*Original Research Article*

# Phenotypic and genotypic detection of carbapenem resistant nosocomial infection in patients attending tertiary care hospital


## ABSTRACT

**Aims:** Nosocomial infection the major cause of mortality. ESKAPE organisms are the main cause of nosocomial infection, these organisms are more or less has carbapenem resistant. This study aimed to isolate and identify the etiological agents responsible for causing nosocomial infection and determine the carbapenemase producing organism by phenotypic test and genes responsible in producing carbapenemase by genotypic detection.
**Study design:** The study design is cross sectional study.
**Place and Duration of Study:** The study was conducted in the Department of Microbiology, at Index Medical college, Indore, between January 2020 and January 2022.
**Methodology:** Total of 246 samples was collected from the patients who develop symptoms after 48- 72 hrs of hospitalization. Samples were processed for identification of etiological agents. Gram negative organisms were selected and further identified for carbapenemase enzyme. Screening was done by Kirby Bauer disc diffusion test. Phenotypic detection was done by Modified Hodge test, Combined Disc test, Double Disc Synergy Diffusion test and Carbapenem Inactivation method Genotypic detection was done by using multiplex polymerase chain reaction for KPC, NDM and OXA-48 gene.
**Results:** Total of 107 gram negative organisms, 19 (17.75%) were carbapenem resistant. Among 19 carbapenem resistant GNR 13% were MHT, 15% CCDT, 17% DDST and 17% mCIM. The sensitivity and specificity of MHT, CCDT, DDST, mCIM were 74%/100, 84%/100,95%/100,95%/100respectively. The genotypic detection show highest percentage of NDM 74% which is followed by OXA-48 31% and KPC 26%.
**Conclusion:** Hospitals have become the hotspot; wide ranges of microorganism causing nosocomial infection are getting carbapenem resistance due to irrational use of antibiotics. Antimicrobial stewardship can be one of the effective measures which could minimize the resistance. Proper universal precaution can also minimize, spread of resistance in organism. If the last resort drug get resistant than it could be the challenge for the clinician to treat their patients. Hospitals should have regular HAI meeting and antibiogram should be release to know the pattern of these notorious organism invading like termites.



*Keywords: Nosocomial infection, Phenotypic, Molecular detection, Antimicrobial resistance, Carbapenem resistance*


## 1. INTRODUCTION

Nosocomial infections are defined as the infection that develops in 48 to 72hour after admission of patient; the infection which was not present or not incubating when the patient is hospitalized [1]. Urinary tract infection is the most common nosocomial infection, followed by skin and soft tissue infection, blood stream infection and ventilator associated pneumonia [2]. The organisms responsible in causing nosocomial infection are defined as ESKAPE. It includes *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter species* other includes *Escherichia coli , Proteus , Citrobacter*, Members of *Enterobacterals* [3,4]. These bacteria are most common in individual critically ill, immunocompromised and who are multi drug resistance ,which lead to life-threatening nosocomial infections[5]. The listed organisms are associated with high mortality and morbidity.[6] According to World Health Organization in 2017 carbapenem resistant *Enterobacterales*, carbapenem resistant *Pseudomonas aeruginosa* and carbapenem resistant Acinetobacter baumannii were ranked as critical high priority [7].Per year antimicrobial resistance increasing, as expected that there will be increment of ten times of antimicrobial resistance by 2050 with the projected scenario of mortality among different continents [8]. Carbapenem have been considered as a potent group of antibiotic to treat extended spectrum beta lactamase producing bacteria in past decade. It is widely prescribed for regimen for multidrug resistant gram negative bacteria , which are capable in causing systemic infection [9,10,11]. Carbapenem resistance is associated with the production of enzyme carbapenemase. It is encoded by carbapenemase encoding genes, which were classified into four group of classes[13].
Class A carbapenemase which include *Klebseilla pneumoniae* carbapenemase (bla $_{KPC}$), imipenem hydrolyzing beta lactamase (bla $_{IMI}$) and *Serratia marcescens* enzyme (bla $_{SME}$). Carbapenemase B are Metallo beta lactamase which include New Delhi metallobeta lactamase (bla $_{NDM}$), Verona Integron Metallo beta lactamase (bla $_{VIM}$), Imipenemase (bla $_{IMP}$),German Imipenemase (bla $_{GIM}$) , and Sao Paulo Metallo beta lactamase (bla SPM). Class D carbapenemase include Oxacillinase consist of bla $_{OXA-48}$, bla $_{OXA-181}$, bla $_{OXA-23}$, bla $_{OXA-204}$ , bla $_{OXA-162}$, and bla $_{OXA-24}$[13,14] Carbapenem resistance can also occur by other modes like porin mutation leads to reduction of outer membrane permeability, over expression of efflux pump, binding sites (penicillin binding protein), and plasmid encoding carbapenem which can be through horizontal plasmid mediated transmission [9,15]. There are various methods for the detection of carbapenem [16,17].The detection of carbapenemase is essential for antimicrobial stewardship to get updated about resistance pattern of antibiotics. There are various range of methologies for detection of carbapenem resistance from conventional Kirby Bauer disc diffusion method to phenotypic methods like modified hodge test , combined disc diffusion test , double disc synergy test and Modified carbapenem inactivation method. Genotypic detection was done by polymerase chain reaction. Antimicrobial stewardship is crucial to minimize the rate of nosocomial infection, the isolates and identification of etiological agent and their resistance pattern are essential perquisite, to prevent further dissemination of infection and reduce hospital burden. As carbapenem resistance have increased globally, in this regard this study aimed to screen carbapenem producing Gram negative resistant Nosocomial infection using both phenotypic and genotypic method.

