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Study the Prevalence and Risk Factors of Metallo-Betalactamase Producing *Pseudomonas aeruginosa* from Teriary Care Centre

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Abstract: ***Background- P.aeruginosa is an important hospital pathogen and usually difficult to treat as it is resistant to most commonly used antibiotics.When this organism acquires the gene Metallo-betalactamase(MBL) production, the treatment options become even more limited. It is thus necessary to identify such organisms at the earliest. This is important to prevent the spread of such pathogens in the hospital environment resulting in resistant hospital acquired infections. However, currently CLSI does not recommended any guidelines for a quick phenotypic identification of Metallo-betalactamase producing organisms.[9]So,the present study was done to identify the prevalence of Metallo-betalactamase producing P.aeruginosa causing infections in a large tertiary care centre and to identify the risk factors associated with these highly resistant infections in a health care setting. Material and method- In this study we studied prevalence,following standared methods of isolation and identification techniques of these bacteria from all clinical samples such as Pus, Sputum, Urine, Blood, CSF etc.For detection of Metallobetalactamase production we were use different phenotypic methods i.e Modified Hodge Test, Im-EDTA disc diffusion method, I m-2MPA double disc synergy test and E test strips.Results- A total of 770 clinical isolates of P.aeruginosa were obtained from various clinical samples over a period of two years.265 (34.4%) of these isolates were imipenem resistant. The prevalence of MBL producing Ps. aeruginosa in the present study was computed to be 32.9 %. Conclusion- To overcome this problem, regular monitoring of the incidence of such organisms in various critical areas of hospital is important. So that isolation and barrier nursing of these patients would prevent the spread of drug resistance in the hospital.***

Keywords: Metallo-betalactamase, *Pseudomonas aeruginosa*

1. Introduction

Bacterial resistance to antimicrobial treatment is an emerging as one of the major public health threats of this century.The wide spread use and in some cases misuse of antimicrobials in all health care settings over the past several decades has been cited as a contributing factor in the development of drug resistance in virtually all bacterial species.[1] The widespread increasing resistance attributable to production of beta lactamase and an increasing number of new enzymes could eventually spell the end of the cephalosporin era. Thus, leaving us with a very narrow formulary for severe infections.[1] Carbapenems have a broad spectrum of antibacterial activity. However there is alarming increase in reports of carbapenem resistance in most gram negative bacteria including *P.aeruginosa*. This carbapenem resistance is mainly due to the most worrisome mechanism of resistance i.e Metallo-betalactamase production.[2]

P.aeruginosa is an important hospital pathogen and usually difficult to treat as it is resistant to most commonly used antibiotics.When this organism acquires the gene Metallo-betalactamase production, the treatment options become even more limited. It is thus necessary to identify such organisms at the earliest. This is important to prevent the spread of such pathogens in the hospital environment resulting in resistant hospital acquired infections. However, currently CLSI does not recommended any guidelines for a quick phenotypic identification of Metallo-betalactamase producing organisms.[3]

So,the present study was done to identify the prevalence of Metallo-betalactamase producing *P.aeruginosa* causing infections in a large tertiary care centre and to identify the risk factors associated with these highly resistant infections in a health care setting.

2. Materials and Methods

The present study was conducted in the microbiology department of a large tertiary care hospital

Study design

Laboratory based prospective of 2 year duration. All clinical samples such as Pus, Sputum, Urine, Blood, CSF received in the laboratory were included in the study. All these samples were collected using strict aseptic precautions and immediately transported to the laboratory. These samples were inoculated on Blood agar, MacConkey's agar. After overnight incubation agar plates were examined for non lactose fermenter colonies. *P.aeruginosa* isolates were confirmed by Oxidase test, Biochemical reactions and growth on Cetrimide agar as per standard techniques.[4] *P.aeruginosa* isolates were subjected to antibiotic sensitivity testing by using Modified Kirby Bauer disc diffusion method as per CLSI guidelines[5]

