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CTX-M TYPE EXTENDED SPECTRUM β-LACTAMASES IN *ESCHERICHIA COLI* ISOLATES FROM CLINICAL SAMPLES FROM KALABURAGI REGION

Jahanara Kudsi and Prof. Kelmani Chandrakanth R.*

[1]Medical Biotechnology and Phage Therapy Laboratory, Department of Post Graduate Studies and Research in Biotechnology, Gulbarga University, Gulbarga-585 106, Karnataka, India.

***Corresponding Author: Prof. Kelmani Chandrakanth R.**
Medical Biotechnology and Phage Therapy Laboratory, Department of Post Graduate Studies and Research in Biotechnology, Gulbarga University, Gulbarga-585 106, Karnataka, India.

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ABSTRACT

The β-lactam antibiotics, along with amino glycosides are widely prescribed antibiotics. However resistance to these drugs continues to increase due to their injudicious use. In recent years, resistance in the Indian *E.coli* population has increased and mostly showing complex mechanisms like extended spectrum β-lactamases (ESBLs) specifically blaCTX-M. The reason may be increased intracontinental movement of the human population. So it should be better to know the prevalence and resistance pattern of these strains, well in advance, to design a systematic policy for empirical therapy. **Methods:** 150 isolates of *Escherichia coli* obtained from 170 non-repeat samples of urine, blood, stool, us etc were studied and their resistance rates and patterns were noted. The isolates were screened for prevalent cephalosporin's resistance (ESBLs) by antibiotic susceptibility test. Minimum inhibitory concentration (MIC) for cephotaxime and ceftazidime was done by HiComb MIC-test strips ESBLs were confirmed by NCCLS-ESBL phenotypic confirmatory test (Combined disc diffusion test) and E-test. Test was carried out using cephotaxime (30µg), ceftazidime (30µg), cephotaxime/clavulanicacid (30/10µg) and ceftazidime/clavulanic acid (30/10 µg) discs. Genotypic conformation for presence of ESBL (CTX-Type) was established by PCR. **Results:** Resistance to 5 beta-lactam antibiotics tested was varying between 70 to 80 percent i.e. approximately 120 isolates. MICs of ESBL producing isolates ranged from 8 to > 240 µg/ml for both ceftazidime and cephotaxime. 110(92%) of above 120 resistant strains were positive for ESBL production both by the E -test for ESBL and ESBL detection kit (Combination disk method). The PCR band after amplification at 544 bp was visualized.

KEYWORDS: *E.coli*, ESBLs, CTX-M, Clinical Samples.

INTRODUCTION

The b-lactam antibiotics, especially the cephalosporin's and b-lactam-b-lactamases inhibitor combinations are major drug classes used to treat infections caused by *E. Coli*.[1] Among *E coli*, b-lactamase production remains the major contributing factor to b-lactam resistance. Extended spectrum b-lactamases are one of the major source of resistance to oxyimino-cephalosporins in Enterobacteriaceae.[2] Most of ESBLs are mutants of TEM and SHV enzymes, but CTX-M enzymes are the newly emerging ESBLs[3] and are increasingly prevalent worldwide among *E.coli* bacteria. The CTX-M enzymes are wide group with more than 30 alleles categorised into five distinct phylogenetic groups, evolved because of genetic escape and mutation of the chromosomal b-lactamase genes of *Kluyvera spp*.[2,3] The CTX-M family, first described in 1992,[4] is known as most dominant non-TEM, non-SHV ESBL among Enterobacteriaceae.

It is identified as a rapidly growing family of ESBLs that selectively prefer to hydrolyze cephotaxime and most of them are active against ceftazidime.[5] Further the incidence of Urinary tract infections (UTI) by ESBL producing *E. coli* was found to be the highest in India (60%) followed by Hongkong (48%) and Singapore (33%).[6] Previous studies from India have reported ESBL production varying from 28% to 84%.[7] On the whole prevalence of ESBL producers was found to vary in different geographical regions and in different institutes. CTX-M enzymes have been the predominant ESBLs in Argentina for >10 years[8] and have prevalence in many parts of the world.[3,9] including Europe.[10,11] Therefore in the present study we are predominantly focussing on prevalence of CTX-M ESBL producing *E.coli* in our region.

