British Microbiology Research Journal
16(3): 1-7, 2016, Article no.BMRJ.27860
ISSN: 2231-0886, NLM ID: 101608140

SCIENCEDOMAIN *international*
www.sciencedomain.org

Detection and Molecular Characterization of ESBLs in *E. coli* Isolates from a Tertiary Care Hospital in North India with Special Attention to CTX-M-27

Nitika Anand[1*], Ashish K. Asthana[2] and Molly Madan[3]

[1]Department of Microbiology, Subharti Medical College, Meerut-250005, Uttar Pradesh, India.

Authors' contributions

This work was carried out in collaboration between all authors. Author NA designed the study, performed the statistical analysis, wrote the protocol, wrote the first draft of the manuscript and managed literature searches. Authors AKA and MM managed the analyses of the study and literature searches. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/BMRJ/2016/27860
Editor(s):
(1) Abha Sharma, Department of microbiology, GB Pant Hospital, New Delhi, India.
Reviewers:
(1) Gulfem Terek Ece, Izmir University, Turkey.
(2) R. Jasmine, Bishop Heber College, Trichy, India.
Complete Peer review History: http://www.sciencedomain.org/review-history/15704

Original Research Article

Received 22nd June 2016
Accepted 20th July 2016
Published 7th August 2016

ABSTRACT

Background: Extended spectrum beta-lactamases are the main cause of resistance to beta lactam antibiotics in members of Enterobacteriaceae. ESBL associated infections are on a rise worldwide and have become a serious public health problem. We aimed to investigate the molecular epidemiology of ESBL producing *E. coli* isolates recovered from various clinical specimens at a tertiary care hospital and to determine the antibiotic sensitivity profile of ESBL positive isolates.
Methodology: A total of 300 isolates of *E. coli* were collected from various clinical specimens between the study period of 2011 to 2014. Antimicrobial susceptibility testing was done. ESBL detection was carried out by CLSI Phenotypic confirmatory method. Molecular typing of ESBLs was performed by uniplex PCR among 100 ESBL isolates. The *bla* $_{CTX-M}$ strains were genotyped by sequencing of PCR product. Nucleotide sequences were submitted to Gen Bank and accession numbers were obtained.
Results: 61% isolates were found to be ESBL producers. ESBL and non-ESBL producers compared among in- and out-patients gave statistically significant result (*P value=0.002*). All ESBL isolates (100%) were sensitive to imipenem. Overall 93.9% ESBL producers and 67.5% non-

**Corresponding author: E-mail: nitika.anand@rediffmail.com;*

ESBLs were Multi Drug Resistant (Resistance to 3 or more class of antibiotics). The difference was statistically significant (*P* value=0.001). Majority of the typeable isolates harboured two or more ESBL genes (52%). Sequencing was done for 10 randomly selected *bla*CTX-M PCR products and majority (90%) were identified as CTXM-15 belonging to CTX-M Cluster-1 while 1 0f 10 (10%) was identified as CTX-M- 27 belonging to CTX-M Cluster-9 on blast analysis. Deduced nucleotide sequences were submitted to Gen Bank. The accession numbers obtained from Gen Bank are KU946005-KU946009.

Conclusion: Our study shows high ESBL occurrence among *E.coli* isolates and highlights the incidence CTX-M-27 for the first time from North India.

Keywords: Extended spectrum beta lactamases; PCR; SHV; TEM; CTX-M.

1. INTRODUCTION

ESBLs are usually plasmid mediated β-lactamases, most commonly found in *K. pneumoniae, E. coli* and other gram negative bacilli [1]. Extended-spectrum β-lactamases (ESBLs) have spread threateningly in many regions of the world. The beginning of this new century marked a massive distinction in the distribution of ESBLs by the emergence of CTX-M, a new ESBL type. CTX-M was found different from the classical TEM and SHV, as this gene originated in a clinically insignificant genus *Kluyvera* spp., present in the environment [2]. CTX-M was first detected in *E.coli* strains in Germany (1988) and presently, there are more than 65 allelic variants which are known [3,4].

The Asia-Pacific region, particularly India, China and Thailand are marked high-risk countries because of the increased rates of infections, both hospital- and community-acquired, caused by ESBL-producing *E. coli* and *K. pneumonia* [5]. India, with the prevalence >80%, is now said to be the capital of ESBL-producing strains. There is an urgent need for information from continuous surveillance to obtain information that will help formulate appropriate antibiotic policy to manage the growing drug resistance problem.

