acceptable growth (more or equal to 2mm button or definite turbidity ) was seen in the positive control well.Absence of turbidity or a button of less than 2mm diameter in the test well was thus taken as the MIC of the organism under test .

**Polymerase chain reaction:-**

DNA Extraction: Molecular identification of KPC-producing *Klebsiella pneumoniae* was performed by *bla*KPC PCR using bacterial lysates from overnight broths prepared by removal of 200 µl of broth culture, centrifugation (12,000 × *g*; 2 min), resuspension in 200 µl of molecular-grade water, boiling at 95°C for 10 min, and discarding the cellular debris by centrifugation (12,000 × *g*; 2 min at 4°C).PCR analysis for *bla*KPC was performed with 1 µl of cell lysates, using the following primers designed to identify all *bla*KPC genes ($bla_{KPC-1}$ through $bla_{KPC-7}$): KPC forward (ATGTCACTGTATCGCCGTCT). KPC reverse (TTTTCAGAGCCTTACTGCCC). The Reaction was set up in a PCR vial, after adding the master mix, the forward and reverse primers and the extracted DNA. 25µl of Master Mix contained 10X Taq buffer, 2mM Mgcl2, 0.4mM dNTPs mix, and 2U *Proofreading* Taq DNA polymerase. (Thermo SCIENTIFIC ,USA) Lysates derived from *Escherichia coli* ATCC 25922 and *bla*KPC carrying *K. pneumoniae* strain 1705 were used as negative and positive controls,respectively, in each PCR. The PCR conditions were as follows: 15 min at 95°C and 38 cycles of 1 min at 94°C, 1 min at 62°C, and 1 min at 72°C, followed by an extension step of 10 min at 72°C.The PCR products were subjected to electrophoresis on 2% agarose gel stained with ehidium bromide and visualized with UV light . The blaKPC gene gave band at 893bp

## Results:-

Out of the total isolates 55(78.5%) were meropenem resistant and 15(21.5%) were meropenem sensitive. Fig.1

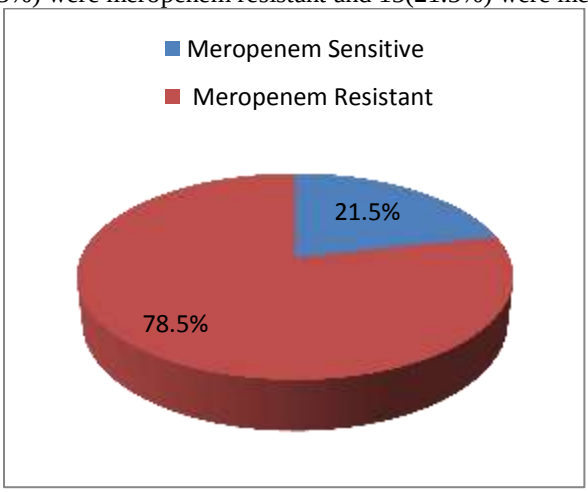


**Fig.1:-**Overall distribution of *Klebsiella pneumoniae* isolates (sensitive and resistant).

Minimum Inhibitory Concentration (MIC) was done on Meropenem resistant isolates by Broth microdilution test. For (36.3%) isolates MIC was ≥256 µg/ml followed by 128 µg/ml in (32.7%) isolates followed by 64 in (20%) isolates and 32 in (10.9%) **.Fig 2**

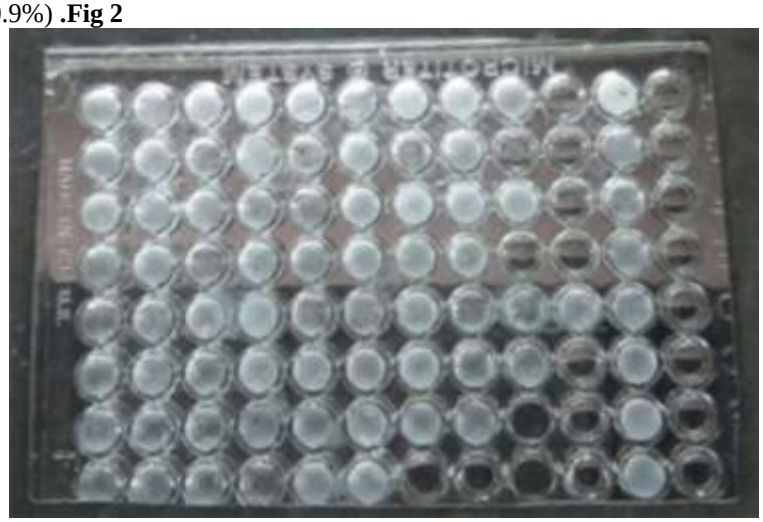

**Fig.2:-**Minimum Inhibitory Concentration (MIC) of meropenem for *Klebsiella pneumoniae* isolates by Broth Microdilution.

On comparison of MIC with PCR for *bla*KPC gene findings it was seen that out of 20 isolates with MIC ≥256 , 18(90%) were positive for *bla*KPC gene , among isolates with MIC 128 µg/ml, 61% were positive for blaKPC gene . whereas isolates with MIC 64µg/ml, 72.7% were positive for this gene and isolates with MIC 32 µg/ml, 66.7% were positive for the blaKPC gene.Table-1,Fig.3

**Table-1:-**MIC(meropenem) of blaKPC positive and negative *Klebsiella pneumoniae* isolates.

| MIC | TOTAL | | PCR POSITIVE | | PCR NEGATIVE |
|---|---|---|---|---|---|
| 32 | 6(10.9) | | 4(66.7) | | 2(33.3) |
| 64 | 11(20) | | 8(72.7) | | 3(27.3) |
| 128 | 18(32.7) | | 11(61.1) | | 7(38.9) |
| ≥256 | 20(36.3) | | 18(90) | | 2(10) |