The antibiotic disc used for this study were (Himedia):

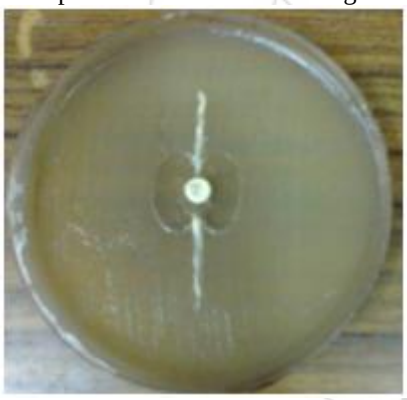
Amikacin	30 µg
Gentamicin	10 µg
Ciprofloxacin	5 µg
Norfloxacin	10 µg
Cefepime	30 µg
Pipercillin –Tazobactam	30 µg
Imipenem/Meropenem	10 µg
Colistin	10 µg
Polymyxin B	50 µg

100 imipenem resistant strains and 100 imipenem sensitive strains of *P.aeruginosa* were collected and formed the study group. Detailed clinical histories of these patients were recorded in standard formats especially with a view to ascertain the risk factors associated with resistance.

The imipenem resistant strains of *P.aeruginosa* were screened for metallo-beta-lactamase production by using the following different phenotypic methods:

1) **Modified Hodge test** [6,7]

Procedure –The surface of Mueller Hinton agar plate was inoculated evenly using a cotton swab with an overnight culture suspension of ATCC E.coli 25922. Which was adjusted to 0.5 Mcfarland standard. After brief drying, an imipenem disc was placed in the center of the plate and imipenem resistant test strain from overnight culture plate was streaked heavily from the edge of disc to the periphery of the plate. The plate was incubated overnight at 37ºC.



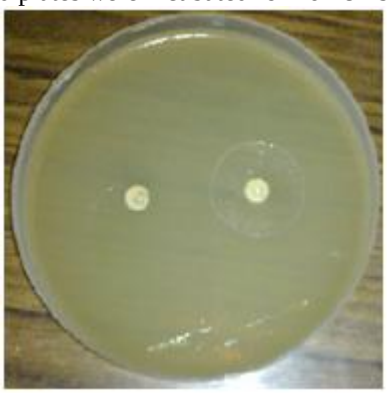
Photograph 1: Modified hodge test showing clover-leaf type indentation at the intersection of the test strain and the standard strain.

Interpretation: When the test strain produces the enzyme carbapenemase, it allows the growth of a carbapenem susceptible strain (E.coli ATCC 25922) towards a carbapenem **disc**. The positive result was taken to be a characteristic cloverleaf indentation. Clover leaf type indentation occurred at the intersection of the test strain and the standard strain.[7]

2) **Imipenem-EDTA disc diffusion test** [8]

Procedure : The suspected strains were inoculated into sterile peptone broth and the turbidity adjusted to 0.5 Mcfarland.

- Sterile cotton swab were dipped in the above broth and plated as a lawn culture on Mueller Hinton agar.
- After drying, two 10 µg Imipenem disc was applied firmly on the surface of agar.
- To one of the imipenem disc 4 µl of 0.5M EDTA was added and plates were incubated for 16-18hrs at 35ºC



Photograph 2: Im-EDTA double disc diffusion test showing difference in the inhibition zones between the two disc is >7 mm represents MBL production

Interpretation: – EDTA is a chelating agent which removes zinc ions from the active site of the Metallo-betalactamase enzyme. This make the enzyme inactive and thus the organism become sensitive to carbapenems. The zone diameter of two imipenem disc were measured and compared. Difference in the inhibition zones between the two discs by ≥7 mm was considered as a positive.