## 2. MATERIAL AND METHODS

### 2.1 Study design

A cross-sectional study was out carried on 246 clinical samples of hospital admitted patients and who develop symptoms after 48 hours. The study was conducted at Index medical college, Hospital and Research Centre , Indore Madhya Pradesh. The study was carried out

from January 2020 to January 2023. All of the methods were under standard guidelines set out by the Clinical and Laboratory Standards Institute (CLSI).

## 3 Bacterial isolation and identification [18]

Gram-negative bacteria were isolated and identified by standard manual conventional method from the culture of the routine clinical samples which include Urine, Blood, Pus, Sputum, Endotracheal Aspirate, HAI's sample (Pleural fluid, Ascitic fluid, Peritoneal fluid , Rectal swab ). The samples were inoculated on blood agar (Himedia),MacConkey agar (Himedia ). Blood and respiratory samples were inoculated also include on chocolate agar (Himedia). Urine sample was inoculated on CLED agar (Himedia). The samples were incubation at 37 °C ± °C, for 24 hours. Colony morphology of growth plates was performed and Gram staining (Himedia) was performed from the samples which show growth. Biochemical identification was performed on the isolates which were gram-negative bacilli. The biochemical tests include Catalase test , Oxidase test Indole test , Methyl red, Voges-Proskauer test ,Citrate utilization test , Urea hydrolysis test , Motility test, Triple sugar iron test , Hugh and Leifson test and nitrate reduction test were performed for identification of gram negative bacilli.

## 4 Carbapenem-resistance screening

All the gram negative bacilli were screened for Carbapenem-resistance. It was done by using Meropenem (10ug) disc, Imipenem (10ug) disc and Etrapenem (10ug) disc. Interpretation of result was done under the guidelines of Clinical and Laboratory Standards Institute .

### 4.1 Phenotypic detection

#### 4.1.2 Detection of carbapenemase production

##### 4.1.2.1 *Modified Hodge test [19]*

According to Clinical and Laboratory Standards Institute (CLSI) (2018 )guidelines, all isolates were subjected to the modified Hodge test. A lawn culture of the 1:10 dilution of 0.5 McFarland suspension of *Escherichia coli* ATCC 25922 was carried out on Mueller Hinton agar plate. Meropenem (10ug) disc was placed in the center of the charged with *Escherichia coli* ATCC 25922 on Mueller Hinton agar plate. A straight line was drawn from the edge of the disk to periphery of the plate with the test organism. A control strains (positive and negative) was also tested on the same plate drawn from the edge of the disk to periphery of the plate. The plates were incubated at 35 °C ± 2 °C for 24 hours. Interpretation of Positive modified Hodge observe by clover leaf-like indentation of the *Escherichia coli* 25922 strain growing along with the test organism growth streak within the disk diffusion zone indicating the production of enzyme carbapenemase and a negative test showed no growth of the *Escherichia coli* ATCC 25922 along the test organism growth streak within the disk diffusion zone. *Klebsiella pneumoniae* (ATCC BAA-1705) and *Klebsiella pneumoniae* (ATCC BAA-1706) were used as positive and negative controls respectively.

