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Original Research Article

Detection of Various Beta Lactamases in *Pseudomonas Aeruginosa* from Various Clinical Samples and Their Co-Existance

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ABSTRACT
In the present study, it's to be detect various type of beta lactamase which affect the growth of Pseudomonas aeruginosa. Pseudomonas aeruginosa is leading nosocomial infection agent. Treatment is more complicated because of high degree of resistance against beta lactamases enzymes. Samples i.e., urine, body fluids, pus, sputum, ear swabs, etc. were collected and Nutrient Agar, Blood Agar and MacConkey agar, oxidase test and their biochmecial reactions used for colony growth and identification. Among various samples, urine, sputum, pus and ET secreation has been detected from ESBL, AmpC and MBL producer Pseudomonas aeruginosa. Maximum ESBL production for 41-50 aged group patients and for AmpC and MBL, the aged group are <10 and 50-60 years respectively. Out of all the positive samples, only one sample i.e., AmpC-MBL, has been isolated for co-existance. It's useful for the treatment against Pseudomonas aeruginosal infection for the physician and restrict the growth of such common and deadly infection.
Keywords: *Beta-Lactamase, ESBL, AmpC, MBL, Pseudomonas aeruginosa.*

INTRODUCTION

Resistance bacteria are emerging world wide as a threat of the favorable outcome of common infection in community and hospital setting.[1] *Pseudomonas aeruginosa* is reported to be amongst leading cause of nosocomial infection. It is known to exhibit intrinsic resistance of *Pseudomonas aeruginosa* to several antimicrobial agents.[2] Treatment of these infections often complicated because of increasing bacterial resistance mediated by varying degree of beta lactamases enzymes such as ESBL, AmpC and MBL.β Lactams are a group of antibiotics acting on the cell wall of a bacterial cell. These include the Penicillins, Cephalosporins, Carbapenems and Monobactams. These bind to and inhibit the carboxypeptidases and transpeptidases. These are the cell wall synthesizing enzymes, also called the penicillin-binding proteins, or PBPs, that catalyze the D-ala cross linkages of the peptidoglycan wall that surrounds the bacterium. As a result, there is weakening of the cell wall structure, leading to cell lysis.[3]In the present study, we have to detect various type of beta lactamases in *Pseudomonas aeruginosa* from various clinical samples and their co-existance.

MATERIAL AND METHODS

In this study, a total 50 positive *Pseudomonas aeruginosa* isolated from various clinical samples

include urine, body fluids, pus, sputum, swabs, ET secretion, ear swabs etc. samples were collected from the patient of Outpatient departments and inpatient department at NIMS hospital. The identification of *Pseudomonas aeruginosa* was based on colony morphology on Nutrient Agar, Blood Agar and MacConkey agar, oxidase test and their biochmecial reactions.

The antibiotic susceptibiity was performed by kirby bauer method against various antibiotic like ampicillin(30µg), ceftazidine(30µg), cefotaxime (30µg), ceftriaxone(30µg), aztreonam(30 µg), amikacin(30µg), tobramycin(10µg), piperacillin/ tazobactum(75µg/10µg), ticarcillin/tazobactum (75µg), cefoperazone/salbactum(75 µg /30 µg), ciprofloxacin (5 µg), imipenam(10 µg), Meropenem(10µg), polymyxin(300 unit)and colistin (10µg). on Muller- Hinton agar.

ESBL detection:- Double disc synergy test

All the isolates of *P.aeruginosa* which showed resistance to ceftazidime were evaluated for ESBL production by using the phenotypic confirmatory test[4]. As per the CLSI 2014 guidelines of ceftazidime disk with or without clavulanate for phenotypic confirmation of the presence of ESBLs production on the confluent growth on Muller Hinton agar. A difference of ≥5 mm between the zone diameters of ceftazidime and its ceftazidime/ clavulanate disks was taken to be phenotypic confirmation of ESBL production. When there was an increase of ≥ 5 mm in inhibition zone diameter around combination disk of Ceftazidime + Clavulanic acid (CAC) versus the inhibition zone diameter around Ceftazidime(CAZ) disk alone, it confirms ESBL production

