www.jmscr.igmpublication.org
Impact Factor (SJIF): 6.379
Index Copernicus Value: 79.54
ISSN (e)-2347-176x ISSN (p) 2455-0450
crossrefDOI: https://dx.doi.org/10.18535/jmscr/v6i11.151

IGM Publication
Journal Of Medical Science And Clinical Research
An Official Publication Of IGM Publication

AMP C Producing Pseudomonas aeruginosa Isolated from a Tertiary Care Centre and its Association with Antibiotic Intake

Authors
Dr Meghana Vijayan P, Dr Joana Mary Magdaline
Department of Microbiology, Government Medical College Ernakulam,
Kalamassery North, Pine- 683503, Kerala
Corresponding Author
Dr Joana Mary Magdaline
Associate Professor in Microbiology, Government Medical College Ernakulam,
Kalamassery North, Pine- 683503, Kerala, India
Phone: 9447196964, Email: *joanamagdaline27@gmail.com*

Abstract
Pseudomonas aeruginosa is a commonly encountered gram negative bacteria which is a potential source of nosocomial infections. Amongst the various acquired mechanisms of resistance, production of beta lactamases is the most common. The failure to detect this may lead to therapeutic failure and spread of resistance .This study was conducted for a period of one year from March 2016 to find out the antibiotic susceptibility pattern of the Pseudomonas aeruginosa isolates obtained from various clinical samples received, to identify the proportion of Amp C producers among the isolates, and to find out whether there is an association between antibiotic intake and the emergence of antibiotic resistance. A total of 125 Pseudomonas aeruginosa isolates were obtained, majority were from respiratory samples. Most of the isolates (90%) were susceptible to Carbapenems. Among the 125 isolates, 110 screened positive as potential Amp C producers. Plasmid mediated Amp C production was confirmed by Boronic acid disk test whereas inducible Amp C was confirmed by disk antagonism test using Ceftazidime and the inducer, Imipenem. Out of the 110, 85 were confirmed to be Amp C producers and majority (81) were inducible Amp C producers and only 4 showed plasmid mediated Amp C production. A statistically significant association between antibiotic resistance and antibiotic usage was demonstrated. Hence steps to prevent inadvertent use of antibiotics should be taken to cut down the problem of resistance.
Keywords: *Pseudomonas aeruginosa; plasmid mediated Amp C; inducible Amp C.*

Introduction

Pseudomonas aeruginosa is the most commonly encountered gram negative bacteria which is not a member of Enterobacteriaceae and is an uncommon member of normal human flora. The organism survives in various environments, in nature as well as in hospital. It is seen in antiseptics, bed pan, respirators, endoscopes in hospital environment and is a potential source of nosocomial infections. Pseudomonas aeruginosa causes a wide spectrum of diseases ranging from superficial skin infections to fatal sepsis[(1)]

Drug resistance poses a therapeutic problem not only in the hospital settings, but also in the community as most of the bacteria have acquired resistance to multiple antibiotics. The increasing

frequency of drug resistance over these years has been attributed to combinations of microbial characteristics, bacterial selection pressure due to overuse of antibiotics and societal and technological changes that enhance the transmission of drug resistance

Pseudomonas shows different types of drug resistance. It shows intrinsic resistance to Sulfonamides, Trimethoprim, Tetracyclines, and Chloramphenicol, the mechanism being lack of uptake. Acquired mechanisms of resistance include enzymatic destruction of antibiotics by beta lactamases, altered antibiotic targets, decreased intracellular uptake and increased cellular efflux of the drug. Amongst these, production of beta lactamases is the most common mechanism of resistance. Pseudomonas producing multiple beta lactamases are also not uncommon. The failure to detect this pose a significant challenge in two ways – therapeutic failure and spread of resistance.