We attempted to characterize the distinct ESBL types (TEM, SHV and CTX-M) produced by *E. coli* isolated from different samples and to determine the type of CTX-M prevalent in this part of country by nucleic acid sequencing. An attempt was also made to assess the in vitro susceptibility of ESBL isolates to currently available antimicrobial classes.

2. MATERIALS AND METHODS

2.1 Samples

A total of 300 consecutive non-duplicate clinical isolates of *E. coli* from various clinical specimens (urine (187), Pus (77), sputum (7), blood (3), body fluids (11) and others(15) were studied prospectively for ESBL production at Chhatrapati Shivaji Subharti Hospital. a tertiary care hospital at Meerut city, north India. The study population included patients of age group 18-50 years and included both male and female population from both Inpatient and Outpatient departments.

2.2 Antimicrobial Susceptibility Testing

The isolates were tested for their antimicrobial susceptibilities by the disc diffusion technique according to the CLSI guidelines [6]. The following antibiotic discs (drug concentrations in µg) were used: Ampicillin (10), Ampicillin/ Sulbactam (10/20), Nitrofurantoin (30), Co-trimoxazole (30), Cefoxitin (30), Amikacin (30), Ciprofloxacin (10), Imipenem (10 µg), Meropenem (10), Cefoparazone/ Sulbactam (75/10).

2.3 Phenotypic Method for Detection of ESBL

ESBL production was confirmed by disk potentiation test using ceftazidime (30 µg) and cefotaxime (30 µg) antibiotic disks with and without clavunalic acid (10 µg) [6]. A ≥ 5 mm increase in the zone diameter around ceftazidime-clavulanic acid, as compared to ceftazidime alone, was considered indicative of ESBL production (Fig. 1). *E. coli* (*ATCC 25922*) was used as ESBL negative and *Klebsiella pneumoniae (ATCC 700603)* was used as ESBL positive control strains throughout the study (Subharti Medical College, Meerut).

2.4 Detection of ESBL Types by PCR

2.4.1 Extraction of genomic DNA

Genomic DNA was extracted by growing ESBL positive strains in 4 ml of BHI broth at 37℃

Table 1. Primers used for molecular characterization of ESBL genes

Target	Primer	Oligonucleotide sequence	ref
bla $_{CTX-M}$	bla $_{CTX-M}$ F	TTTGCGATGTGCAGTACCAGTAA	[7]
	bla $_{CTX-M}$ R	CGATATCGTTGGTGGTGCCATA	
bla_{SHV}	OS-1	TCGGGCCGCGTAGGCATGAT	[8]
	OS-2	AGCAGGGCGACAATCCCGCG	
bla_{TEM}	OT-1	TTGGGTGCACGAGTGGGTTA	[8]
	OT-2	TAATTGTTGCCGGGAAGCTA	

Table 2. Cycling conditions for amplification

	For TEM and SHV	For CTX-M	Cycles
Initial Denaturation	95℃ for 3 min	94℃ for 2 min	
Denaturation	95℃ for 1 min	95℃ for 20 sec	
Annealing	55℃ for 1 min	51℃ for 30 sec	35 cycles
Elongation	72℃ for 5 min	72℃ for 30 sec	
Final Elongation	72℃ for 5 min	72℃ for 3 min	

overnight. 200 µl of overnight grown culture was added to 800 µl of D/W and boiled for 10 min at 100℃. The bacterial suspension was then centrifuged at 12,000×g for 2 min. The supernatant was used as the template DNA for PCR. The purified DNA was stored at -20℃.