3) Double Disc Synergy test [9]-Imipenem with a Thiol compound i.e. 2 Mercaptopropionic acid (Himedia)

Procedure

- The suspected strain was inoculated into sterile peptone broth and turbidity adjusted to 0.5 Mcfarland.
- Sterile cotton swab was dipped in above broth and a lawn culture made on Mueller Hinton agar.
- After drying two 10 µg imipenem disc were applied firmly on the agar and filter paper disc was placed near one of the imipenem disc.
- Filter paper disc was placed 1-1.5 cm away from the imipenem disc.
- Then add 3 µl of 2-Mercapto-propionic acid on the filter disc
- The plate was incubated for 16-18 hrs at 37 ºC.

Photograph 3: Imienem- 2 Mercaptopropionic acid double disc synergy test showing distinct extention of zone of inhibition towards 2MPA.

Interpretation: Inhibition of activity of the enzyme is demonstrated by the use of Thiol compounds i.e. 2

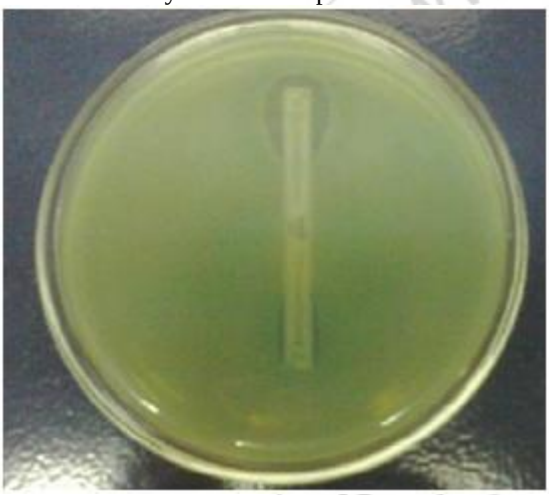
Mercaptopropionic acid.The presence of synergistic zone was interpreted as positive. Synergistic Zone means MBL positive *P.aeruginosa* shows distinct extention of the zone of inhibition towards 2MPA filtered paper disc.

4) E-test (Epsilometer test) (Biomerieux,France) [9,10]

The E test Metallo-betalactamase strip consists of double sided dilution range of Imipenem in one side and Imipenem-EDTA dilution on other side. The E test strip determines the MIC of the antimicrobial agent

Procedure

- The individual colonies of strain were suspended in liquid broth to attain a turbidity matching to 0.5 Mcfarland.
- With sterile cotton swab a lawn culture was made in the same way as for disc diffusion.
- Then E-test strip was placed on the agar with sterile applicator.
- Then plate was incubated for 16-18 hrs at 37 ºC and results of MIC of imipenem and imipenem- EDTA read directly from the strip.



Photograph4: MBL E-test showing MIC ratio of IP (Imipenem) / IPI (Impenem-EDTA) of 8.i.e MBL producing strain of *P.aeruginosa*

Interpretation:
Ratio of Imipenem/Imipenem EDTA ≥8, presence of phantom zone and distortion of ellipse were interpreted as positive results. This test was taken as the gold standard for detection of a Metallo β lactamase producer.

3. Results

A total of 770 clinical isolates of *P.aeruginosa* were obtained from various clinical samples over a period of two years. 265 (34.4%) of these isolates were imipenem resistant.From these 265 isolates 100 were taken in a study for detection of MBL production and identifying different risk factors.

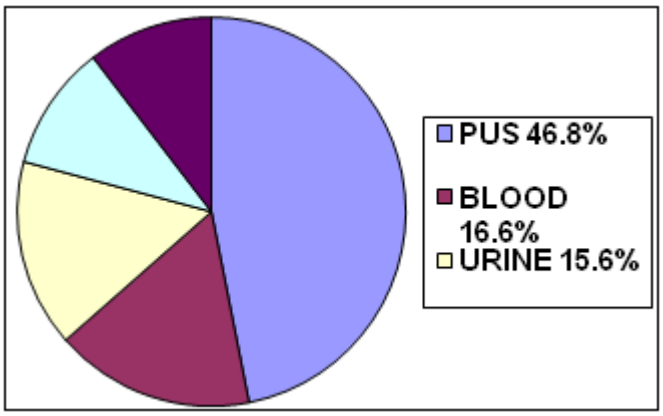


Figure 1: shows sample wise distribution of MBL positive *P.aeruginosa*

Majority of the MBL positive isolates were obtained from Pus samples i.e 46.8% and 16.6% from blood samples.