The primary cause of resistance to beta lactams is the production of enzymes– beta lactamases, that cleave the amide bond of beta lactam and destroy the antibiotic. The β-Lactamases are encoded either by chromosomal genes or by transferable genes located on plasmids and transposons. Besides, β-lactamase genes (bla) are frequently present on integrons, which often carry multiple-resistance determinants

Amp C beta lactamases are clinically important Cephalosporinases that mediate resistance to Cephalosporins, Penicillins, beta lactamase inhibitors but not to Carbapenems and Cefepime. The major mechanisms of Amp C production described are – chromosomal/inducible, and plasmid mediated/transmissible.

Amp C belongs to Ambler class C, while in functional classification of Bush et al. they were assigned to group 1. The first bacterial enzyme reported to destroy penicillin was the Amp C β-lactamase of Escherichia coli, although it had not been named so in 1940. The sequence of the Amp C gene from E. coli was reported in 1981.

The mechanism behind inducible Amp C production is related to various proteins like Amp R and Amp D. Amp R is the protein responsible for repressing transcription of gene for Amp C. The usual load of cell wall degradation products is recycled by Amp D. In presence of Beta lactam antibiotics, the cell wall degradation products are more than that can be handled by Amp D and it interferes with Amp R thus resulting in loss of repression and constitutive production of Amp C enzyme.

Aims and Objectives

1. The susceptibility pattern of Pseudomonas aeruginosa isolates obtained from various clinical samples.
2. The proportion of Amp C producers among the Pseudomonas aeruginosa isolates
3. To find out whether there is an association between antibiotic intake and the emergence of antibiotic resistance.

Various authors from different parts of the world have studied mechanisms of resistance exhibited by Pseudomonas aeruginosa. The study by Tapan Majumdhar et al. from Tripura[2], study by Subbalakshmi Easwaran et al. from Bangalore[3] and the study on burns patients in Iran by Roya Rafiee et al.[4] showed Amp C as the predominant resistance mechanism of Pseudomonas aeruginosa. On the other hand, study conducted on Pseudomonas aeruginosa isolates of France by Pitout et al.[5], Algeria by Meradji et al.[6] and that from ICUs in Punjab[7] showed Carbapenemases as the predominant mechanism.

Relevance of the study

The pre-eminent role of Pseudomonas aeruginosa in hospital infections is due to its resistance to commonly used antibiotics and antiseptics, thereby establishing itself widely in hospitals. These bacteria can survive and multiply if moisture is available, even with minimal nutrients. This extreme adaptability of the organism helps it to survive in equipments like respirators and

endoscopes, bed pans, lotions, ointments, eye drops and stocks of distilled water. A small percent of the population harbors Pseudomonas aeruginosa in the skin of axilla and perineum. Faecal carriage is common following antibiotic intake and hospitalization.

The pathogenesis of Pseudomonas is greatly credited to its ability to develop wide spread resistance to multiple antibiotics and disinfectants in addition to production of a strong array of virulence factors. Major risk factors for these resistance patterns (by beta lactamases) include long term antibiotic exposure with high rates of Cephalosporin and Carbapenem usage, inadequate therapy, prolonged ICU stay, severe illness and instrumentation.[8]

In our lab among the Pseudomonas aeruginosa isolated from clinical samples during the last three years around 45% were resistant to most of the antibiotics like Piperacillin, Piperacillin-Tazobactam and Cephalosporins.

This resistance could be due to any one or combination of mechanisms mentioned above. Routine antibiotic sensitivity testing may not detect the potential inducers of the beta lactamases.

Hence this study is conducted to identify the actual magnitude of the problem of drug resistance among Pseudomonas isolates in our hospital. If required we may consider incorporating screening methods for detection of beta lactamases routinely.

We can also get an idea regarding the risk factors contributing to the resistance patterns seen here. It may help us to formulate an antibiotic policy.

Moreover as resistant isolates have limited treatment options, detection of the above beta lactamases may help to improve the clinical management.

Detection Methods of Amp C

There are no CLSI guidelines for detection of Amp C till date.

a. Screening Test

As per CLSI guidelines, Cefoxitin is not tested for Pseudomonas isolates whereas Ceftazidime is included in the panel. Hence screening was done in our study as per the CLSI guidelines laid down for potential ESBL producers using Ceftazidime disk.

b. Phenotypic Confirmatory Test For Plasmid Mediated AMP C

Since there are no set guidelines for detection of Amp C, different authors have performed their own methods like the inhibitor based methods using Boronic acid derivatives, three dimensional test and its modifications, Amp C disk test. Phenyl boronic acid test was used by many authors for Amp C detection.