MATERIALS AND METHODS

Isolation and identification of *E.coli*

For this report, we analysed data for 150 strains of *E. Coli* from 170 different clinical Samples like urine, stool, blood, pus etc over a period of 3 months in 2013.The clinical samples were collected from hospitals and diagnostic laboratories in Kalburagi region over a period of three months in 2013.Identification was done by culture on EMB agar. Isolation of strains was done by conventional morphological, cultural and biochemical characterisation. Standard strain of *E.coli* MTCC 443 was obtained from Medical and Phage Therapy Laboratory, department of Biotechnology, Gulbarga University, and Kalaburagi.

Antibiotic Sensitivity Test and MIC Determination

Antibiotic sensitivity test was conducted for *E. coli* isolates using disk diffusion techniques as described by Bauer-Kirby method by using standard antibiotic disk.[12] Isolates were tested for resistance against commonly used third generation broad spectrum antibiotics like Cefipime (30 µg), Cephotaxime (30 µg), Cefpodoxime (30 µg), Ceftazidime (30 µg), Ceftriaxone (30 µg). The antibiotic disks were obtained from Hi-Media Laboratories, Mumbai, India. After incubation at 37^{o} C overnight, diameter of the zone of inhibition were measured and results were interpreted according to CLSI guidelines[13] and recorded as susceptible(S), intermediately susceptible(I) or resistant (R) to the antibiotics. MIC's of antibiotics were also determined on Mueller–Hinton Agar using Hicomb MIC strips according to CLSI.[14] Following antibiotics were used: cephotaxime (0.0001 to 240 µg), ceftazidime (0.0001 to 240 µg) (Hi-Media, Mumbai, India).

Combination disk method Test for ESBL production

The Combined disc diffusion test (CLSI Recommended) was followed. Test was carried out using cefotaxime (30 µg), ceftazidime (30 µg) and cefotaxime/clavulanic acid30/10g) and ceftazidime/clavulanic acid (30/10 µg) discs. The discs were obtained commercially from Hi-Media Laboratories, Mumbai, India. Standard strain of E.coli MTCC 443 was obtained from Medical and Phage Therapy Laboratory, department of Biotechnology, Gulbarga University, and Kalaburagi. Positive results were taken when there was a =/> 5mm increase in ceftazidime/clavulanic acid zone diameter.[14]

Confirmation of ESBL using E- test

The confirmation of ESBL was also performed by E-test ESBL strips (E-test Himedia) and the test was performed in accordance to the manufacturer's instructions. Double ended strips containing gradient of ceftazidime and ceftazidime +clavulanic acid, cefotaxime and cephotaxime+clavulanic acid at the other end were tested. The presence of ESBL was confirmed by the appearance of phantom zone below the formation of TZ inhibition ellipse and clavunate caused a more than or equal to three doubling concentration decrease ratio of > 8 in the MIC values of ceftazidime.[14]

Detection bla CTX-M genes

DNA extraction

DNA was isolated from bacterial cells using DNA purification kit (DNA purification kit, Himedia Mumbia). The purified DNA was stored at -20°C. The samples were run on agarose gel and stained with ethidium bromide. The stained gel was examined for presence of bands under UV-light using molecular weight marker.

PCR amplification of bla CTX-M genes

The PCR assay was targeted for the presence of CTX-M genes with specific primers.[15] PCR amplification of bla CTX-M alleles was carried out on 5 resistant isolates with these primers BlaCTX-M 5^{1}-TCCCGCAGATAAATCACC-3^{1} for 3^{1}ATGTGCAGYACCAGTAARGT-5^{1}. A single reaction mixture contained 1 µl of DNA extract, 30 pmol of each primer, 100 µM (each) dNTPs, 1.25 U Taq polymerase and buffer with 1.5 mM MgCl2 supplied along with the kit in a total volume of 50 µl. A thermal cycler was used and the following reaction parameters were used: initial denaturation at 94ºC for 7 min; denaturation at 94ºC for 50 sec, annealing at 50ºC for 40 sec and elongation at 72ºC for 60 sec, repeated for 35 cycles; and final extension at 72ºC for 5 min15. The resulting PCR products were run in 1.6 per cent agarose gels (Himedia, Mumbai) containing 1xtris-acetate EDTA and 0.5 µg of ethidium bromide/ ml and visualized under UV transilluminator and photographed with Gel documentation system.[16]

