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Comparative analysis of detection methods of Extended Spectrum Beta Lactamases in Gram Negative clinical isolates with special reference to their Genotypic Expression

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Abstract:

Antibiotic resistance in gram negative bacteria is increasing worldwide in both outpatients as well as hospitalized patients. Most bacterial isolates carry resistance determinants for extended spectrum beta lactamases (ESBL) production on plasmids that can easily spread from organism to organism. This study was conducted to compare the rate of detection of ESBL positive organisms by different methods, to use the clinical and laboratory standard institute (CLSI) detection methods for detecting ESBLs in bacteria other than Enterobacteriaceae and genotypic characterization of these ESBL strains. A total of 100 non repetitive gram negative isolates, which were resistant to ceftriaxone, cefotaxime or ceftazidime were only included in the study. All the 100 screen test positive isolates were tested for ESBL production using the different phenotypic detection methods. Molecular characterization of the strains was done at the Rajiv Gandhi Centre for Biotechnology, Trivandrum. Among total 100 gram negative isolates which were 3rd generation cephalosporin resistant, 79(79%) were ESBL positive by using CLSI phenotypic confirmatory test. Of this 79%, majority of isolates were detected in blood samples (38%). Of the different methods employed, E-test detected additional 3% and 11% ESBL positive *Escherichia coli* and *Klebsiella pneumoniae* respectively. CLSI phenotypic confirmatory test also detected ESBL positive *Pseudomonas sp*, *Acinetobacter sp* and *Enterobacter cloacae*. The genotype characterization of 52 isolates showed 29 with CTX-M and 13 with TEM genes. Thus, the study shows a significant rate of ESBL production in gram negative bacteria emphasizing the need for enhanced infection control and antibiotic stewardship programs to limit the spread of these organisms.

Key words: ESBL, Gram negative bacteria, E-test, Genotype.

Introduction:

Extended spectrum beta lactamases (ESBLs) are beta-lactamases that hydrolyze extended-spectrum cephalosporins with an oxyimino side chain. These cephalosporins include cefotaxime, ceftriaxone, and ceftazidime, as well as the oxyimino-monobactam aztreonam[1, 2] .The majority of ESBL producing strains are *Klebsiella pneumoniae*, *Klebsiella oxytoca* and *Escherichia coli* (*E.coli*). They have also been found in *Pseudomonas aeruginosa* and other Enterobacteriaceae strains like *Enterobacter spp*, *Citrobacter spp*, *Proteus spp*, *Morganella morganii*, *Serratia marcescens*, *Salmonella spp*. and *Shigella dysenteriae*.[2]

The problems which are associated with ESBLs include multi-drug resistance, difficulty in detection and treatment and increased mortality. These resistant bacteria are associated with severe infections such as bacteraemia, intra-abdominal infections, urinary tract infections (particularly in the community setting) and respiratory tract infections. Thus the present study was conducted to compare the rate of detection of ESBL positive organisms by different methods and genotypic characterization of the ESBL strains. Even though, clinical and laboratory standard institute (CLSI)

screening and confirmatory tests apply only to *Escherichia coli*, Klebsiella spp. and *Proteus mirabilis*, we have used this method to detect ESBLs in other gram negative bacteria also.

Materials and Methods:

A total of 100 non repetitive isolates obtained from clinical specimens of the patients admitted in Government Medical College, Kozhikode were included in the study. The study was conducted from March 2011 to February 2012 for a period of one year. Specimens such as pus aspirate, blood, cerebrospinal fluid, sputum, urine, pleural fluid, corneal scrapings and ascitic fluid were included.

Inclusion criteria: Gram negative bacteria which were resistant to1st, 2nd and 3rd generation cephalosporins.

Exclusion criteria: All gram negative bacteria sensitive to 3rd generation cephalosporin.