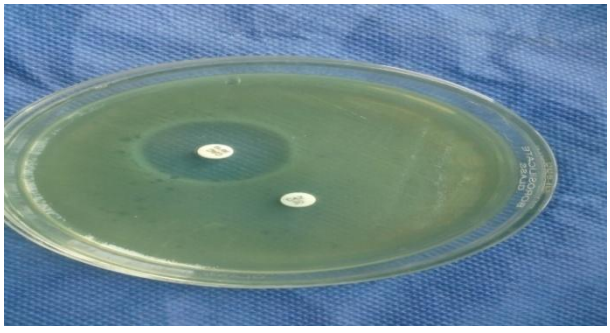

Figure 1:- Phenotypic Confirmatory Test with combination disc using ceftazidime disc 30 µg and ceftazidime clavulanate disc 30/10 µg.(ESBL Positive).

AmpC detection

Screening test

Cephoxitin disk test:- Screening of AmpC was done by cephoxitin disk test. Isolated zone of diameter less than 14 mm was taken as screen positive and was subjected to confirmatory testing.

Disk antagonism test:- Ceftazidine and cephoxitin disc was placed 20 mm apart from centre to centre. After 8-24 hrs. isolates showed blunting of ceftazidine zone of inhibition adjacent to cephoxitin disc was considered screen positive and ampC β-lactamase inducibility was recognized.

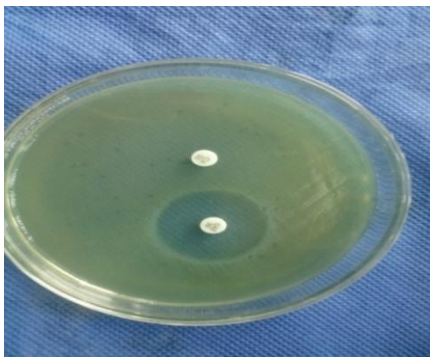

Figure 2:- Screening test of Ampc detection by Disc antagonism test (Ceftazidime and Cefoxitin disc)

Confirmatory test:-

- **Preparation of disc:** AmpC disks were prepared in house by applying 20µl of a 1:1 mixture of saline and 100X Tris-EDTA to sterile filter paper disks, allowing the disks were to dry and storing them at 2-8°C.
- **Procedure**

1. The surface of a Muller Hinton agar plate was inoculated with a lawn of cefoxitin susceptible to *E. coli* ATCC 25922 according to the standard disk diffusion method.
2. A 30µg cefoxitin disk was placed on the inoculated surface of the Muller Hinton agar.

3. Immediately prior to use, AmpC disks was rehydrated with 20µl of saline and several colonies of each test organism was applied to the disk.
4. The inoculated AmpC disk with the test organism is inverted and then placed on agar plate almost touching the cefoxitin antibiotic disk. The plate is then inverted and inoculated overnight at 35°C in ambient air.

- **Interpretation**

  Positive result: Plates were examined for either an indentation or a flatting (distortion) of the zone of inhibition around cefoxitin antibiotic disk, indicating enzymatic inactivation of cefoxitin.

  Negative result: The absence of distortion was indicating no significant inactivation of cefoxitin.

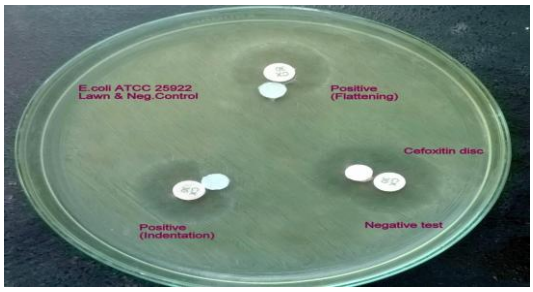

Figure 3:- Confirmatory test of AmpC by AmpC disk method

MBL detection

❖ **Screening test (Combined disc test):-** An overnight broth culture of the test strain was used to inoculate a plate of Muller Hinton agar (MHA). An Imipenem disk 10µg was initially placed on MHA. Another imipenem disk which EDTA [10µl] {5% (that is a concentration of 500µg/disc} was added and placed on the plate. Plates were incubated at 37°C for 24 hrs. An increase of the zone of IMP-EDTA by 5 mm or more as compared to IMP zone of IMP-EDTA by 5 mm or more as compared to IMP disc alone was considered to be an MBL producer.