Table 1: shows the Age wise distribution of patients with MBL +ve and -ve *P.aeruginosa* infection

Age in years	MBL +ve n (%)	MBL –ve n (%)
<1	0 (0)	09 (9)
1-10	13 (13.5)	05 (5)
11-20	02 (2.08)	04 (4)
21-30	26 (27)	19 (19)
31-40	24 (25)	23 (23)
41-50	09 (9.3)	17 (17)
51-60	10 (10.4)	**10 (10)**
61-70	06 (6.2)	09 (9)
71-80	06 (6.2)	04 (4)
Total	96	100

P>0.05. Chi square =3.15

Age in yrs	OR
Up to 20	1
21-40	1.43
>40	0.09

Age group 21-40 yrs had 1.43 times more risk of acquiring MBL +ve *P.aeruginosa* infection than other age groups.

Table 2: Shows hospital stay of patients in the study groups at the time of infection

Duration in Days	Patients with MBL +ve *P.aeruginosa* infection n (%)	Patients with MBL – ve *P.aeruginosa* infection n (%)
1-10	45 (46.8)	63 (63)
11-20	47 (48.9)	37 (37)
21-30	3 (3.1)	0 (00)
31-40	0 (00)	0 (00)
41-50	0 (00)	0 (00)
51-60	1(01)	0 (00)
Total	96	100

P <0.01 Chi square = 8.05 OR=1.7

Patients with a duration of hospital stay >10 days had 1.7 times more risk of have MBL positive *P.aeruginosa* infection than those with a lesser duration of hospital stay.

Patients were also evaluated for the presence of underlying diseases such as Hypertension Diabetes mellitus and other immunosuppressive conditions at the time of acquisition of infection.

Table 3: Shows the patients having an associated underlying disease at the time of admission.

	Pts with underlying diseases No (%)	Pts without underlying diseases No (%)
MBL +ve n=96	57 (59.3)	39 (40.6)
MBL –ve n=100	24 (24)	76 (76)

P < 0.0001 Chi square =22.82 OR = 4.6

59.3% of patients with MBL producing *P.aeruginosa* infection had an underlying associated disease. In MBL –ve only 24% of patients showed underlying diseases. According to statistical analysis this table shows presence of underlying diseases is a significant risk factor in acquisition of MBL *P.aeruginosa* infection.

Previous history of taking carbapenem may precipitate resistance. This factor was evaluated. The antibiotics which were commonly used were Meropenem or Imipenem.

Table 4: Shows distribution of patients taking carbapenem group of antibiotics in the study groups

	Patients with h/o taking carbapenems No (%)	Patients with no h/o taking carbapenem No (%)
MBL +ve n=96	39 (40.6)	57 (59.3)
MBL - ve n=100	2 (2)	98 (98)

P < 0.0001 Chi square =45.06 OR=33.52

40.6% patients with MBL+ve *Ps aeruginosa* infection were on the carbapenem group of antibiotics usually at the time or in the two weeks before acquisition of the infection. 98 % of MBL negative patients had not received carbapenem group of antibiotics. According to statistical analysis this table shows patients taking carbapenem group of drugs during hospitalization was a highly significant risk factor in the acquiring a resistant *Pseudomonas* infection.

Table 5: Shows association of *P.aeruginosa* infection with exposure to various invasive procedures.