RESULTS

In the present study 150 isolates of *E. coli* from 170 non-repeat samples of urine, blood, stools, pus etc were isolated. Out of 150 isolates of *E. coli* around 120 (70-80%) (Table 1) (Fig 1) isolates were resistant to third generation antibiotic like Cefipime (30 µg), Cefotaxime (30 µg), Cefpodoxime (30 µg), Ceftazidime (30 µg), Ceftriaxone (30 µg). 110(92%) of above 120 resistant strains were positive for ESBL production both by the E -test for ESBL and ESBL detection kit (fig 2).

According to cephalosporins susceptibly test of *E.coli* sample in Kalaburagi region 70 to 80 percent of strains were resistant. This was in accordance to others studies in India. In a recent study in north-western India by Kaur and Aggarwal,[17] 45.8% isolates were found to be ESBL producers. In south e Asian region, particularly India, China and Thailand are marked as high-risk countries because of the increased rates of infection caused by ESBL-producing *E. coli* and *K. Pneumonia*.[18] India, with the prevalence >80%, is now said to be the centre of ESBL-producing strain.[19] Another study from south India reported 79.4% of phenotypic positive nosocomial isolates positive for CTX-M genes, of which 63.7% were *E. coli* and all were positive for the CTX-M-1 group.[20] Suhkla et al.[21] have reported that 72 per cent of isolates were found to be resistant to all 3rd generation cephalosporin's. These reports are evidence for high

prevalence of CTX-M type ESBL in almost all parts of India. MICs of ESBL producing isolates ranged from 8 to > 240 µg/ml for both ceftazidime and cefotaxime, majority of the isolates (120 i.e. 80%) had MIC > 32 µg/ml and few (40%) >240 µg/ml (fig 3).In the phenotypic confirmatory test for esbl using cephalosporin/clavulanate combination discs, 110(92%) strains showed enhanced susceptibility to ceftazidime and cefotaxime in the presence of clavulanic acid, thus indicating ESBL production in them (fig 2). Five random ESBL positive isolates were tested for genotypic conformation for blaCTX-M gene amplification. Amplification of blaCTX-M produced a band at 544 bp in all five samples.

Table 1: Percentage of resistance among E.*coli* isolates.

Sl no	Antibiotic Used	% of resistance	No of isolates
1	Cefipime(30 µg),	80	72
2	Cefotaxime(30 µg)	76	63
3	Cefpodoxime(30µg)	76	63
4	Ceftriaxone (30µg).	70	63
5	Ceftazidime(30	74	66

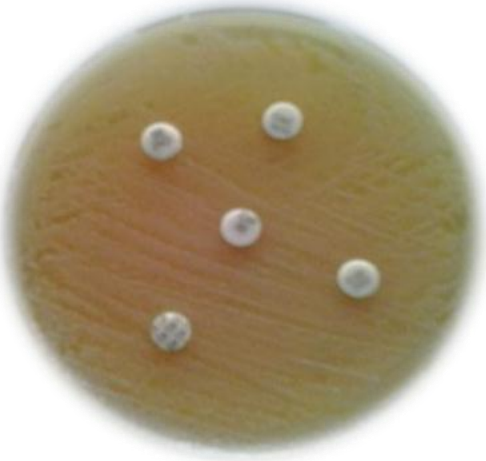

Figure 1: Antibiotic Resistance to cephalosporins antibiotic.

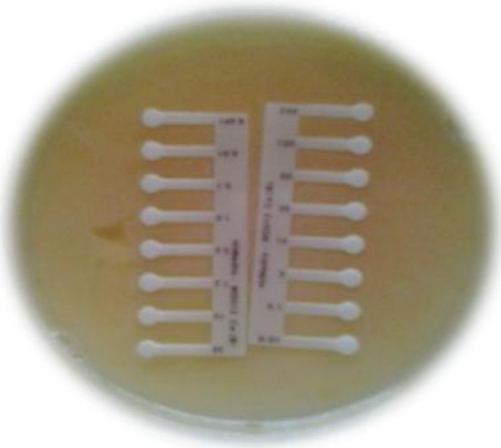

Figure 2: MIC for Resistant Strains

Figure 3: ESBL Detection Test.