##### 4.1.2.2 *Modified Carbapenem Inactivation Method (mCIM) [19]*

The mCIM method was led down by Clinical and Laboratory Standards Institute (CLSI) guidelines with use of imipenem disc. A loopful of bacterial isolate from an overnight culture

plate, was emulsified in 2 mL of Tryptone Soya Broth (TSB). A imipenem disc (10µg) was immersed in Tryptone Soya Broth. Tryptone Soya Broth (TSB) containing imipenem disc was incubated at 35 °C ± 2 °C for two -four hours. 0.5 McFarland suspension of *Escherichia coli* ATCC 25922 in nutrient broth was made with the help of cotton swab lawn culture on a Mueller Hinton agar. *Escherichia coli* ATCC 25922 will act as susceptible strain for potent imipenem disc. Now allow the plates to dry for 3-10 minutes. Imipenem disc from TSB-imipenem disc suspension was picked before applying on the plate, pressed the excess fluid with help of sterile inoculating loop. With the help of loop removed the disc from the tube and then placed on the Mueller Hinton agar plate previously charged with Escherichia *coli* ATCC 25922 imipenem susceptible. Plates were incubated at 35 °C ± 2 °C for 18-24 hours. Interpretation of positive result show zone between 6-15 mm or presence of colonies within a 16-18 mm zone. Presence of enzyme carbapenemase, the imipenem disc will be hydrolysed and there will be no inhibition of the imipenem-susceptible *Escherichia coli* ATCC 25922. Negative result show zone more than 19 mm. Absence of enzyme carbapenemase, the imipenem disc will not be hydrolysed and will inhibit growth of the imipenem-susceptible E. coli ATCC 25922. Indeterminate show zone between 16-18 mm. *Klebsiella pneumoniae* ATCC BAA-1705 and *Escherichia coli* ATCC 25922 were used as positive and negative controls respectively.

## 5 Detection of metallo-β-lactamase

### 5.1 Combined Disc Test (CDT) [20]

The test organisms were inoculated on Mueller Hinton agar plate as per Clinical and Laboratory Standards Institute (CLSI) guidelines. After drying, two 10 µg Meropenem discs were placed on the lawn culture with 20 mm distance from centre to centre of the discs. A 10 µl of 0.5 M EDTA (Himedia) was added to one of the Meropenem discs and incubated overnight. Incubate at 35 °C ± 2 °C for 24 hour. The inhibition zones of Meropenem and Meropenem plus EDTA were compared after incubation. Interpretation of positive result show increase in zone of diameter for Meropenem plus EDTA of ≥5 mm than Meropenem alone.

### 5.2 Meropenem EDTA double disc synergy test (DDST) [21]

0.5 McFarland was inoculated on Mueller Hinton agar plate. After drying, a Meropenem disc (10 µg) and a blank filter paper disk was placed 10mm apart from edge to edge, 10 µl of 0.5 M EDTA (Himedia) solution was then applied to the blank filter paper disc, to achieve the concentration of 750 µg . The plates were incubated at 37°C for 24 hour. Interpretation of positive is indicated as enhancement in the zone of diameter of > 5 mm.

## 6 Genotypic detection [22]

DNA was extracted by using Qiagen miniprep kit as per the brochure provided in the kit. The DNA of each isolates were subjected to multiplex PCR of bla $_{NDM}$, bla $_{KPC}$ , bla $_{OXA\ 48}$ were according to Martha F. Mushi et al. 20 µL PCR reaction mixture include 10 µL of master mix , 0.5 µL of primer and 4 µL nuclease free water, 2 µL Q buffer, and 200 ng of purified DNA template. The PCR amplification was done using Applied Biosynthesis thermal cycler. A total 30 cycles were programmed with the initial denaturation cycle for 10 minute at $95^0$C, followed by 30 seconds denaturation at $94^0$C , Annealing for 30 seconds at $55^0$C and Extension for 1 minute at $72^0$C for bla $_{NDM}$ bla $_{KPC}$ , bla $_{OXA\ 48}$ . Additional final extension is required for 7 minute at $72^0$C.