Identification and Detection of ESBL: Identification of microorganisms was performed according to standard procedures[3]. Gram negative isolates were screened for potential ESBL producers by Kirby-Bauer disc diffusion method on Mueller-Hinton Agar (MHA). Isolates that showed resistance to one or all of the following antibiotics were considered as potential ESBL producers and were only included in the study (**Table I**). All resistance breakpoints were according to clinical and laboratory standard institute (CLSI) guidelines (document M100-S20).[4]
Confirmation of ESBL production: All 100 screen positive isolates were then subjected for confirmatory test by CLSI phenotypic confirmatory test (cephalosporin/clavulanate combination disc test) (PCT)-(**Figure I**), double disc diffusion test (DDST)-(**Figure II**) and E-test-(**Figure III**). E-test was done by using E-test strips, one end of strip contains a gradient of ceftazidime(TZ) (minimum inhibitory concentration(MIC) test range of 0.5 to 32μg/ml) & the other end with a gradient of ceftazidime plus a constant concentration of clavulanate(TZL) (4μg/ml), manufactured by biomerieux. The presence of ESBL is confirmed by the appearance of a phantom zone or deformation of ceftazidime (TZ) ellipse (Figure IV) or when either the MIC of TZ is reduced by ≥3 $\log_2$dilutions in the presence of clavulanic acid.
Molecular characterizations of ESBLs by PCR: Molecular characterization of 52 isolates, determined as ESBL positive by phenotypic confirmatory test, were done at the Rajiv Gandhi Centre for Biotechnology, Trivandrum. Polymerase chain reaction was carried out to detect the presence of the most common ESBL genes-*bla*TEM (bla-beta lactamase) and *bla*CTX-M. PCR was done in Eppendorf Master Cycler, Gradient (Eppendorf, Germany).

Table I: ESBL screen test (CLSI Recommended)

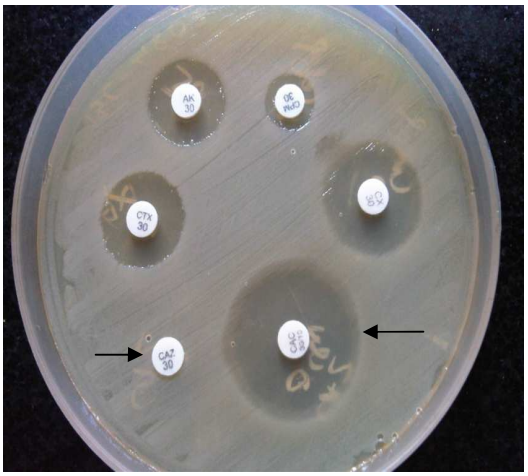
Antibiotic agent	Disc content	Resistant
Ceftazidime	30 µg	≤ 22 mm
Cefotaxime	30 µg	≤ 27 mm
Ceftriaxone	30 µg	≤ 25 mm

Results:

Maximum number of patients was in the elderly age group of >50yrs (47%) and majority were males (54%). Of the 100 study samples, majority were cases of sepsis (24%). 21% of cases were urinary tract infections while abscess contributed to 12% of cases. Isolates from blood samples constituted 39% followed by urine (22%). Of the 22 urine isolates, 20 were ESBL producers (90%), while only 30 (76%) out of 39 blood isolates were ESBL producers. Of this 20 ESBL positive urine isolates, 13 (65%) were from catheterized samples.
Of the 100 ESBL screen positive isolates, 79 isolates were detected as ESBL

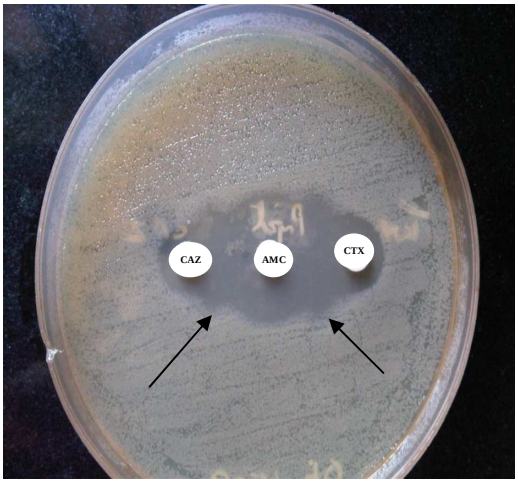
producers by CLSI phenotypic confirmatory test.