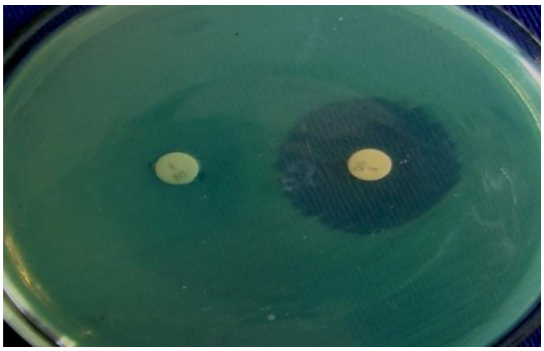

Figure 4:- Screening test of MBL by Imipenem and Imipenem+EDTA

❖ **Confirmatory test (Modified Hodge's test):-** According to CLSI, this confirmatory test for carbapenemase production is performed for epidemiological or infection control purposes (CLSI 2009, Lee et al., 2001).

Principle: An indicator strain (*E.coli* ATCC 25922) is used in this assay. If the test strain produces carbapenemase enzyme, it will diffuse in the culture medium and the sensitive indicator strain will grow even in the presence of Imipenem in the vicinity of test strain producing an indentation. This method has been validated by CLSI for Enterobacteriaceae members.

Inoculation

- The surface of the Mueller Hinton agar plate is inoculated with 1:10 dilution of a freshly prepared 0.5 Mc Farland suspension of *E. coli* ATCC 25922.
- Meropenem (10µg) disc is placed at the centre of the inoculated plate.
- With an inoculation loop, 3 – 5 colonies of the test organism are streaked from the edge of the disc to the edge of the plate in a straight line upto a length of 20-25 mm. Up to 4-5 organisms can be tested on the same plate with one drug.
- The plate is incubated overnight at 35°C ± 2°C in ambient air for 16–24 hours.

Interpretation

After incubation, the plate is examined for a clover leaf-type indentation at the intersection of

the test organism and the *E. coli* 25922, within the zone of inhibition of the carbapenem susceptibility disc.

MHT Positive test has a clover leaf-like indentation of the *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 zone.

Figure 5:- Confirmatory test of MBL by Modified hodge's test.

RESULT & DISCUSSION

Out of 50 isolates of 70% male and 30% female patients. *Pseudomonas aeruginosa*, show maximum sensitivity against Polymyxin 98%, Colistin 98, Imipenem 89% , Cefeparazone/ Salbactum 86% , Meropenem 84%, Pipercillin / Tazobactum 82%, ciprofloxacin 72%, Cefepime 80%, amikacin, colistin 62% each, tobramycin 60%, ceftazidime 42%, Ceftriaxone and aztreonam 40% each, least cefotaxime 38% and Ampicillin show no sensitivity. In all isolates of pseudomonas aeruginosa 4 ESBL, AmpC producer and 3 MBL producer.

Table 1: No. of Isolation among various Beta-Lactamase Producer

Total no. of isolates	ESBL	AmpC	MBL
50	4	4	3

Figure 6: Isolation among various Beta-Lactamase Producer

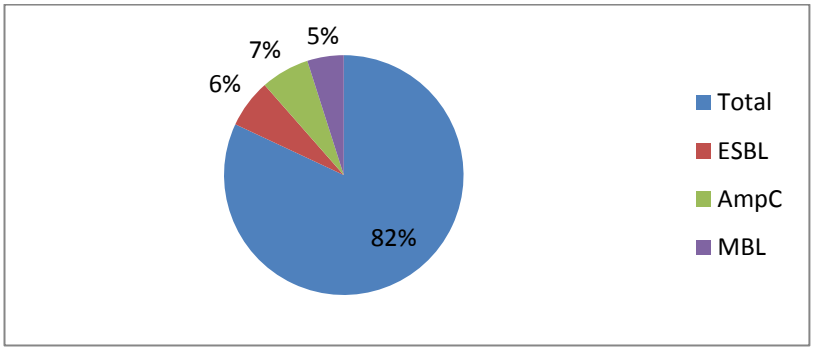

Table 2: ESBL, AmpC and MBL positive pseudomonas aeruginosa From Various clinical specimens:

Specimens	Isolates (n=15)	ESBL No. (%)	Amp C No (%)	MBL No (%)
Pus	15	1 (6.66)	2(13.33)	0 (0.00)
Ear swab	13	0 (0.00)	0(00)	0 (00)
Urine	9	2 (22.22)	0(00)	2 (22)
Sputum	7	0 (0.00)	2 (28.57)	1 (14.28)
ET secretion	2	1 (50)	0(0.00)	0 (0.00)
Blood	2	0 (0.00)	0 (0.00)	0 (0.00)
Foley's tip	1	0 (0.00)	0 (0.00)	0 (0.00)
Catheter tip	1	0 (0.00)	0 (0.00)	0 (0.00)

As shown in table out of 50 isolate of P. aeruginosa, 4 ESBL producer P. aeruginosa detected from clinical samples i.e 2 from urine, 1 from pus and 1 from ET secretion. 4 AmpC producer P. aeruginosa detected 2 from pus and 2 from sputum sample. 3 MBL producer P. aeruginosa detected 2 from urine and 1 from sputum sample

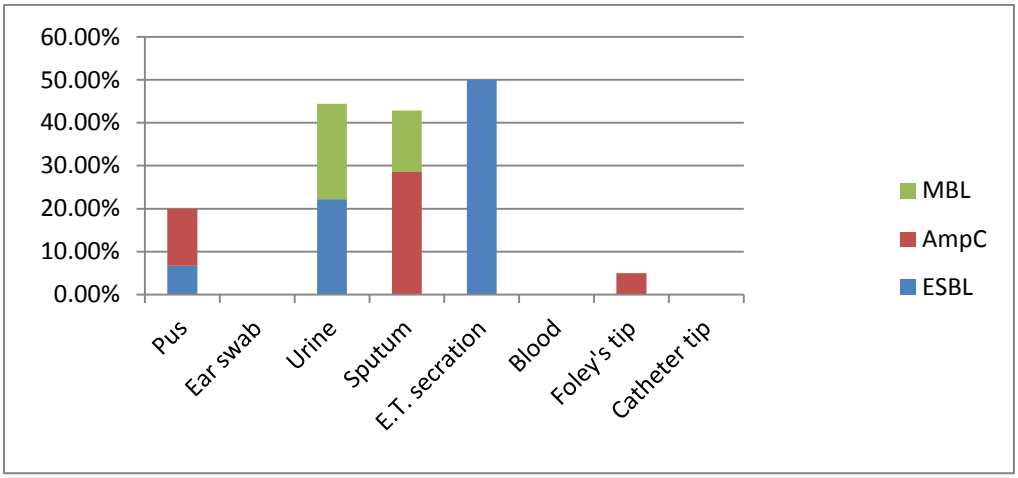

Figure 7: ESBL, AmpC and MBL positive pseudomonas aeruginosa From Various clinical specimens

Table 3:- Distribution of ESBL, AmpC and MBL producer P.aeruginosa in relation of age group

Age(yrs)	Isolate of P.aeruginosa (n=50)	ESBL no.(%)	AmpC no.(%)	MBL no.(%)
<10	2	0 (0.00)	1(50)	0(0.00)
11-20	11	0(0.00)	0 (0.00)	1(9.99)
21-30	6	0 (0.00)	0(0.00)	0(00)
31-40	9	1 (11.11)	1(11.11)	0(00)
41-50	5	2(40)	0(0.00)	1(20%)
51-60	9	1 (11.11)	2(22.22)	0(0.00
>60	9	0 (0.00)	0(0.00)	1(11.11)