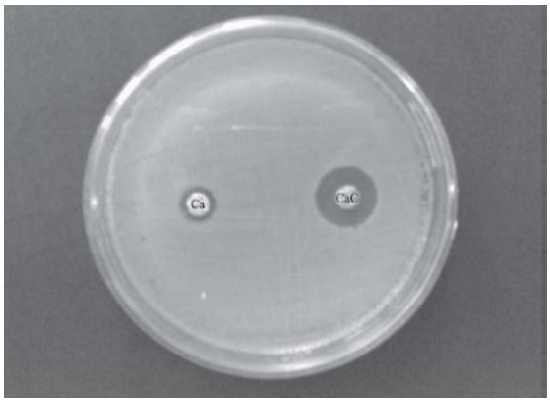

Fig. 1. Positive phenotypic confirmatory test for ESBLs

2.4.2 PCR amplification for β-lactamase encoding genes

Among 183 ESBL producing isolates, 100 isolates were selected randomly for molecular detection by PCR. Uniplex polymerase chain reaction was carried out for detection of TEM, SHV and CTX-M genes. Amplification reaction was performed in a reaction mixture containing 12.5 µl master mix (10 X PCR buffer + DNTP's+Taq DNA polymerase), 1 µl of each primers, 9.5 µl of DNAse free water and 1 µl of DNA template. The primers were obtained from Genei^{TM}. Primer which was used has been indicated in Table 1. Amplification was performed in a Thermocycler (Applied biosystems 2720) with cycling conditions as mentioned in Table 2. Agarose Gel Electrophoresis was done at 50 volt for 2.5 hour.

The gel was visualized under UV transilluminator (lab India life sciences). A 100 bp ladder molecular weight marker (Roche, USA) was used to measure the molecular weights of amplified products. The images of ethidium bromide stained DNA bands were digitized using a gel documentation system (Uvitec Cambridge, Genei)

2.5 Gene Sequencing Analysis

DNA sequencing of 10 randomly selected CTX-M positive PCR amplicons was performed at Chromous Biotech Pvt Ltd. Bangalore by Sanger's method using Big Terminator Version 3.1 kit and ABI 3500Xl Genetic Analyzer.

2.6 Statistical Analysis

Chi-square test was used to make comparisons among categorical variables. A p -value of less than 0.05 was considered statistically significant.

3. RESULTS

Of the 300 *E. coli* isolates, a total of 183(61%) isolates were phenotypically identified as ESBL producers. ESBL isolates were obtained from various clinical specimens as documented in Table 3. ESBL production was quite high among the urine samples (59.5%), followed by pus

samples (26.7%). Among 183 ESBL producing strains, 138 (75.4%) belonged to IPD patients and 45 (24.5%) belonged to OPD patients.(Fig 2). ESBL and non-ESBL producers compared among in- and out-patients gave significant result (*P value= 0.002*).

The overall susceptibility of ESBL isolates to various antibiotics was as follows: Nitrofurantoin (77.06%), Amikacin (73.7%), Meropenem (72.1%), Cefoparazone/sulbactam (46.4%), Ampicillin (9.2%) and Ciprofloxacin (16.3%).All the isolates were sensitive to imipenem (100%). However, among non-ESBL producers the susceptibility to the various antibiotics was higher than ESBL producers (Table 4). The statistical analysis showed significant difference (*P* value <0.05) in the resistance for antibiotics like Ampicillin, Ampicillin/Sulbactam, Amikacin, Ciprofloxacin, Cefoxitin, Cotrimoxazole, Cefoparazone/Sulbactam among ESBL producers and non-ESBL producers. Overall 93.9% ESBL producers and 67.5% non-ESBLs were MDR (*P* value=0.001).

100 randomly selected phenotypically confirmed ESBL producers were genotyped. The molecular characterization revealed that 75% isolates possessed TEM gene, 58% isolates possessed CTX-M gene and 21% possessed SHV gene either alone or in combination. 10(10%) out of 100 isolates did not carry any of three genes. The negative amplification in the 10 isolates may be because of presence of other ESBL types which were not further investigated. Two or more genes for ESBL were present in 52 (52%) of ESBL isolates, bla_{TEM} + bla_{CTX-M} being the most common combination(34%) followed by bla_{TEM}+bla_{SHV}+bla_{CTX-M} (11%), bla_{SHV}+bla_{CTX-M} (4%) and bla_{TEM}+bla_{SHV} (3%) (Table 5). On sequence analysis of 10 randomly selected CTX-M amplified products,9 were identical to CTX-M-15 belonging to Cluster- 1 while 1 gene was identical to CTX-M-27 belonging to Cluster-9.