A study on "Evaluation of phenotypic tests for the detection of AmpC beta-lactamase in clinical isolates of Escherichia coli" by Deepika Hande in Meerut compared the boronic acid disk test and three dimensional test for Amp C detection. Both tests showed comparable results. Hassan et al. in this study found that the boronic acid disk test method highly sensitive (88%) and specific (92%) for Amp C detection.

Moreover it is a simple phenotypic method that is easy to perform and interpret. Hence we selected the Boronic acid disk test for confirmation of plasmid mediated Amp C.

c. Phenotypic Confirmatory Test For Inducible AMP C

CLSI guidelines are not available for confirmation of inducible Amp C also.

Disk antagonism test was used by many authors and proved useful for detection of inducible Amp C. Different inducers like Imipenem and Cefoxitin were tried to induce Amp C production and both the methods gave comparable results as per the study conducted at Bangalore.[3]

In our study we had used Imipenem as the inducer in the disk antagonism test

Materials and Methods

All the clinical samples for bacteriology culture and sensitivity from inpatients received in the Microbiology laboratory for a duration of 1 year starting from March 2016 were processed immediately as per the lab guidelines.

Sampling method – universal sampling

Ethical clearance was obtained from the Institutional Ethical Committee on 25/01/2016. (IEC 05/16)

Isolates were identified by various biochemical reactions according to standard algorithms. Identification of Pseudomonas aeruginosa was done upto the species level.

1. Antibiotic susceptibility testing

Antibiotic sensitivity testing for all Pseudomonas aeruginosa isolates was done by Kirby Bauer disk diffusion method. The panel of antibiotic disks put up for sensitivity according to CLSI guidelines.[9]

All the antibiotic disks were procured from Microexpress company and stored at 4°c.

Quality control check for antibiotic disks was done using ATCC Pseudomonas 27853

3. Detection of resistance mechanism by Amp C

All the pseudomonas aeruginosa isolates were subjected to the screening test for Amp C production. The isolates which turned out positive for screening test were further subjected to the confirmatory tests for plasmid mediated and inducible Amp C production.

a. Screening test

Isolates with zone size of Ceftazidime less than 22mm and that of Cefotaxime less than 27mm were considered as potential Amp C producers.[9]

They were subjected to confirmatory test for Amp C production (both inducible and plasmid mediated)

b. Confirmatory test for plasmid mediated or transmissible Amp C

Test done by: Boronic acid disk test method.

Test method: Disk diffusion

Medium: Mueller Hinton Agar

Inoculum: Turbidity of inoculum was matched to 0.5 McFarland standard. (standard disc diffusion recommendations)

Antibiotic disks used: Ceftazidime disks(30µg). The antibiotic disk was obtained from Microexpress company and stored at 4°C.

Reagent used: Freshly prepared phenyl boronic acid. This was prepared by dissolving 0.15g of phenyl boronic acid powder in 10ml of distilled water. It was then autoclaved at 121°c for 15 minutes at 15lbs pressure. Phenyl boronic acid was procured from Hi Media, Mumbai, in powder form and stored in dark at room temperature.

Incubation conditions: 35°c +/- 2°c, ambient air

Incubation length: 16-18 hours

Principle: Phenyl boronic acid inhibits Amp C production.

Method: The prepared inoculum of the isolate was swabbed onto Mueller Hinton Agar plate and two Ceftazidime disks (30µg) were placed 25mm apart. After that 20µl of freshly prepared phenyl boronic acid was added (with sterile precautions) to one of the disks and the plate was incubated.[10,11]

Interpretation An isolate was confirmed to be an Amp C producer if it showed 5mm or more increase in zone diameter for Ceftazidime disk with phenyl boronic acid versus the Ceftazidime disk alone.

c. Confirmatory test for inducible/chromosomal Amp C

Test done: Disk antagonism test.