	Patients who underwent invasive procedure No (%)	Patients with no invasive procedure No (%)
MBL +ve (n=96)	96 (100)	0 (0)
MBL – ve (n=100)	66 (66)	34 (34)
Total	162	34

P<0.0001 chisquare=39.53 OR=50.9

This table shows that undergoing an invasive procedures during hospitalization was a highly significant risk factor in getting infected with MBL positive *P.aeruginosa.*

Table 6: shows ward wise distribution of Patients infected with *P.aeruginosa*

Ward	No. of patients with MBL +ve *P.aeruginosa* n(%)	No. of patients with MBL -ve *P.aeruginosa* n(%)
Medicine	26 (27)	31 (31)
Surgery	25 (26)	15 (15)
TB. Chest	10 (10.4)	22 (22)
Pediatrics	10 (10.4)	08 (8)
Skin	10 (10.4)	11 (11)
ENT	03 (2)	03 (3)
OBGY	02 (2)	04 (4)
Ortho	00 (0)	05 (5)
Burns	00 (0)	01 (1)
ICU	10 (10.4)	00 (0)
Total	96	100

MBL +ve isolates were more commonly isolated from infections in the Medicine and surgical disciplines Surprisingly the Burns ward did not show significant presence of *P.aeruginosa* infections.
100 Imipenem resistant isolates were tested for MBL production by Four different Phenotypic methods.

E test was taken as the gold standard. and used to Evaluate the performance of Modified Hodge test, Im-EDTA disc diffusion test and Im-2 Mercaptopropionic acid double disc synergy test

Table 7: Shows performance of the different Phenotypic methods for detection of Metallo-betalactamase.

	Modified Hodge test	Im-EDTA disc diffusion test	Im-2Mercapto-propionic acid double disc synergy test
Positive	100	100	69
Negative	00	00	31
Sensitivity	100%	100%	69%

Modified Hodge test picked up all the MBL producers. Im-EDTA disc diffusion test was 100% sensitive for MBLs.Whereas the Im- 2 Mercaptopropionic acid double disc synergy test showed the least sensitivity.

Table 8: shows Antimicrobial susceptibility pattern in Metallo-beta-lactamase and Non Metallobetalactamase producing *P.aeruginosa* isolates.

Antimicrobial agents	MBL +ve *P.aeruginosa* (%)	MBL -ve *P.aeruginosa* (%)
Amikacin	51 (53.1)	63 (63)
Gentamicin	41 (42.7)	64 (64)
Ciprofloxacin	43 (44.7)	43 (43)
Cefepime	23 (23.9)	58 (58)
Ceftazidime	47 (48.9)	52 (52)
Pipercillin+Tazobactam	59 (61.4)	NT
Colistin	82 (85.4)	NT
Polymyxin B	93 (96.8)	NT

NT : not tested

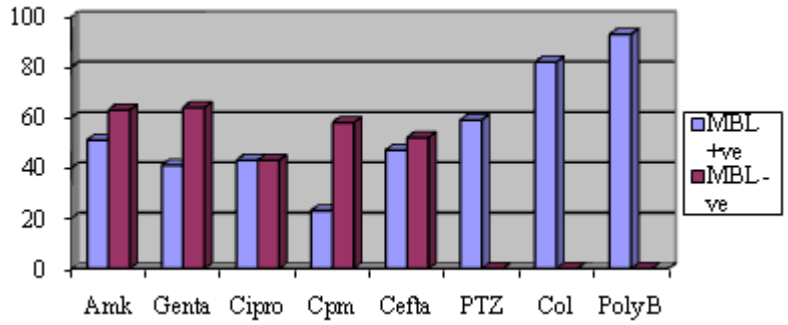


Figure 2:.Shows comparison of sensitivity pattern among MBL positive and MBL negative *P.aeruginosa* .

MBL positive isolates were more resistant to multiple antibiotics as compared to MBL –ve isolates. They were highly susceptible to the Colistin and Polymyxin B. These were not tested for in the MBL –ve isolates.