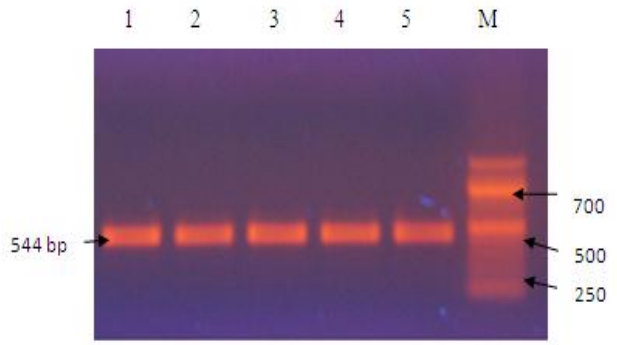

Figure 3: PCR Amplification of bla CTX-M gene.

Lane M: 1Kb DNA ladder; **Lane 1-5**: ESBL positive strains.

DISCUSSION

The present study clearly highlight that most of the isolated *E.coli* were resistant to third generation cephalosporin's like Cefipime, ceftazidime, cefapodoxime, cefatoxime and cefuroxime. *E. coli* possessing CTX-M gene causing UTI's began to be reported from earlier this decade from USA, Saudi Arabia, India, Japan, Nepal, China and Brazil.[36] Sundram Medical Foundation India has reported an ESBL positivity rate between 26.9% and 48.3 %.[22-25] The present study clearly shows that there is high prevalence of ESBL *E. coli* in clinical isolates and its resistance to commonly used antibiotics in this region. ESBL producers do not respond well to the usually prescribed empirical therapy. Also, there is an rising risk of associated mortality and high cost of therapy when the patients are put on the standard empirical therapy.[26] Recent surveys have shown that ESBLs in Enterobacteriaceae in India range from 70–90%[27] consequently making the use of reserved antibiotics such as carbapenems necessary.[28,29] Though the rates of cephalosporin resistance are lower in other countries in comparison to India but the growing prevalence of ESBL producers is sufficient to drive a greater dependence on carbapenems. Along with time the selection pressure for

carbapenem resistance in Enterobacteriaceae will increase leading to its widespread prevalence. This will be a worldwide public health concern, since there are very few antibiotics beyond carbapenems.[30] Already *Klebsiella pneumonia* clones with KPC carbapenemase are a major problem in the USA, Greece, and Israel, and plasmids encoding the VIM metallo-carbapenemase have disseminated among *K pneumoniae* in Greece.[30] Most investigators in India have used phenotypic methods, and have reported prevalence ranging from 6.6 to 88.8 per cent prevalence of ESBLs. There are a quite a few reports of molecular identification of beta lactamases in India. In south India Sekar et al.[31] reported the prevalence of blaCTX-M gene in the 39 selected clinical isolates. The prevalence of blaCTX-M was reported by Shahid et al., 2006[32] and 72 (77.4%) of the 93 *E.coli* isolates were found to be CTX-M group -1 positive by PCR in north Indian isolates.

In conclusion, Non-prescription sale and use of antibiotics in India is leading to selection pressure on ESBL producing *E.coli*.[33] This increase in resistance is of great public health concern because there only few antibiotic in reserve.[34] More importantly, failure to initiate appropriate antibiotic therapy from the start appears to be responsible for higher patient mortality (35) to avoid this indiscriminate use of antibiotic and to control the selection pressure over ESBL producing *E.coli* population, knowledge of local and recently susceptibility trends is useful. There should be constant vigilance over the local ESBL prevalence as there is variation in report of ESBL positive samples in different parts of India. Our report gives brief insight of susceptibility pattern of *E.coli* in Kalaburagi urban areas. A proper empirical therapy can be designed using this data.

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Conflict of interest statement
We declare that no conflict of interest.