Test method: Disc diffusion

Medium Mueller Hinton Agar

Inoculum: Turbidity of inoculum was matched with 0.5 McFarland standards. (standard disc diffusion recommendations)

Antibiotic disks used: Ceftazidime (30µg), Imipenem (10µg). The antibiotic disks were procured from Microexpress company and stored at 4°C.

Incubation conditions: 35°c +/- 2°c, ambient air

Incubation length: 16-18 hours

Principle: On exposure to an inducer beta lactam antibiotic (Imipenem used here), the normally repressed gene for beta lactamase enzyme expresses to a high level which is sufficient to produce significant resistance.

Method: The prepared inoculum of isolate was swabbed onto Mueller Hinton Agar plate. A Ceftazidime disk (30µg) and an Imipenem disk (10µg) were placed at a distance of 20mm apart and incubated.

Interpretation: An isolate was confirmed to be an inducible Amp C producer if a blunting of zone for Ceftazidime was observed adjacent to Imipenem disk.

Data collection and entry

Clinical data and details regarding antibiotic usage were collected from clinical case records and entered into the proforma. The same were numerically coded and entered into Microsoft Excel spread sheet.

The identification of the Pseudomonas aeruginosa isolates, susceptibility of the same to each antibiotic, the results of screening and confirmatory tests for the mechanism of resistance were also coded and entered into the excel spread sheet.

Data analysis

The data entered in the excel spread sheet was analyzed using Statistical Package for Social Sciences (SPSS) software 16.0. Qualitative variables were summarized using frequency or percentage. Chi square test was used in the analysis of study variables. The level of statistical significance was taken as p value <0.05.

Results

A total of 125 Pseudomonas aeruginosa isolates were obtained.