## 7. RESULTS

A total of 246 clinical samples were collected during the study period from Jan 2020 to Jan 2022. Out of 246, 107 (43.49%) were gram negative bacilli. From 107 gram negative bacteria, the highest percentage were of *Escherichia coli* (32.55%) followed by, *Klebseilla pneumoniae* (17.5%), *Citrobacter species* and Pseudomonas aeruginosa (8.52), *Acientobacter Species* (7.55%) , Proteus mirabilis (4.56%) and *Enterobacter species* (3.87%). On performing Kirby Bauer disc diffusion method 52(59.09%) male patient were sensitive and 12 (63.15%) were resistant to carbapenem where as 36(40.90%) were sensitive and 7(36.84%) were resistant to carbapenem. The statistical analysis of *p*-value = .00001 for both carbapenem resistant and carbapenem sensitive as shown in Table no. 1

Table no.1 **Bivariate Associations for Demographics presentation of carbapenem resistant and carbapenem sensitive patient**

| **Characteristic** | **Frequency N%** | **Carbapenem Sensitive N%** | **Carbapenem Resistant N%** |
|---|---|---|---|
| Gender | | | |
| Male | 64(59.8) | 52(59.09) | 12(63.15) |
| Female | 43(40.1) | 36(40.90) | 7(36.84) |
| Residence | | | |
| Urban | 34(31.7) | 29(32.9) | 5(26.3) |
| Rural | 73(68.2) | 59(67.0) | As |
| Age group (in year) | | | |
| less than10 | 6 (5.6) | 5(4.6) | 1(5.2) |
| 11_ 20 | 17(15.8) | 13(14.7) | 4(21.0) |
| 21-30 | 32(29.9) | 24(27.2) | 8(42.1) |
| 31-40 | 22(20.5) | 20(22.7) | 2(10.5) |
| 41-50 | 14(13.0) | 12(13.6) | 2(10.5) |
| > 50 | 16(14.9) | 14(15.9) | 2(10.5) |
| Wards | | | |
| Medicine | 55(51.4) | 49(55.6) | 6(6.8) |
| Surgery | 23(21.4) | 18(20.4) | 5(5.6) |
| Pediatrics | 8(7.4) | 6(6.8) | 2(2.2) |
| Obstetrics & Gynecology | 6(5.6) | 4(4.5) | 2(2.2) |
| Intensive care unit | 14(13.0) | 11(12.5) | 2(2.2) |
| Previous history of admission | | | |
| Yes | 32(29.9) | 29(32.9) | 3(15.7) |
| No | 75(70.0) | 59(67.0) | 16(84.2) |
| New antibiotic | | | |
| Yes | 23(21.4) | 15(17.04) | 8(42.1) |
| No | 84(78.5) | 73(82.9) | 11(57.8) |
| Length of hospital days | | | |
| <5 days | 65(60.7) | 54(61.3) | 11(57.8) |
| 5-7 days | 37(34.5) | 31(35.2) | 6(31.5) |
| > 7 days | 5(4.6) | 3(3.4) | 2(10.5) |

Out of 19 carbapenem resistance 14 isolates were show MHT positive result where as 16 were CDDT positive, 18 were DDST and mCIM positive. Among 107 gram negative bacteria 19 (17.75%) were screened by Kirby Bauer. Among the phenotypic methods for detection of Carbapenem was done by MHT and mCIM. For Metallo beta lactamase detection was done by CDDT and DDST. The sensitivity of MHT, mCIM, CDDT, DDST within CI 95% were 74%, 95%, 84%, 95% respectively shown in table no. 2

Table no. 2 **Comparison between different phenotypic methods of carbapenem resistance among gram negative bacteria**

| Organism | No. of isolates | Disc test | Diffusion | MHT | CDDT | DDST | mCIM |
|---|---|---|---|---|---|---|---|
| *Escherichia coli* | 42 | | 4 | 2 | 2 | 3 | 4 |
| *Klebseilla pneumoniae* | 22 | | 4 | 3 | 4 | 4 | 4 |
| *Citrobacter species* | 11 | | 3 | 3 | 3 | 3 | 2 |
| *Enterobacter species* | 5 | | 1 | 0 | 1 | 1 | 1 |
| *Proteus species* | 6 | | 1 | 1 | 1 | 1 | 1 |
| *Pseudomonas aeruginosa* | 11 | | 3 | 3 | 3 | 3 | 3 |
| *Acientobacter Species* | 10 | | 3 | 2 | 2 | 3 | 3 |
| *TOTAL* | 107 | | 19 | 14 | 16 | 18 | 18 |