Table 3. Sample wise distribution of ESBL producing isolates

Clinical samples	ESBL positive (%)
Urine	59.5
Pus	26.7
Blood	0.5
Sputum	3.2
Body fluids	4.3
Others	5.4

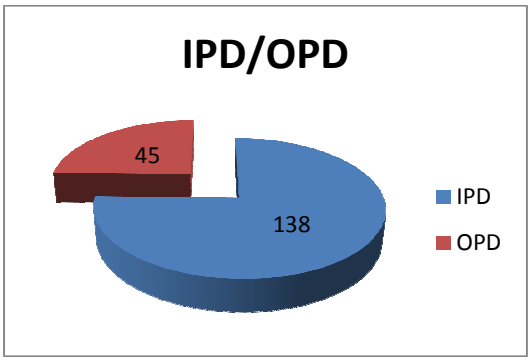

Fig. 2. Distribution of ESBL isolates among IPD /OPD patients

4. DISCUSSION

In our study prevalence of ESBLs in *E. coli* was high (61%).Various other studies from India have also reported high ESBL production ranging from 41.0% to 63.5% [9-11]. The high rate of ESBL prevalence in India and its widespread dissemination is a cause for worry.

In the present study urine samples (59.5%) were the major source of ESBL producing strains. A study conducted by Shanthi et al. [12] reported urine as the major source of ESBL producers. Iraj et al. [13] from Bangladesh also reported similar findings in their study.

Table 4. Antibiotic susceptibility profile of ESBL and non-ESBL isolates

Antimicrobials	ESBL	NonESBL	P value
Ampicillin	9.2%	18.8%	0.001*
Ampicillin/Sulbactam	35.5%	42.7%	0.002*
Amikacin	73.7%	82%	0.027*
Ciprofloxacin	16.3%	29%	0.001*
Cefoxitin	34.4%	51.2%	0.013*
Cotrimoxazole	21.8%	31.6%	0.008*
Imipenem	100%	100%	-
Cefoparazone/Sulbactam	46.4%	59.8%	0.036*

Table 5. Extended spectrum β-lactamase (ESBL) genotypes in *E. coli*

Positive by PCR for ESBL gene	Number amplified
A. Two or more ESBL gene	**52**
blaTEM+bla_{SHV}+bla_{CTX-M}	11
bla_{TEM}+bla_{CTX-M}	34
bla_{TEM}+bla_{SHV}	3
bla_{SHV}+bla_{CTX-M}	4
B. Single ESBL gene	
bla_{TEM}	26
bla_{SHV}	3
bla_{CTX-M}	9

In present study high prevalence of ESBL producing *E. coli* among hospitalized patients (75.4%) was observed and is relevant with the data of other investigators [14-16].

Antibiotic susceptibility pattern of ESBL isolates in our study revealed poor susceptibility to Ampicillin (9.2%), Ciprofloxacin (16.3%),Co-trimoxazole (21.8%), Cefoxitin (34.4%), Ampicillin/Sulbactam (35.5%) while susceptibility to Imipenem was 100%. These findings correlated well with other studies [9,14,17,18]. In the present study 93.9% ESBL isolates were found to be Multidrug resistant. However, various studies have reported 100% Multidrug resistance among ESBL positive isolates [12,14,16].

Majority of the ESBLs are derivatives of TEM, SHV or CTX-M enzymes and these enzymes are most often found in *E. coli* and *K. pneumonia.* Phenotypic tests for ESBL detection only confirm whether an ESBL is produced or not but cannot detect the ESBL subtype. Therefore Genotypic identification of these enzymes is essential for determining their epidemiology. In the light of this scenario, we performed molecular characterization of 100 randomly selected ESBL positive strains. Of the 100 ESBL positive isolates, the most commonest gene was blaTEM(75%) followed by blaCTX-M(58%) and blaSHV, (21%). These findings are in accordance with a study conducted by Lim et al. [19] in Malaysia who reported TEM as the commonest ESBL gene in *E. coli* isolates (88%) followed by CTX-M(20%) and SHV(8%). Another study conducted by Varkey et al. [20] in Vellore reported TEM being the most prevalent ESBL gene among *E. coli* isolates (75%). On the contrary various studies [14,21,22] have reported blaCTX-M as the most commonest gene among *E. coli* isolates.

During the past decade CTX-M has emerged in many countries of the world [23]. In the present study prevalence of CTX-M was 58% which is quite similar to the findings of a recent study conducted in north-western India by Kaur and Aggarwal, in which 59.32% of *E. coli* possessed the CTX-M genes [11].