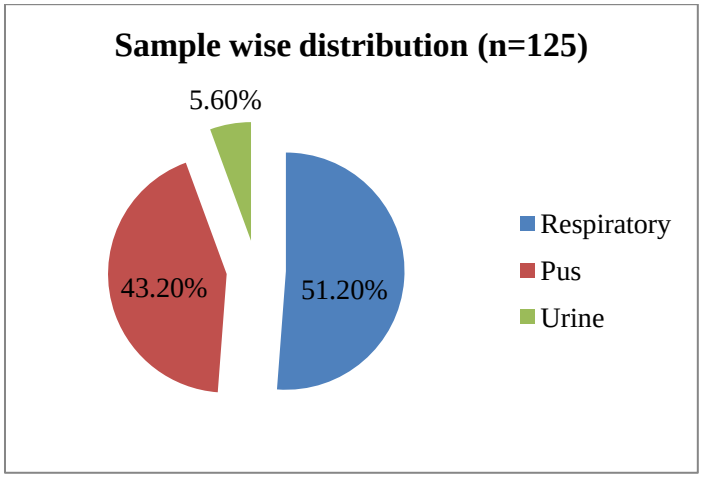

Fig 1 – sample wise distribution of Pseudomonas aeruginosa

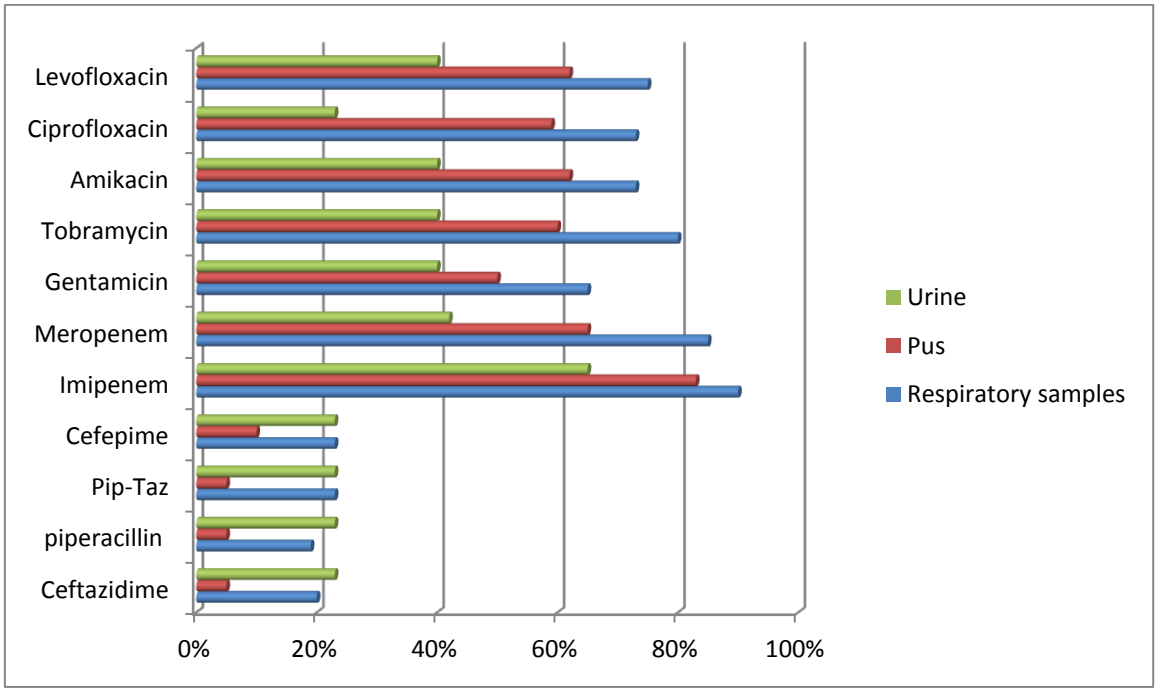

Fig 2 – susceptibility pattern of Psudomonas aeruginosa isolates

Majority of the isolates (90%) were susceptible to Imipenem followed by Meropenem, but resistant to beta-lactams and beta-lactam/beta-lactamase inhibitor combinations.

Resistance mechanisms of Pseudomonas aeruginosa

A total of 110 Pseudomonas aeruginosa isolates were found to be positive in the screening test which were then subjected to confirmatory tests. Out of which 81 (64.8%) were found to be inducible Amp C producers and 4 (3.2%) were plasmid mediated Amp C producers. The remaining 25(20%) isolates which showed resistance might be due to other mechanisms like ESBL production, porin loss, increased membrane permeability and so on.

Inducible Amp C was the major mechanism of resistance.

Table 3: Antibiotic Usage and Resistance

Mechanism of resistance	Cephalosporin	Aminoglycoside	Fluoroquinolone	Multiple antibiotics including cephalosporins	Penicillin	others	Total
Plasmid AmpC	1	0	0	3	0	0	4
Inducible AmpC	30	1	1	46	1	2	81

68.4% of the patients on Cephalosporins either as monotherapy or in combination with others had been found to develop inducible Amp C.

Chi square test was applied and the p value obtained was .009 (< .05)

Hence the association between emergence of inducible Amp C& antibiotic intake was found to be statistically significant.

Antibiotic Usage and Resistance

Mechanism of resistance	Cephalosporin	Aminoglycoside	Fluoroquinolone	Multiple antibiotics including cephalosporins	Penicillin	others	Tot
Plasmid AmpC	1	0	0	3	0	0	4
Inducible AmpC	30	1	1	46	1	2	81

Table 1: antibiotic use and resistance

Discussion

Sample Wise Distribution of Pseudomonas aeruginosa

Our study yielded maximum isolates of Pseudomonas aeruginosa from respiratory samples followed by pus samples. A smaller quantity of isolates were obtained from urine sample.

The studies from inside and outside India varied in the distribution of the Pseudomonas isolate from various clinical samples. The study at New Delhi[12] had obtained the maximum number of isolates from respiratory samples.

Susceptibility Pattern of Pseudomonas aeruginosa

Our results were comparable with other studies done by Bose et al.[13] in a rural area of Central India and study done by Tapan Majumdar et al[2]. which also showed maximum susceptibility to Imipenem and least susceptibility to Cephalosporins.