Figure I: Combination disc test



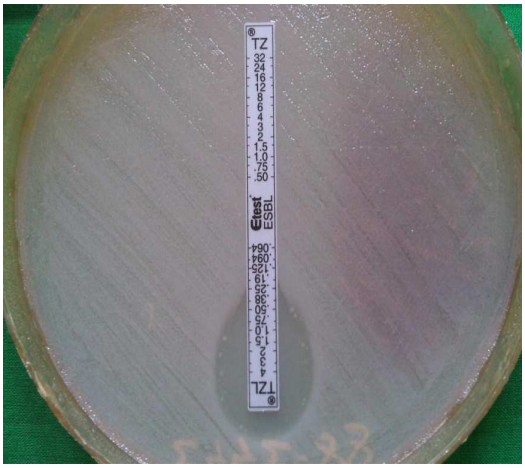
Ceftazidime (CAZ) → 6 mm &
Ceftazidime+ Clavulanate(CAC)→24 mm
(≥ 5mm increase in zone diameter for the disc in the presence of clavulanate)

Figure II: Double Disc Diffusion Test (DDST) for ESBL detection



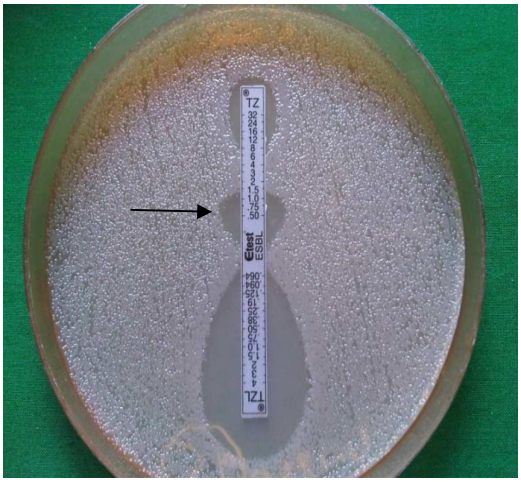
CAZ – Ceftazidime
AMC – Amoxycillin + Clavulanate
CTX – Cefotaxime
(A clear extension of the edge (Synergy) of the CAZ/CTX inhibition zone towards the disc containing clavulanate.)

Figure III: E-Test for the detection of ESBL



MIC Of Ceftazidime (TZ)→32
MIC Of Ceftazidime+Clavulanate (TZL)→0.25
(MIC of TZ is reduced by ≥ 3 $\log_2$ dilutions in the presence of clavulanic acid.)

Figure IV: E-Test - Phantom zone indicative of ESBL



Microbial profile: Of the total 79 ESBL positive isolates, 32 were *E.coli* which is 40.5% of ESBLs. Percentage of *Klebsiella pneumoniae* producing ESBL was 34.1%. **Table II** shows the percentage detection of ESBL positive isolates.

Table II: Organisms Positive for ESBL production

Organism	Total Number	ESBL Positive
Acinetobacter baumanii	5	5
E.coli	37	32
Enterobacter cloacae	6	4
Klebsiella pneumoniae	36	27
Pseudomonas aeruginosa	13	8
Proteus mirabilis	3	3
Total	**100**	**79**

Comparison of different methods of ESBL detection: E-test detected additional 3% ESBL positive *E.coli* and 11% ESBL positive *Klebsiella pneumoniae* when compared to combination disc method. Additional 17% ESBL positive *Enterobacter cloacae* were also detected by E-test compared to combination disc method. Both methods were equal in detection in case of *Proteus mirabilis*. Combination disc method was superior in detection in case of *Pseudomonas aeruginosa* and *Acinetobacter baumanii*. **Table III** shows the percentage detection of ESBL positive isolates by different techniques.