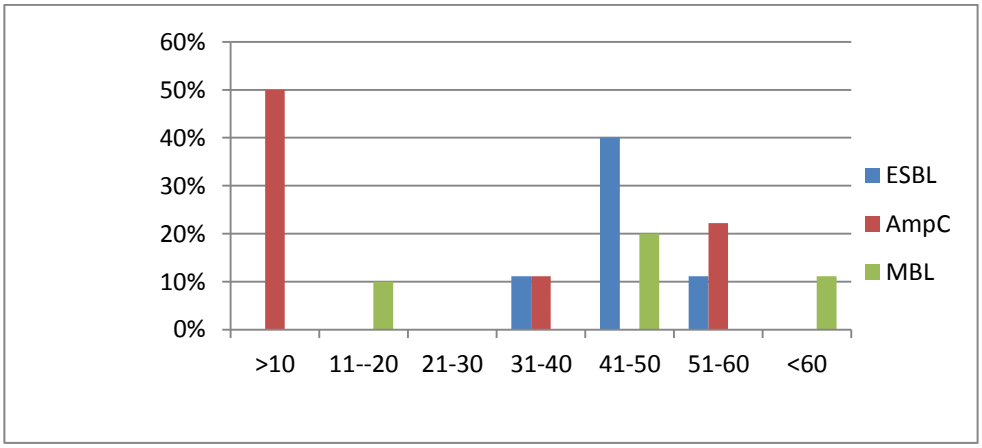

Figure 8:- Distribution of ESBL, AmpC, MBL in according to age group

Above graph seen 11.11% ESBL production in 31-40 and 51-60 and 40% in 41-50 age group. In AmpC 50% in <10, 11.11% seen in 31-40 and 22.22% seen in 51-60 age group. 9.99% in MBL in 11-20, 20% in 50-60% and 11.11% in <60 age group. Figure- Co-existance between ESBL – AmpC, AmpC-MBL, ESBL-MBL and ESBL-AmpC- MBL from P. aeruginosa isolates

Table 4:- Distribution of ESBL, AmpC and MBL in relation to sex

Sex (n=50)	ESBL no. (%)	AmpC no. (%)	MBL no.(%)
Male (n=34)	4 (11.76)	3 (8.82)	3 (8.82)
Female (n=16)	0 (0)	1 (6)	0 (0)

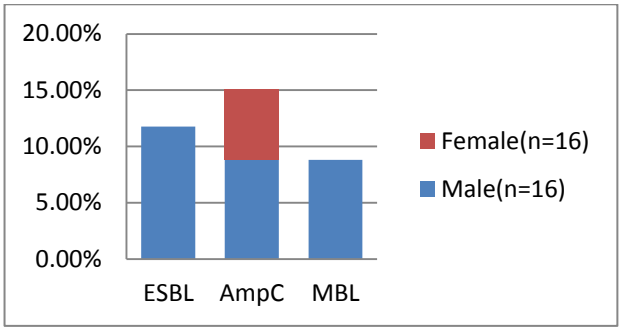

Figure 9:- Distribution of ESBL, AmpC and MBL in relation to male and female

In this in ESBL in male is 11.76%, AmpC in female is 6% and in MBL is 8.82.

Table 4:- Co-existance between ESBL – AmpC, AmpC-MBL, ESBL-MBL and ESBL- AmpC- MBL from P. aeruginosa isolates

Isolate (n=50)	ESBL-AmpC No.(%)	AmpC-MBL No.(%)	ESBL-MBL No.(%)	ESBL-AmpC-MBL No. (%)
1	(0.00)	1 (2%)	0 (0.00)	0 (0.00)

As shown table out of 50 *Pseudomonas aeruginosa*, only in 1 isolate AmpC-MBL coexistence exist while none of Co-existance between ESBL – AmpC, ESBL-MBL and ESBL- AmpC- MBL exist.

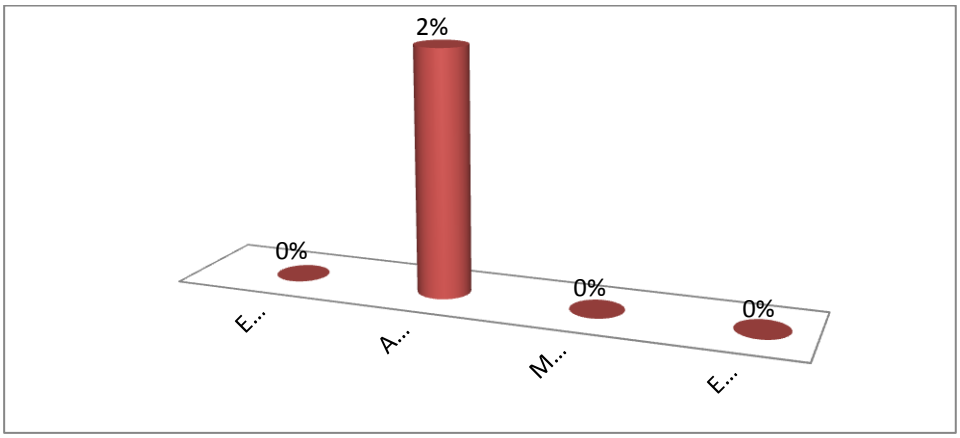

Figure 10: Co-existance between ESBL – AmpC, AmpC-MBL, ESBL-MBL and ESBL- AmpC- MBL from *P. aeruginosa* isolates