4. Discussion

P.aeruginosa is most common hospital acquired pathogen and infection due to these are difficult to treat due to antibiotic resistance. Recently, Metallo-betalactamase (MBL) producing *P.aeruginosa* have emerged which have the capacity to hydrolyse virtually all β lactam agents including the carbapenems.[11] so for infection control management it is important to identify MBL positive isolates of *Pseudomonas aeruginosa.*

In this study a total of 770 isolates of *P.aeruginosa* were obtained from various clinical samples over a two year period. These isolates were screened for imipenem resistance by Modified Kirby-Bauer disc diffusion test according to CLSI guidelines 2010.[5] Out of the 770 *P.aeruginosa* isolates 265 (34.4%) were found to be imipenem resistant. Imipenem resistance amongst *P.aeruginosa* in various studies across the country have been seen to range from 10 to 42%.In study done at a tertiary care center from Pondicherry Kanungo R et al reported 10.9% of resistance in the year 2006.[12] Kumar et al found 19.5% of carbapenem resistance in 2011 from tertiary care hospital in North India.[1]

The prevalence of MBL producing *Ps. aeruginosa* in the present study was computed to be 32.9 %. Indian studies have shown a varying range of a prevalence of MBL producing *P.aeruginosa* from 5% to 54.5% [13,14,15,16]. The prevalence of Metallo-betalactamase producing *P.aeruginosa* from other countries ranges from 6% to 46%.[17,18,19,20,21] According to the above studies,the prevalence of MBL in *P.aeruginosa* varies from country to country. These differences may be because every institute uses different antibiotic prescribing policies.

In present study Majority of the MBL positive isolates were obtained from Pus samples i.e 46.8% and 16.6% from blood samples and only 10.4% of MBL positive isolates obtained from sputum samples. Various risk factors could make a patient more prone to developing MBL producing Pseudomonas infections in the hospital environment. In the present study that duration of hospital stay, patient under going invasive procedures ,use of carbapenem group of antibiotics during treatment, and the presence of underlying diseases such as Diabetes mellitus, hypertension, asthma were evaluated for the role they played in the acquisition of MBL *Ps. aeruginosa.*

In our study we observed that the age group 21-40yrs had 1.43 times more risk of acquiring MBL +ve *Ps. aeruginosa* infection than other age groups.(Table 1). The studies by Bashir et al in 2011.[8] Varaiya *et al* in 2008,and De et al in 2012 did not show age as a significant risk factor for acquiring MBL positive *P.aeruginosa* infection.[15,22]

In our study the duration of hospital stay >10 days having 1.7 times risk of acquisition of MBL *P.aeruginosa* infection than duration of hospital stay ≤10 days.(Table 2) In the study by Varaiya *et al* in 2008 the average hospital stay of patients with MBL production was 32 days, and a significant risk factor in acquisition of drug resistance.[15]The study by De et al in 2012 showed a hospital stay of >8 days as a risk factor.[22,] So duration of hospital stay is the most important risk factor for acquiring MBL positive *P.aeruginosa* infection. This may be due to the fact that patients with critical illness are the group of patients with a long duration of hospital stay.

Patients were also evaluated for other risk factor i.e the presence of underlying diseases such as Hypertention, Diabetes Mellitus and other immunosuppressive conditions at the time of acquisition of infection. We found that presence of underlying diseases is a significant risk factor in acquisition of MBL *P.aeruginosa* infection. Hirakata et al. (1998, 2003) suggested that malignancy is a risk factor for acquisition of MBL producing *P. aeruginosa.*[23] In the study by De et al in 2012 previous use of any antibiotics was a significant risk factor in acquiring a drug resistant infection.[22] Bashir et al in 2011 showed previous use of beta lactam agents and carbapenems were significant risk factors.[8]