Molecular characterization of ESBLs: Fifty two clinically significant, phenotypically ESBL positive isolates were tested by PCR, to detect the presence of *bla*TEM and *bla*CTX-M genes. The most common genotype found was CTX-M. **Table IV** shows the percentage of ESBL genotypes detected.

Resistance genes in different organism: Out of 23 *E.coli* tested, 17 (73.9%) isolates showed the presence of CTX-M gene. 5(21.7%) isolates showed the presence of TEM gene. CTX-M gene predominated (71.4%) in *Klebsiella pneumoniae* also. One (16.6%) *Pseudomonas aeruginosa* showed the presence of TEM gene. Both CTX-M & TEM genes were not isolated in *Acinetobacter baumanii* and *Proteus mirabilis.* **Table V** shows the percentage detection of resistance genes in different organisms.

Discussion:

Understanding the impact of drug resistance is of critical importance as the changing trend of antibiotic resistance has a large impact on the empirical therapy of different infections[5]. Antibiotic resistance varies according to geographic locales and is directly proportional to the use and misuse of antibiotics. The prevalence of ESBL production among Enterobacteriaceae varies greatly from country to country and among the institutions within the country.

Table III: Comparison of different methods of ESBL detection

Organism	Combination Disc Method(PCT)(%)	DDST(%)	E-Test(%)	Total
E.coli	32(86.4)	23(62.1)	33(89.1)	37
Klebsiella pneumoniae	27(75)	23(63.8)	31(86.1)	36
Pseudomonas aeruginosa	8(61.5)	1(7.6)	2(15.3)	13
Enterobacter cloacae	4(66.6)	3(50)	5(83.3)	6
Acinetobacter baumanii	5(100)	0(0)	1(20)	5
Proteus mirabilis	3(100)	2(66.6)	3(100)	3
Total	**79(79)**	**52(52)**	**75(75)**	**100**

Table IV: Molecular Characteristics

Genes	Number of Isolates	Percentage (%)
CTX-M Only	19	36.5
TEM Only	3	5.8
CTX-M & TEM	10	19.2
None	20	38.5
Total	**52***	

*29 out of 52 isolates showed the presence of *bla*CTX-M (55.7%). 13 isolates were positive for *bla*TEM(25%). *bla*CTX-M predominated in the isolates.

From our study, ESBLs detected among 3rd generation cephalosporin resistant gram negative bacteria was found to be 79%. High percentage of ESBL s were reported by Jain Amitha et al (86%) [6] and Rodrigues et al (53%) [7]. Of the total 79 ESBL positive isolates majority was from blood sample (38%) followed by urine (25%). Of the 22 urine isolates, 20 were ESBL producers (90%). Of this 20 ESBL positive urine isolates, 13 (65%) were from catheterized samples. In a study by Khalid Mubarak et al, urine was found to be the major source of ESBL isolates[8]. Kader et al have reported a high incidence of ESBL production among catheterized patients.[9]

Microbial profile: In the present study, 40.5% of ESBL producers were E.coli while ESBL positive *Klebsiella pneumoniae* was 34.1% (**Table II**). However, most of the past studies have shown ESBL producing Klebsiella strains to be more prevalent than ESBL producing *E.coli.* In accordance with our study, similar results were obtained by Ananthakrishnan et al [10] (*E.coli* 56.2%, *Klebsiella pneumoniae* 21.8%) and B Jorn Blomberg et al[11] (*E.coli* 25%, *Klebsiella pneumoniae* 17%) with highest percentage of ESBLs among *E.coli*.