Among the CTX-M-type ESBLs, CTX-M-15 is now the most widely distributed in *E. coli* which became a major cause of infections in both community and hospitals [24,25]. In view of the present scenario, we made an attempt to determine the CTX-M subgroup prevalent in our region by performing nucleic acid sequencing of 10 randomly selected CTX-M positive isolates. On blast analysis, majority of the isolates,9 of the 10(90%) were identical to CTX-M-15 belonging to Cluster-1. George and Jesudasan from vellore in 2015 reported CTX-M-15 as the predominant ESBL type among *E. coli* isolates [26] which is quite similar to our findings. Another study conducted by Padmini et al. [18] from Coimbatore in 2008 reported the presence of CTX-M-15 gene on nucleic acid sequencing. These reports document evidence of the high prevalence of CTX-M-15 type ESBLs in almost all parts of India.

In the present study, 1 of 10 isolate (10%) matched with CTX-M-27 like ESBL gene belonging to CTX-M-9 group. To the best of our knowledge, this is the first report from North India about the detection of the $bla_{CTX-M-27\ like}$ gene, as earlier report of detection of $bla_{CTX-M-27\ like}$ gene has been from National Institute of Cholera and Enteric Diseases from Kolkata [27]. In this study, Roy et al reported to have isolated CTX-M-27 like and CTX-M-14 like ESBL gene from *E.coli* and *Klebsiella* isolates from neonatal stool specimens. There have been few reports on detection of CTX-M-27 worldwide. A study conducted by Bonnet et al from France reported isolation of CTX-M-27 producing strain that produced a novel D240G variant designated CTX-M-27 and derived from CTX-M-14 as well as CTX-M-19 [28]. CTX-M-27, which harbours the Asp240Gly substitution, confers eightfold higher levels of resistance to ceftazidime than its parental enzyme, CTX-M-14 [2].

5. CONCLUSION

In conclusion, the high occurrence of ESBLs in our region cannot be ignored. There is an urgent need to adopt effective infection control measures to combat the spread of such strains. Our findings emphasizes the increasing role of the bla_{CTX-M} β-lactamases in almost all parts of

India which highlights the need for their epidemiological monitoring and prudent use of antibiotics. The detection of CTX-M-27 from this part of the country is indeed alarming. More such molecular studies must be conducted to determine the common ESBL enzymes and their resistance pattern which will help clinicians formulate appropriate antibiotic regimen for treatment.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Emery CL, Weymouth LA. Detection and clinical significance of extended- spectrum β-lactamases in a tertiary-care medical center. J Clin Microbiol. 1997;35:2061-7.
2. Bonnet R. Growing group of extended spectrum beta-lactamases: The CTX-M enzymes. Antimicrob Agents Chemother. 2004;48:1-14.
3. Bradford PA. Extended spectrum beta-lactamases in the 21st century, characterization, epidemiology and detection of this important threat. Clin Microbiol Rev. 2001;14:933–51.
4. Tzouvelekis LS, Tzelepi E, Tassios PT, Legakis NJ. CTX-M type β-lactamases: An emerging group of extended- spectrum enzymes. Int J Antimicrob Agents. 2000;14:137–43.
5. Hawkey PM. Prevalence and clonality of extended-spectrum beta-lactamases in Asia. Clin Microbiol Infect. 2008;14:159-65.
6. Clinical and Laboratory Standards Institute (CLSI). Performance standards for antimicrobial susceptibility testing. CLSI approved standard M100-S16 16th informational supplement. Clinical and Laboratory Standards Institute, Wayne, Pa; 2006.
7. Edelstein M, Pimkin M, Palagin I, Edelstein I, Stratchounski L. Prevalence and molecular epidemiology of CTX-M extended-spectrum β-lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae* in Russian hospitals. Antimicrob Agents Chemother. 2003;47: 3724-32.
8. Arlet G, Philippon A. Construction by polymerase chain reaction and intragenic DNA probes for three main types of transferable β-lactamases (TEM, SHV, CARB). FEMS Microbiology Letters. 1989;82:19–26.
9. Goyal A, Prasad KN, Prasad A, Gupta S, Ghoshal U, Ayyagiri A. Extended spectrum β-lactamases in *Escherichia coli* & *Klebsiella pneumonia* & associated risk factors. Indian J Med Res. 2009;129:695-700.
10. Jain A, Roy I, Gupta MK, Kumar M, Agarwal SK. Prevalance of extended spectrum β-lactamase-producing gram negative bacteria in septicaemic neonates in a tertiary care hospital. J Med Microbiol. 2003;52:421-5.
11. Kaur M, Aggarwal A. Occurence of the CTX-M, SHV and the TEM genes among the extended spectrum β-lactamase producing isolates of Enterobacteriaceae in a tertiary care hospital of North India. J Clin Diagn Res. 2013;7(4):642-645.
12. Shanthi M, Sekar U. Extended spectrum beta lactamase producing *Escherichia coli* and *Klebsiella pneumoniae*: Risk factors for infection and impact of resistance on outcomes. J Assoc Physicians India. 2010;58 Suppl:41–4.
13. Iraj A, Nilufar YN. Antibiogram of extended spectrum beta-lactamase (ESBL) producing *Escherichia coli* and *Klebsiella pneumoniae* isolated from hospital samples. Bangladesh J Med Microbiol. 2010;04(01):32–6.
14. Sharma M, Pathak S, Srivastava P. Prevalence and antibiogram of extended spectrum β-lactamase (ESBL) producing gram negative bacilli and further molecular characterization of ESBL producing *Escherichia coli* and Klebsiella spp. J Clin Diagn Res. 2013;7(10):2173–2177.
15. Kulkarni R, Dohe V, Ghadge D, Bhore A. A study of extended spectrum beta-lactamase (ESBL) producers in clinical isolates. Medical Journal of Western India. 2013;41(1):18-22.
16. Abhilash KP, Veeraraghavan B, Abraham OC. Epidemiology and outcome of bacteremia caused by extended spectrum beta-lactamase (ESBL)-producing *Escherichia coli* and Klebsiella Spp. in a tertiary care teaching hospital in South India. J Assoc Physicians India. 2010; 58 Suppl:13–7.
17. Umadevi S, Kandhakumari G, Joseph NM, Kumar S. Prevalance and antimicrobial susceptibility pattern of ESBL producing gram negative bacilli. Journal of Clinical