REFERENCE

1. Pitout JD. Infections with extended-spectrum beta-lactamase-producing Enterobacteriaceae: changing epidemiology and drug treatment choices. Drugs, 2010; 70: 313-33.
2. Bradford, P. A. Extended-spectrum b-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. Clinical Microbiology Reviews, 2001; 14: 933–51.
3. Bonnet, R. Growing group of extended-spectrum b-lactamases: the CTX-M enzymes. Antimicrobial Agents and Chemotherapy, 2004; 48: 1–14.
4. Bauernfeind, A., J. M. Casellas, M. Goldberg, M. Holley, R. Jungwirth, P. Mangold, T. Rohnisch, S. Schweighart, and R. Wilhelm. A new plasmatic cefotaximase from patients infected with *Salmonella typhimurium*. Infection, 1992; 20: 158–163.
5. Poirel, L., Kampfer, P. & Nordmann, P. Chromosomeencoded Ambler class a b-lactamase of *Kluyvera georgiana*, a probable progenitor of a subgroup of CTX-M extended spectrum blactamases. Antimicrobial Agents and Chemotherapy, 2002; 46: 4038–40.
6. Hsueh PR, Hoban DJ, Carmeli Y, et al. Consensus review of the epidemiology and appropriate antimicrobial therapy of complicated urinary tract infections in Asia-Pacific region. J. Infect, 2011 Aug; 63(2): 114-23.
7. Shobha, K.L., G.S. Rao, S. Rao and C.K. Sreeja, Outcome of cephalosporin treatment for serious Prevalence of extended spectrum beta-lactamases in infections due to apparently susceptible organisms urinary isolates of *Escherichia coli*, *Klebsiella* and producing extended-spectrum -lactamases: *Citrobacte*r species and their antimicrobial Implications for the clinical microbiology laboratory. Susceptibility pattern in a tertiary care hospital. J. Clin Microbiol., Indmedica – Ind. J. Pract Doctor, 3(6). 22. Cognacs, S., L. Gualco, S. Roveta, S. Mannelli, 2007; 39: 2206-2212.
8. Radice, M., Power, P., Di Conza, J. et al. Early dissemination of CTX-M-derived enzymes in South America. Antimicrobial Agents and Chemotherapy, 2002; 46: 602–4.
9. Walther-Rasmussen, J. & Hoiby, N. Cefotaximase (CTX-M-ases), an expanding family of extended-spectrum b-lactamases. Canadian Journal of Microbiology, 2004; 50: 137–65.
10. Baraniak, A., Fiett, J., Sulikowska, A. et al. Countrywide spread of CTX-M-3 extended-spectrum b-lactamase-producing microorganisms of the family Enterobacteriaceae in Poland. Antimicrobial Agents and Chemotherapy, 2002; 46: 151–9.
11. Bou, G., Cartelle, M., Tomas, M. et al. Identification and broad dissemination of the CTX-M-14 b-lactamase in different Escherichia coli strains in the northwest area of Spain. Journal of Clinical Microbiology, 2002; 40: 4030–6.
12. Bauer AW, Kirby WMM, Antibiotic susceptibility testing of a standardized single disk method. Am J Clin Pathol, 1966; 36: 493-496.
13. Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing; Approved Standards M2-A7, Eighteenth Informational Supplement. Wayne, PA: CLSI document M100-S18, 2008.
14. Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing; Nineteenth Informational Supplement. Wayne, PA: CLSI document M100-S19, 2009.