ESBL production has also been found in other members of the Enterobacteriaceae family [12]. CLSI guidelines which are only for *E.coli*, *Klebsiella pneumoniae*, and *Proteus mirabilis*, were extended for other gram negative bacteria in the present study. We observed ESBL production in *Pseudomonas aeruginosa* (10.1%), *Acinetobacter baumanii* (6.3%), *Enterobacter cloacae* (5%) and *Proteus mirabilis* (3.7%) also (**Table II**). In *Pseudomonas aeruginosa*, ESBL production is less as compared to Enterobacteriaceae, because their resistance is mediated by various other mechanisms such as the production of metallo-betalactamases, lack of drug penetration due to mutations in the porins and the loss of certain outer membrane proteins and efflux pumps.[13,14]

Different methods of ESBL detection: In the present study, presence of ESBLs was determined with CLSI phenotypic confirmatory test (PCT), double disc diffusion test (DDST) and E-test (Table-III). PCT detected 79 (79%) isolates, DDST detected 52 (52%) isolates and E-test detected 75(75%) isolates. Thus a higher rate of ESBL detection was found with CLSI phenotypic confirmatory test.

In this study, for *E.coli*, PCT detected 86.4% of ESBLs. DDST detected 62.1% of ESBLs (Table-III). Additional 24% cases were detected by CLSI phenotypic confirmatory test than DDST method. In *Klebsiella pneumoniae*, PCT detected 75% of ESBLs. DDST detected 63.8% of ESBLs. Additional 12 % cases were detected by CLSI phenotypic confirmatory test than DDST method. Thus in this study, it was observed that ESBL production among *E.coli* and *Klebsiella pneumoniae* isolates were more frequently detected by the combination disc (PCT) method than the double disc approximation test. The clinical laboratory standards institute (CLSI) also recommends the use of the combination disc method for the phenotypic confirmation of ESBL production among Enterobacteriaceae[4].Umadevi S et al from Pondicherry also observed in their study that ESBL production among *E. coli* and

Klebsiella pneumoniae isolates were more frequently detected by the combination disc method than the double disk approximation test.[15]

E-test detected additional 3% and 11% of ESBL cases of *E.coli* and *Klebsiella pneumoniae* respectively than CLSI phenotypic confirmatory test (**Table III**). In a study by Martin G. Cormican, Steven A. Marshall and Ronald N. Jones, similar results were obtained with higher sensitivity for E-test for ESBL detection in *E.coli and* Klebsiella *sp.*[16]

In the present study, PCT was superior in detection of ESBLs in case of *Pseudomonas aeruginosa* and *Acinetobacter baumanii* than DDST and E-test (**Table III**). However, CLSI recommends the double disc approximation method for testing ESBL production among the *Pseudomonas aeruginosa* isolates.[4] Failure to detect the better performance of the double disc approximation test as compared to the combination disc method for the detection of ESBL production among the *Pseudomonas aeruginosa* in this study could be due to the relatively small number of isolates which were tested. Even though, the phenotypic confirmatory tests are also highly sensitive and specific, there are a number of instances when these tests may be falsely positive or falsely negative. False positives may occur if the isolate lacks ESBL but produces an excess of TEM-1 or SHV-1. On the other hand, isolates harbouring both ESBLs and AmpC type β-lactamases may result in false-negative results.

Molecular characterization of ESBLs: Of the 52 ESBL positive isolates subjected for polymerase chain reaction to look for the presence of blaTEM and blaCTX-M genes, only 32 (61.5%) could be typed for one or more genes (**Table IV**). The negative amplification in the remaining isolates may be due to presence of other ESBL genes, which were not explored further. Twenty nine isolates (55.7%) were positive for CTX-M gene and thirteen isolates (25%) positive for TEM gene. Ten isolates showed the presence of both CTX-M and TEM genes. The blaCTX-M was the most common and was present either alone or in combination with other ESBL type(s). These findings support the hypothesis that CTX-M is emerging as the dominant ESBL type in clinical isolates.[3] Pournaraset al[17] reported 87 % prevalence of CTX-M enzyme among ESBL producers in a tertiary care hospital of Greece. Data from the last 10 years establishes CTX-M genotype as the predominant ESBL in Europe and East Asia. Another study by Jemima and Varghese from Chennai found that 15.83% of ESBL positive isolates had blaCTX-M gene[18].They also noted that among those CTX-M producing isolates, CTX-M-1gene was positive in 47.3% isolates.