Low susceptibility of Ceftazidime and Cefepime was matching with study done by Vinita Rawat et al. "Detection of different beta lactamases and their co-existence by using various disk combination methods" in india. [14]

Mechanism of Resistance

Our study results were in agreement with the results of the study of Tapan Majumdar et al.[2] from Tripura which showed 76% inducible Amp C, 12% plasmid mediated Amp C production in Pseudomonas. Study conducted in Bangalore by Subbalakshmi Easwaran et al. [3] also showed a higher percentage of inducible Amp C than plasmid mediated which was in accordance with our study. Study from burns patients in Iran by Roya Rafiee et al.[4] also showed a predominance of Amp C (68.6%) in Pseudomonas.

In the study conducted in Iran by Davood Kalanthar, 65% were MBL producers. Study from Algeria by Meradji et al. showed 18.75% Carbapenem resistant strains. Pitout et al. from France in their study had 46% MBL producers. [5,6]

In India, studies from Varnasi had 45.5% isolates which showed combination of Amp C and MBL as the predominant mechanism of resistance.[15] The study conducted in ICUs of Punjab revealed MBL (26.6%) as the major resistance mechanism [7]. The predominant mechanisms of resistance in a study from Aligarh[16] was found to be ESBL (59.4%). A study conducted from different parts of India showed that ESBL (45.6%) was the major resistance mechanism in Pseudomonas aeruginosa. [17]

Since studies at various places showed differences in the prevalence of the resistance mechanisms in Pseudomonas, it is essential that we conduct a study at our place to find out the type and prevalence of resistance among the isolates.

Antibiotic Usage and Resistance

There is a paucity of studies undertaken to find out statistically significant association between antibiotic intake and resistance mechanism among Pseudomonas isolates.

We have found a significant association between antibiotic intake and resistance mechanism. Hence we have to discourage long term antibiotic use especially Cephalosporins while formulating antibiotic policy.

The study on "Epidemiology and risk factors for infections due to Amp C beta lactamase producing E coli" by Vanesa Pascual et al. in Spain found a statistically significant association between previous intake of Fluoroquinolones and Amp C resistance mechanism.[18]

Our study tried to find an association between fluoroquinolone intake and emergence of resistance and a p value of 0.05 was obtained. Since this is a border line value, we may have to include more number of Pseudomonas isolates before finding an association between emergence of resistance and Fluoroquinolone therapy.

Limitations

The study was conducted based on phenotypic methods alone. Each method has its own shortcomings leading to false positive and false negative results

The simultaneous expression of multiple mechanisms by Pseudomonas like enzymatic alteration, decreased intracellular uptake, increased cellular efflux of the drug, altered target sites also adds to the confusion.

These could be overcome by comparing the results with a gold standard method like PCR

Conclusion

Majority of the Pseudomonas isolates (90%) were susceptible to Carbapenems.

The majority (64.8%)of the Pseudomonas aeruginosa isolates were inducible Amp C producers.

The association between antibiotic intake and emergence of resistance was found to be statistically significant for Pseudomonas aeruginosa.

Sources of support – nil

References

1. Patricia M Tille. Bailey and Scott's diagnostic microbiology. 13th ed. South Dakota: Elsevier; 2014. 336-339 p.
2. Tapan Majumdar, Shibabrata Bhattacharya, and Raunak Bir. Prevalence

of Extended Spectrum β-Lactamase and AmpC β-Lactamase among Enterobact-eriaceae and Pseudomonadaceae Isolated at Tertiary Care Set up in Tripura, India. RRJMB. 2014 Jun;3(2):19–26.