In the present study, *Pseudomonas aeruginosa* was common in male patients i.e 70% as campared to female patients i.e 30 %.. Similar observation of male prepondance was made Arora D et al (2010)[5], Flegoo et al(2014)[6] and Anurave K et al (2013).[7] *Pseudomonas aeruginosa* showed no were sensitive to Ampicillin which was correlate to Kalantar et al -2013[8] and Farida A et al. (2010)[9] Imipenem show 89% sensitivity which is correlate to Kumar V et al 2013[10] which show 100% sensitivity and also similar to Jacobson et al 1995.[11] *Pseudomonas aeruginosa* showed sensitivity 42% against Ceftazidime which was comparable to other study 41% by Sharma et al 2010.[12] The higher sensitivity were shown in the studies Usha K et al 2013[13] to be 55%, Kumar V et al.[10] 2011 to be 70%. Lower sensitivity were shown by Franco et al 2010[14] to be 14.5%

Pipercillin/Tazobactum show 82% sensitivity against *Pseudomonas aeruginosa* higher sensitivity was shown by Kumar V et al[10] 2012 100%. Polymyxin show 98% sensitivity against *Pseudomonas aeruginosa*. Lower sensitivity shown by Farida et al 2010[9] had reported 80%.*Pseudomonas aeruginosa* showed sensitivity 86% against Cefeparazone/Salbactum. Lower sensitivity shown by Juyal D et.al,2013[15] 39.36%. *Pseudomonas aeruginosa* showed sensitivity 84% against Meropenem which was correlate to Rajput A et al 2013.[16]

In the present study,7% ESBLs production by the phenotypic confirmatory test combined disk diffusion test in this test use ceftazidime and ceftazidime clavulanic acid because clavulanic acid inhibit the ESBL production. The present study similar with Jacoboson K L et al (1995)[17] was also produced 7.7% ESBLs Upadhyays et.al2010[18] reported very low incidence of ESBL among *P. aeruginosa* (3.3%), which contrasts in present study which showed 8%of ESBL production. ESBL production co-relate to Preshattiwar et.al 2011[19] showed that among the 126 *Pseudomonas aeruginosa* isolates, 28 [22.22%] were ESBL producers, which was similar to 20.27 % ESBL producing isolates of *P. aeruginosa* which was reported by Aggarwal et al[20].Uma et al 2011[21] (77.33%) and Mathur et al 2001[22] (64%) much higher then present study.In the present study, sensitivity of ceftazidime (, Cefotaxime, ceftriaxone and Aztreonam produce higher resistant.In present study produce only 6% AmpC production by Disk antagonism test, in this test used Cefoxitin and ceftazidime disc (screening test) and confirm by confirmatory test (AmpC disc test). In this study 4 test is positive in confimatory. Present study correlate with Shoorashetty R M et al (2011)[23] in this out of 200 samples 12 (6.00%) Ampc produce. Rawat v et al 2010[24] study also correlate with present study. Others study like Salamni F et al 2012[25] was much higher than present study is 81% AmpC produce.

MBL production out of 50 isolates seen 5% detected by the screening and confirmatory test. In screening test used Imipenem and Imipenem + EDTA and Meropenem and Meropenem + EDTA and confirmatory test by Modified Hodge's test. Bash et al 2011 correlate to present study in that out of 283 isolates 33 (11.6%) produce MBL production. Co-existance of ESBL, AmpC and MBL were studied. In present study co-existanceseen only in AmpC+MBL only 2%, and no any co-existance seen in ESBL+ AmpC and ESBL + MBL due to low production of ESBL, AmpC and MBL. Goel V et al 2013 study show co-existance between AmpC+ MBL in 3 (11.9%) thas is correlate to present study. Salami F et al 2012[25]study also correlate with present study in that AmpC+ MBL 4.6%.

CONCLUSION

The study is most valuable for the patients resist from *Pseudomonas aeruginasal* infections and guide the physician for better treatment against it and it reduced the rate of such infection. Government should take the appropriate steps against P.aeruginosal infections as it is common now-a-days and make the country free from such deadly and commonest infections.

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