Table V: Resistance genes in different organisms

Organism	PCR				Total
	Only CTX-M	Only TEM	Both CTX-M & TEM	None	
Acinetobacter baumanii	0	0	0	3	3
E.coli	13	1	4	5	23
Enterobacter cloacae	1	0	1	2	4
Klebsiella pneumoniae	5	1	5	3	14
Pseudomonas aeruginosa	0	1	0	5	6
Proteus mirabilis	0	0	0	2	2
Total	19	3	10	20	52

Of the 23 *E.coli* tested in this study, 17(73.9%) isolates showed the presence of CTX-M gene, 5(21.7%) isolates showed the presence of TEM gene (**Table V**). Of the 14 *Klebsiella pneumoniae* tested, 10(71.4%) isolates showed the presence of CTX-M genes, 6(42.8%) isolates showed the presence of TEM gene. One(16.6%) *Pseudomonas aeruginosa* showed the presence of TEM gene. Both CTX-M & TEM genes were not isolated in *Acinetobacter baumanii* and *Proteus mirabilis*. Sekhar et al reported that 44.4% of *E. coli* and 35.29% of *K. pneumoniae* strains were found to be positive for blaCTX-M gene by PCR.[19]

The drawback of the present study is that the sample size was small and we were unable to do molecular characterization for all the isolates.

Conclusions:

The rise of antibiotic resistance emphasizes the importance of judicious use of antibiotics. Although infection control procedures continue to play a central role, changes in antibiotic policy may play an even greater role in this setting. The regular detection of ESBLs by standard detection methods should be carried out in every lab where molecular methods cannot be performed, as genotyping is not more informative for the treatment. Patients' culture and sensitivity report must state that the isolate is a suspected or proven ESBL producer and also include a note stating that ESBL production may predict therapeutic failure with penicillins, aztreonam and all cephalosporins (except cephamycins), irrespective of their in vitro susceptibility.

References:

1. Bush K, Jacoby GA, Medeiros AA. A functional classification scheme for beta-lactamases and its correlation with molecular structure. Antimicrob Agents Chemother 1995; 39:1211-33.

2. Bradford PA. Extended Spectrum beta lactamases in 21st century: Characterization, epidemiology and detection of this important resistance threat. Clin Microbiol Rev 2001; 14 (4): 933-951; http://dx.doi.org/10.1128/CMR.14.4.933-951.2001

3. Paterson DL, Bonomo RA. Extended Spectrum Beta–lactamases: a clinical update. Clin Microbiol Rev 2005; 18:657-86; http://dx.doi.org/10.1128/CMR.18.4.657-686.2005

4. Clinical Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing; 20th informational supplement ed. CLSI document M100-S20. Wayne, PA: CLSI; 2010.

5. Gales AC, Jones RN, Gordon KA, Sader HS, Wilke WW, Beach ML, et al. Activity and spectrum of 22 antimicrobial agents tested against urinary tract pathogens in hospitalized patients in Latin America: report from the second year of the SENTRY Antimicrobial Surveillance Program (1998). J Antimicrob Chemother 2000; 45:295-303; http://dx.doi.org/10.1093/jac/45.3.295

6. Jain A, Roy I, Gupta MK, Kumar M and Agarwal SK. Prevalence of extended spectrum beta-lactamase producing gram negative bacteria in septicaemic neonates in a tertiary care hospital. J Med MicrobioL 2003; 52:421-25.