15. Woodford, N., E.J. Fagan and Ellington, M.J. Multiplex PCR for rapid detection of genes encoding CTX-M extended-spectrum -lactamases. J Antimicrob Chemother, 2006; 57: 154-155.
16. Jemima SA, Varghese S. Molecular characterization of nosocomial CTX-M type β-lactamase producing Enterobacteriaceae from a tertiary care hospital in South India. Indian J Med Microbiol, 2008; 26: 365–368. doi: 10.4103/0255-0857.43581. [PubMed] [Cross Ref]
17. Kaur M, Aggarwal A. Occurrence 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: 642-5.
18. Hawser SP, Bouchillon SK, Hoban DJ, Badal RE, Hsueh PR, Paterson DL. Emergence of high levels of extended-spectrum-β-lactamase-producing gram-negative bacilli in the Asia-Pacific region: data from the Study for Monitoring Antimicrobial Resistance Trends (SMART) Program. Antimicrob. Agents Chemother, 2009; 53: 3280–3284.
19. Hawkey PM. Prevalence and clonality of extended-spectrum beta-lactamases in Asia. Clin Microbiol Infect, 2008; 14: 159-65.
20. Mohamudha Praveen R, Manivannan S, Harish BN, Parija SC. Study of CTX-M type of Extended Spectrum β-lactamase among Nosocomial Isolates of *Escherichia coli and Klebsiella pneumoniae* in South India. Indian J Microbial, 2012; 52: 35-40.
21. Shukla I, Tiwari R, Agrawal M. Prevalence of extended spectrum-lactamase producing *Klebsiella pneumoniae* in a tertiary care hospital. Indian J Med Microbiol, 2004; 22: 87-91.
22. Livermore DM, Canton R, Gniadkowski M, et al. CTX-M: changing the face of ESBLs in Europe. J Antimicrob Chemother, 2007; 59: 165–74.
23. Harish BN, Menezes GA, Shekatkar S, Parija SC. Extended spectrum beta-lactamase-producing *Klebsiella pneumoniae* from blood culture. J Med Microbiol, 2007; 56: 999–1000.
24. Livermore DM. Has the era of untreatable infections arrived? J Antimicrob Chemother, 2009; 64(suppl 1): i29–36.
25. Nordmann P, Cuzon G, Naas T. The real threat of *Klebsiella pneumoniae* carbapenemase-producing bacteria. Lancet Infect Dis, 2009; 9: 228–362622.
26. Moland ES, Kim S-Y, Hong SG, Thomson KS. Newer β- lactamases: clinical and laboratory implications, Part II. Clin Microbiol Newsl, 2008; 30: 79-85.
27. Menon T, Bindu D, Kumar CP, Nalini S, Thirunarayan MA.Comparison of double disc and three dimensional methods to screen for ESBL producers in a tertiary care hospital. Indian J Med Microbiol, 2006; 24: 117-20, 21.
28. Harish BN, Menezes GA, Shekatkar S, Parija SC. Extended spectrum beta-lactamase-producing *Klebsiella pneumoniae* from blood culture. J Med Microbiol, 2007; 56: 999–1000.
29. Livermore DM. Has the era of untreatable infections arrived? J Antimicrob Chemother, 2009; 64(suppl 1): i29–36.
30. Nordmann P, Cuzon G, Naas T. The real threat of *Klebsiella pneumoniae* carbapenemase-producing bacteria. Lancet Infect Dis, 2009; 9: 228–3626.
31. Sekar B, Shwetha R, Arunagiri K, Menaka K, Lalitha P Aparna V, et al. Detection and characterization of bla CTX-M gene by PCR-RFLP analysis among third generation cephalosporin resistant Gram negative isolates. Proceedings of MICROCON 2006. XXX National Congress of Indian Association of Medical Microbiologists, 2006 October 27-29; Government Medical College, Nagpur. MICROCON, 2006; OB-17: 27.
32. Shahid M, Singhal M, Malik A, Shukla I, Khan HM. ESBL phenotypes and prevalent genotype of CTX-M type beta lactamases in clinical isolates of E.coli in a North Indian tertiary care hospital. Proceedings of MICROCON 2006. XXX National Congress of Indian Association of Medical Microbiologists, 2006 Oct 27-29; Government Medical College, Nagpur. MICROCON, 2006; 46: 70.
33. Ghafur AK. An obituary—on the death of antibiotics! J Assoc Physician India, 2010; 58: 143–44.
34. Woodford N, Ward ME, Kaufmann ME, Turton J, Fagan EJ, James D, et al. Community and hospital spread of *Escherichia coli* producing CTX-M extended-spectrum beta-lactamases inThe UK. J Antimicrob Chemother, 2004; 54: 735–43.
35. Tumbarello M, Sanguinetti M, Montuori E, Trecarichi EM, Posteraro B, Fiori B, et al. Predictors of mortality in patients with bloodstream infections caused by extended-spectrum-beta-lactamase-producing Enterobacteriaceae: importance of inadequate initial antimicrobial treatment. Antimicrob Agents Chemother, 2007; 51: 1987-94.
36. Rumana Mowla KM, Imam AH, Asaduzzaman M, Raihan SZ, Chowdhury AKA, Nasrin N. Emergence of Multidrug Resistant Extended Spectrum Beta Lactamase producing Escherichia coli associated with Urinary tract infection in Bangladesh. J of Basic and Clin Pharm, 2011; 3(1): 225-228.