Several isolates carried more than one gene. About 52% of gene carried $bla_{NDM}$ alone which was followed by 5% of $bla_{OXA-48}$ and $bla_{KPC}$. The coexisting genes include the highest prevalence of 21% $bla_{NDM+OXA-48}$ followed by $bla_{NDM+KPC}$ 11% and 5% were found in all three genes.. The highest prevalence of gene was seen *Escherichia coli* 26% which is followed by *Klebseilla pneumoniae* 21% and 16% of *Citrobacter species*, *Pseudomonas aeruginosa* and *Acientobacter Species* with least prevalence of 5% in *Enterobacter Species* The prevalence of resistance genes in *Enterobacterals* were dominant in *E. coli* were $bla_{NDM}$ , $bla_{OXA-48}$, co-exitance of $bla_{NDM+OXA-48}$ and blaNDM+KPC. *Klebseilla pneumoniae* to be second dominant organism consist of $bla_{NDM}$, $bla_{KPC}$ and $bla_{NDM+OXA-48+KPC}$. *Citrobacter species* and *Enterobacter species* consist of only $bla_{NDM}$. Whereas *Proteus species* do not contain any resistant gene. In non-fermenter *Pseudomonas aeruginosa* and *Acientobacter Species* contain $bla_{NDM}$ and $bla_{NDM+OXa-48}$. is mentioned in Fig. 1