Chi square 6.56 ,p value 0.087

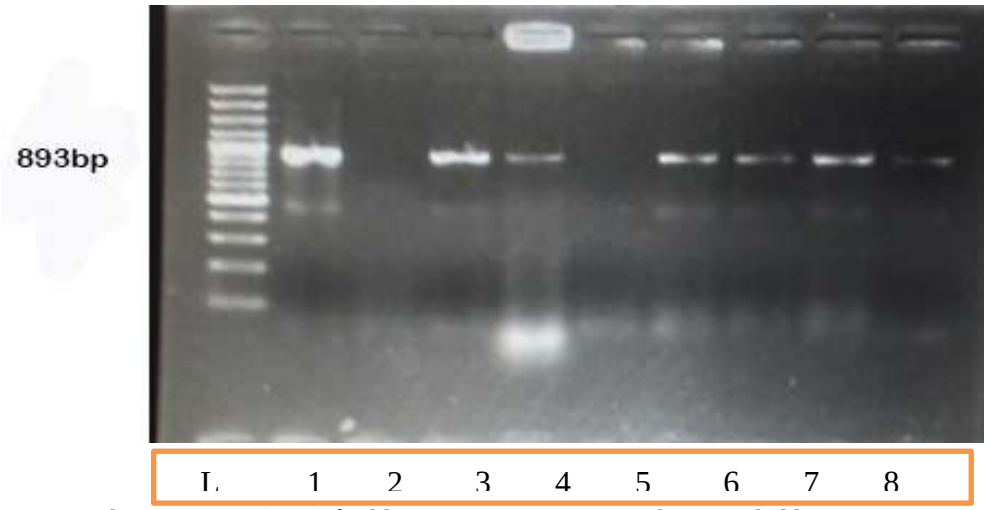


**Fig. 3:-**Poiymerase Chain Reaction(PCR) for blaKPC gene: Lane-L 100 bp DNA ladder, Lane-1, Positive Control; Lane-2, Negative Control; Lanes 3-9,Test strains

## Discussion:-

Out of the total isolates 55(78.5%) were meropenem resistant and 15(21.5%) were meropenem sensitive .our study results are similar with study conducted by Marquez P et al according to which 83% isolates were carbapenem resistant *Klebsiella pneumonia*.[15]According to a study conducted by Shanmugam P et al.(2013), Forty three (93.4%) out of the 46 isolates were resistant to Meropenem.[16] Seibert et al in their study in Brazil found *K. pneumoniae* was the microorganism that presented with the greatest resistance to carbapenems ( 62.0%).[17] Praveen *et al.*, 2010 studied K. pneumoniae, from 134 clinical isolates about 43.6% percent isolates were resistant to Meropesnem. (Praveen *et al.*, 2010).[18]

On comparison of MIC with PCR for *bla*KPC gene findings it was seen that out of 20 isolates with MIC ≥256 , 18(90%) were positive for *bla*KPC gene , among isolates with MIC 128 µg/ml, 61% were positive for blaKPC gene . whereas isolates with MIC 64µg/ml, 72.7% were positive for this gene and isolates with MIC 32 µg/ml, 66.7% were positive for the blaKPC gene . According to a study conducted by Giani et al ,Carbapenem MICs of the 234 carbapenem-nonsusceptible *K. pneumoniae, MIC of* >32 µg/ml was found in 94% isolates with ( KPC) carbapenemase, whereas isolates with (VIM ) carbapenemase ,MIC range was 2 to >32 , and those with (OXA) type carbapenemase MIC was 1 to 8 µg/ml.[19] Also according to Parveen et al. 43.6% were resistant to meropenem. The MIC of meropenem showed a varied range. Eight isolates showed a maximum MICs of>128µg/mL, nine were MIC at 128µg/mL. Similarly 6 isolates showed MIC of 16µg/mL and five had MIC of 32µg/mL. None of the *K. pneumoniae* was found to produce MBL by EDTA-meropenem disk approximation test. [18]

As per a study conducted by Castenheira et al,among 271 *Klebsiella* spp., carbapenemase encoding genes were observed in 62 (22.9%) strains. Strains carrying KPC genes displayed MIC results against all carbapenems at ≥4 µg/ml, whereas strains carrying *bla*VIM-and *bla*OXA-48-like had MIC values from ≤0.12 to >8 µg/ml for different

carbapenem compounds. [20] According to Endimiani A et al among carbapenems, $MIC_{50/90}$s were 4/64 mg/L for imipenem and meropenem, 4/32 mg/L . Genetic analysis revealed that the isolates possessed the following *bla* genes: $bla_{KPC-2}$ (59.5%), $bla_{KPC-3}$ (40.5%), $bla_{TEM-1}$ (90.5%), $bla_{SHV-11}$ (95.2%) and $bla_{SHV-12}$ (50.0%). PFGE demonstrated that 32 (76.2%) isolates were clonally related (type A KPC).[21] Also Spyros Pournaras et al investigated Meropenem heteroresistance in six apparently meropenem-susceptible, *Klebsiella pneumoniae* carbapenemase (KPC)-producing *K. pneumoniae* (KPC-KP) clinical isolates, compared with that in carbapenemase-negative, meropenem-susceptible controls. In population analyses, the KPC-KP isolates grew at meropenem concentrations of 64 to 256 µg/ml. Heteroresistant colonies had significantly elevated expression of the $bla_{KPC}$ gene compared with the native populations. [22]

## Conclusion:-

All the *Klebsiella pneumoniae* resistant isolates by vitek 2 were also having MIC in resistant range by BMD method and isolates positive for blaKPC gene are having higher MIC ranges, as all resistant isolates were having MIC ≥32µg/ml.

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