3. Subbalakshmi Easwaran, Ranjani Chittur Yerat Rajendran Ramaswamy,. A study on detection of extended-spectrum beta-lactamases (ESBLs) and comparison of various phenotypic methods of AmpC detection in Pseudomonas aeruginosa from various clinical isolates in a tertiary care teaching hospital. Muller J Med Sci Res. 2016;7:35–9.
4. Roya Rafiee,Fereshteh Eftekhar,Seyyed Ahmad Tabatabaei, Dariush Minaee Tehrani. Prevalence of Extended-Spectrum and Metallo β-Lactamase Production in AmpC β-Lactamase Producing Pseudomonas aeruginosa Isolates From Burns. Jundishapur J Microbiol. 2014 Sep;7(9).
5. Pitout JDD, Gregson DB, Poirel L, McClure J-A, Le P, Church DL. Detection of Pseudomonas aeruginosa Producing Metallo-β-Lactamases in a Large Centralized Laboratory. J Clin Microbiol. 2005 Jul 1;43(7):3129–35.
6. S Meradji, B Abouddihaj, Z Khalid, D Mazouz, H Chettibi, N Elmdaghri, M Timinouni . Epidemiology of carbapenem non-susceptible Pseudomonas aeruginosa isolates in Eastern Algeria. Antimicrobial resist and Infection Control.2015;4;27
7. Loveena OberoiNachhatarjit singh, Poonam Sharma, Aruna Aggarwal. ESBL,MBL and Ampc β Lactamases Producing Superbugs – Havoc in the Intensive Care Units of Punjab India. J Clin Diagn Res.2013Jan;7(1):70-73
8. P.N.A. Harris,J.K. Ferguson. Antibiotic therapy for inducible AmpC Beta lactamase-producing Gram-negative bacilli: what are the alternatives to carbapenems, quinolones and aminoglycosides? International Journal of Antimicrobial Agents. 2012;40:297–305.
9. CLSI. Perforamnce standards for antimicrobial susceptibility testng.26th ed. CLSI supplement M100. Wayne, PA:Clinical and Laboratory Standards Institute; 2010.
10. Pandey A, Rawat A, Handa S, Thakuria B, Handa D, Asthana A. Evaluation of phenotypic tests for the detection of AmpC beta-lactamase in clinical isolates of Escherichia coli. Indian J Pathol Microbiol. 2013;56(2):135.
11. Hassan A, Usman J, Kaleem F, Gill MM, Khalid A, Iqbal M, et al. Evaluation of different phenotypic methods for detection of Amp C Beta-lactamase producing bacteria in clinical isolates. J Coll Physicians Surg Pak. 2013;23(9):629–32.
12. Behera B, Mathur P, Das A, Kapil A, Sharma V An evaluation of four different phenotypic techniques for detection of metallo-β-lactamase producing Pseudomonas aeruginosa .IJMM 2008;26(3):233-237
13. Bose S,Mallick SK BS Khodke M. Inducible AmpC Beta-Lactamase Producing Pseudomonas Aeruginosa Isolated In A Rural Hospital Of Central India. Journal of Clinical and Diagnostic Research. 2009 Dec;3:1921–7.
14. Vinita Rawat, Monil Singhai, Pankaj Kumar Verma. Detection of Different β-Lactamases and their Co-existence by Using Various Discs Combination Methods in Clinical Isolates of Enterobacteriaceae and Pseudomonas spp. Journal of Laboratory Physicians. 2013 Jun;5(1):21–5.
15. Kumar V, Sen M R, Nigam C, Kumari S . Burden of different beta-lactamase classes among clinical isolates of AmpC-producing Pseudomonas aeruginosa in burn patients: A prospective study. Indian

Journal of Critical Care Medicine. 2012 Jul;16(3):136-140

16. Mehvash Haider, Meher Rizvi, Nazish Fatima, Indu Shukla, Abida Malik. Necessity of detection of extended spectrum beta-lactamase, AmpC and metallo-beta-lactamases in Gram-negative bacteria isolated from clinical specimens. Muller J Med Sci Res. 2014 Jun;5(1):23–8.
17. Chaudhary M, Payasi A. Rising Antimicrobial Resistance of Pseudomonas aeruginosa Isolated from Clinical Specimens in India. Journal of Proteomics & Bioinformatics. 2013;6:005-009
18. Vanesa Pascual,Gabriel Ortiz,Maria Simo,Noemı´ Alonso,Javier Garau,Esther Calbo. Epidemiology and risk factors for infections due to AmpC b-lactamase-producing Escherichia coli. J Antimicrob Chemother. 70:899–904.