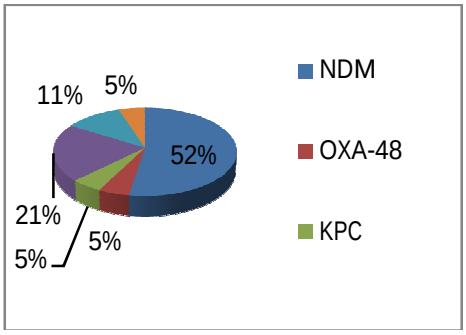

Fig. no. 1 **Distribution of Genes**

## 8. DISCUSSION

A study by Ph. Henkhoneng *et al*. [23] found 30% resistance to Meropenem and 26% of Doripenem. This may be due to over use of Meropenem. A study by Mohamudha R.P *et al* [24]found Meropenem resistance of 45 isolates, Imipenem and Ertapenem resistance of 33 and 21 respectively and Gupta E et al [25] found 22.16 % of overall, resistance to Meropenem and 17.32 % Imipenem. In this study out of 19 carbapenem resistant microorganisms, 14 isolates show MHT positive result where as 16 were CDDT positive, 18 were DDST and mCIM positive. Among 107 gram negative bacteria 19 (17.75%) were screened by Kirby Bauer. The phenotypic methods for detection of Carbapenem were done by MHT and mCIM. For Metallo beta lactamase detection was done by CDDT and DDST. The sensitivity of MHT, mCIM, CDDT, DDST within CI 95% were 74%, 95%, 84%, 95% respectively. Similar study reported by Naim H,*et al* [26] show CDDT 84.81%. In contrast to Naim H,*et al* [26] sensitivity of MHT, and DDST show 97.41% and 84.81%. A study by Cury et al.[27], reported 35.5% MHT positive *Enterobacterals*. Several isolates carried more than one gene. New Delhi metallo-β-lactamase 52% was the pre-dominant gene in this study, which was similar to that reported by Naim et al. [26] which is followed by 5% of bla $_{OXA-48}$ and KPC which was similar to that reported by Naim et al.[26] Similar study by Codjoe, et al [28] reported highest percentage of bla $_{NDM}$ 83% , bla $_{OXA-48}$ 75% , bla $_{VIM}$ 49% and bla $_{IMP}$ 43%, while reported least percentage of bla KPC. Another study by Nachimuthu, R. et al[29]; Kazi, M. et al.[30]; Okoche et al [31]; and Mushi et al [32];Braun S.D., et al[33] also reported highly prevalence of $bla_{NDM}$ and $bla_{OXA}$. Namrata Kumari [248] reported $bla_{NDM}$ (4.6%), $bla_{OXA}$ (2.3%), and blaVIM (1.6%). In contrast Kollenda H., et al [34] reported high prevalence of $bla_{OXA\ -48}$ as compare to bla $_{NDM}$.
The coexisting genes include the highest prevalence of 21% $bla_{NDM+OXA-48}$. In contrast to one of the study, the co-existence of $bla_{VIM}$ and $bla_{NDM}$ was 39.6%, whereas Mohanam et al [35] reported 14.6% and Ellappan et al [36] reported a 17.3% co-existence genes. In our study it was followed by $bla_{NDM+KPC}$ 11% and 5% were found in all three genes. The highest prevalence of gene was seen *Escherichia coli* 26% which is followed by *Klebseilla pneumoniae* 21% and 16% of citrobacter species, *Pseudomonas aeruginosa* and *Acientobacter Species* with least prevalence of 5% in *Enterobacter Species*. KPC is endemic in Israel , while $bla_{VIM}$, $bla_{IPM}$,$bla_{NDM}$ and $bla_{OXA-48}$ are endemic in Greece ,Japan , India and Turkey and it is disseminated around the world [37] In a study by Bourafa N., et al.[37], Kollenda H et al.[34], Shokri D., et al.[39], Mahrach Y., et al .[40] and Okoche D., et al.[31] reported carbapenemases genes in various multi drug resistant Gram-negative bacilli bacteria. [194, 38, 41,40,31]. In our study also $bla_{KPC}$ was least detected which was similar to Deldar Abad Paskeh M., et al [42] and Okoche D., et al.,[31]. In some studies there is no detection of gene $bla_{KPC}$ .[43,44]. According to one of the study there is less detection of gene bla KPC among Carbapenemase A as it also contain other genes like GES, SME, NMC-A and IMI[45]. The genes in the blood isolated were 1 $bla_{NDM+blaOXA-48}$ and 1 $bla_{NDM}$. The genes in urine were 5 $bla_{NDM}$ followed by 1 $bla_{OXA-48}$. The gene in pus the genes were 1 $bla_{NDM+OXA-4}$, 1 $bla_{NDM}$. In sputum 1 $bla_{NDM+KPC}$ , 1 $bla_{NDM}$ and 1 $bla_{KPC.}$ In Endotracheal aspirate gene were 2 $bla_{NDM\ +OXA-48}$ followed by 1 $bla_{NDM}$. In other samples from hospital associated infection include 1 $bla_{NDM}$ and 1 $bla_{NDM+OXA-48}$. Study by Vamsi et al [46]and Garg et al[47] reported least co-existence of $bla_{NDM}$ and $bla_{OXA-48}$ of about 1.4%, 20.0%. In our study $bla_{NDM}$ and $bla_{OXA-48}$ co-existence were reported in *Escherichia coli* and *Pseudomonas aeruginosa*. In contrast Grag *et al* [47] identified the $bla_{NDM}$ and OXA-48 co-existence pre-dominantly in *Escherichia coli*, followed by *Klebseilla pneumoniae*, and *Enterobacter*. In our study none of the non fermenter consist $bla_{OXA-48.}$ Similar study was reported by Vamsi et al

[46] which also reported no gene of $bla_{OXA\ -48}$ were detected. Several studies reported co-harbouring of carbapenemase [48, 49]. Coexistence of carbapenemase genes is a therapeutic challenge for clinicians. It is due to restricted treatment options and the potential for world-wide spread by horizontal transfer [50].

## 8. CONCLUSION

In our study it is found that phenotypic determinant have sensitivity of MHT, mCIM, CDDT, DDST within CI 95% was 74%, 95%, 84%, 95% respectively. It is important to perform genotypic detection to avoid false positive results as there is a call of carbapenem resistance in hospital settings. In our study 52% of gene was carried by blaNDM alone which is followed by 5% of blaOXA-48 and blaKPC. The coexisting genes include the highest prevalence of 21% blaNDM+OXA-48 followed by blaNDM+KPC 11% and 5% were found in all three genes.. The highest prevalence of gene was seen E.coli 26% which is followed by Klebseilla pneumoniae 21% and 16% of citrobacter species, Pseudomonas aeruginosa and Acientobacter Species with least prevalence of 5% in Enterobacter Species The carbapenem nosocomial infection can be stopped by regular update of antimicrobial susceptibility surveillance, If the measures followed are continuously updated and upgraded the infection level can be fur every hospital should have infection control meeting and release antibiogram for the clinician to get updated about the antimicrobial resistance pattern, which will also help the society. Hand hygiene is the key to break the spread of infection and control most of the multidrug resistant organism.

## 9. ETHICAL APPROVAL

The study was approved by Ethics committee MU/MM/BNS/2020/51(a)

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