and Diagnostic Research. 2011,5(2):236-239.

18. Padmini S, Raju B, Mani KR. Detection of Enterobacteriaceae producing CTX-M extended spectrum β-lactamases from a tertiary care hospital in South India. Indian Journal of Medical Microbiology. 2008;26(2):163-6.
19. Lim K, Yain R, Yeo C, Puthucheary S. Characterization of multidrug resistant ESBL-producing *Escherichia coli* isolates from hospitals in Malaysia. Journal of Biomedicine and Biotechnology. 2009;10. Article ID: 165637.
20. Varkey D, Balaji V, Abraham J. Molecular characterisation of extended spectrum Beta-l actamase producing strains from blood sample. International Journal of Pharmacy and Pharmaceutical Sciences. 2014;6(3):276-278.
21. Vaida S, Marius L, Aurelija B, Justas P, Rita P, Agne G, et al. Molecular characterization of extended-spectrum b-lactamase producing *Escherichia coli* and *Klebsiella pneumoniae* isolates from hospitals in Lithuania. Journal of Medical Microbiology. 2010;59(10):1263–65.
22. Shahid M, Singh A, Sobia F, Rashid M. blaCTX-M, blaTEM, and blaSHV in Enterobacteriaceae from North-Indian tertiary hospital: High occurrence of combination genes. Asian Pacific Journal of Tropical Medicine. 2011;101-105.
23. Bonnet R. Growing group of extended-spectrum β-lactamases: The CTX-M enzymes. Antimicrob Agents Chemother. 2004;48:1-14.
24. Cantón R, González-Alba JM, Galán JC. CTX-M enzymes: Origin and diffusion. Front Microbiol. 2012;3:110.
25. Poirel L, Bonnin RA, Nordmann P. Genetic support and diversity of acquired extended-spectrum β-lactamases in gram-negative rods. Infect Genet Evol. 2012;12:883–893.
26. George EA, Jesudasan MV, Nandagopal B, Sankar S, Sudandiradoss C. Molecular characterization of CTX-M type extended spectrum beta lactamase producing *E. coli* isolated from humans and the environment. Indian Journal of Medical Microbiology. 2015;33(5):73-79.
27. Roy S, Mukherjee S, Singh AK, Basu S. CTX-M-9 group extended-spectrum β-lactamases in neonatal stool isolates: Emergence in India. Indian Journal of Medical Microbiology. 2011;29(3):305-308.
28. Bonnet R, Recule C, Baraduc R, Chanal C. Effect of D240G substitution in a novel ESBL CTX-M-27. Journal of Antimicrobial Chemotherapy. 2003;52(1):29-35.

Peer-review history:
The peer review history for this paper can be accessed here:
http://sciencedomain.org/review-history/15704