Previous history of taking carbapenems may precipitate resistance. This factor was evaluated in our study and we found that patients taking carbapenem group of drugs during hospitalization was a highly significant risk factor in the acquiring a MBL Pseudomonas infection.(Table4) Zavascki et al.in 2006 showed exposure to β-lactams is a significant risk factor for MBL Pseudomonas infection.[24] Study by R.C. Ceza´rio et al.in 2009 also showed similar findings.[25] In the study by De et al in 2012 previous use of any antibiotics was a significant risk factor in acquiring a drug resistant infection.[22]

In our study we found that patients undergoing various invasive procedures during hospitalization was a highly significant risk factor in getting infected with MBL positive *Ps. aeruginosa*.(Table5) Various studies by Zavascki et al.in 2006,[24] R.C. Ceza´rio et al.in 2009,[25] Bashir et al in 2011,[8] de et al have also reported invasive procedures as significant risk factor for MBL positive infections.The role of indwelling catheter for acquisition of MBL producing *P.aeruginosa* infections has been especially documented. [22]

Different areas of the hospital may have different rates of drug resistant pathogens.. This will essentially vary depending on the efficacy of infection control procedures followed and the use of antibiotics in that area. When analyzing the isolation of MBL positive *P.aeruginosa* from different wards We found that 27% and 26% of MBL positive *P.aeruginosa* obtained from the Medicine and Surgical ward.respectively.(Table 6) This may be due to the more number of invasive procedures occur in these wards and a longer duration of hospital stay due to admission of patients with chronic illnesses in the medical wards. Surprisingly we got only 10.4% of MBL positive isolates from ICUs and no patients from the Burns ward with MBL +ve Pseudomonas infections(Table6).

All the 100 *P.aeruginosa* isolates showing carbapenem resistance by Modified Kirby bauer disc diffusion test were further screened by different phenotypic methods for detection of MBL. Many reports suggest that using meropenem resistance rather than imipenem may increase the detection of MBL production but in the present study we used imipenem resistance to prelimnary detect MBL producers.[26] Since MIC measurements are more accurate and quantitative methods E test was taken as the gold standard [27] for evaluating three different phenotypic methods for screening for MBL production which can be routinely performed in most laboratories. Four isolates were E test negative. Thus 96% of imipenem resistant isolates were definite MBL producers.

Antibiotic susceptibility profile of the isolates varies from center to center depending on the type of antibiotics used in the particular center. In our study we found that MBL positive P.aeruginosa showed highest susceptibility to Polymyxin B (93%) and Colistin (82%) (Table8) These two drugs are the main treatment options for MBL positive *Ps. aeruginosa* infections. However strains resistant to these antibiotic are emerging which will leave us with almost no treatment options for these bacteria ie. the“ Super Bug” is emerging.[28]

As expected the antibiotic susceptibility of MBL negative strains was more encouraging. Similarly , Bhongle et al showed a high susceptibility to Colistin 96.55% and to polymyxin B 86%.[29] Another study by Kumar et al showed highest susceptibility to PTZ i.e 95%[1] So PTZ is a good treatment option for MBL positive *Ps. aeruginosa*. Kotgire et al showed 100% susceptibility to polymyxin B and only 50 % susceptibility to PTZ[3]

Treatment of these multidrug resistant organisms is difficult as a limited options are available. There are number of Metallo-enzyme inhibitors like EDTA, but they have only been used in vitro ,and none can be used in humans. The only alternative is the use of polymyxin and colistin which showed promising outcome so far against gram negative bacilli.

Thus it is advisable to prevent these spread of drug resistant organisms rather than try to treat them. Preventing these spread of resistance in the hospital entails monitoring of antibiotic resistance and isolating patients with multiple drug resistance. Enforcing hand hygiene and and environmental decontamination will help to prevent the spread of these drug resistance gene from patient to patient.

Controlling the use of antibiotics in the hospital environment by formulating antibiotic policy and monitoring the appropriate use of antibiotics in the hospital, the community, and in veterinary practice will result in decrease in antibiotic resistance.

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