7. Rodrigues C, Joshy P, Jani SH, Alphonse M, Radhakrishnan R, Mehta A. Detection of beta-lactamases in nosocomial gram negative clinical isolates. Indian J Med Microbiol 2004; 22: 247-50.

8. Bindayna KM, .Senok AC, Jamsheer AE. Prevalence of extended spectrum beta-lactamase producing Enterobacteriaceae in Bahrain. Journal of Infection and Public Health 2009; 2: 129-135; http://dx.doi.org/10.1016/j.jiph.2009.04.005

9. Kader AA, Angamuthu K. Extended-spectrum beta- lactamases in urinary isolates of *Escherichia coli*, *Klebsiella pneumoniae* and other gram-negative bacteria in a hospital in Eastern province, Saudi Arabia. Saudi Med J 2005; 26: 956-59.

10. Ananthakrishnan AN, Reba Kanungo, Kumar A, Badrinath S. Detection of extended spectrum beta-lactamase producers among surgical wound infections and burns patients

in JIPMER. Indian J Med Microbiol 2004; 18(4): 160-165.
11. Blomberg B , Jureen R, Manji KP, Tamim BS, Mwakagile DS, Urassa WK. et al. High rate of fatal cases of paediatric septicaemia caused by Gram negative bacteria with Extended Spectrum Beta-lactamases in Dar Es Salaam, Tanzania. J Clin Microbiol 2005; (43):745-49; http://dx.doi.org/10.1128/JCM.43.2.745-749.2005
12. Navon-Venezia S, Hammer-Munz O, Schwartz D, Turner D, Kuzmenko B, Carmeli Y. Occurrence and phenotypic characteristics of extended spectrum beta-lactamases among members of the family Enterobacteriaceae at the Tel-Aviv Medical Centre(Israel) and evaluation of diagnostic tests. J Clin Microbiol 2003; 41:155-8; http://dx.doi.org/10.1128/JCM.41.1.155-158.2003
13. Singhal S, Mathur T, Khan S, Upadhyay DJ, Chugh S, Gaind R, etal. Evaluation of methods for AmpC beta-lactamase in gram negative clinical isolates from tertiary care hospitals. Indian J Med Microbiol 2005; 23:120-4.
14. Walsh TR, Toleman MA, Poirel L, Nordmann P. Metallo-beta-lactamases:the quiet before the storm? Clin Microbiol Rev 2005; 18: 306-25; http://dx.doi.org/10.1128/CMR.18.2.306-325.2005
15. Umadevi S, Kandhakumari G, Joseph N M, Kumar S, Easow JM, Stephen S, Singh U K. Prevalence and antimicrobial susceptibility pattern of ESBL producing Gram Negative Bacilli. Journal of Clinical and Diagnostic Research 2011; 5:236-39.
16. Cormican MG, Marshall SA and Jones R N. Detection of Extended-Spectrum b-Lactamase (ESBL)-producing Strains by the Etest ESBL Screen. J Clin Microbiol Aug. 1996; 34:1880–1884.
17. Pournaras S, Ikonomidis A, Kristo I, Tsakris A, Maniatis AN. CTX-M enzymes are the most common extended-spectrum beta-lactamases among *E.coli* in a tertiary Greek hospital. J Antimicrob Chemother 2004; 54:574-5; http://dx.doi.org/10.1093/jac/dkh323
18. Jemima SA and Verghese S. Molecular characterization of nosocomial CTX-M type β-lactamase producing Enterobacteriaceae from a teriary care hospital in South India. Indian J Med Microbiol 2008; 26(4):365-8; http://dx.doi.org/10.4103/0255-0857.43581
19. Sekar B, Arunagiri K. Detection and characterization of bla-CTX-M gene by PCR-RFLP analysis among third generation cephalosporin resistant gram negative isolates. Proceedings of the 30th National Congress of Indian Assoc of Med Microbiologists, Microcon 2006.Oct27-29; Nagpur, India 2006. P.27.

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