

Status of extended spectrum beta lactamase producing *Escherichia coli* and *Klebsiella* species in urinary tract infection

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Background: Production of Extended Spectrum Beta Lactamases (ESBLs) is an important mechanism of beta-lactam resistance in Enterobacteriaceae. And the occurrence of ESBL producing isolates has increased worldwide.

Methods: The study was carried out in bacteriology laboratory of TUTH from March 2005 to May 2005 that included 371 midstream urine samples (msu). During these three months period, the MSU samples were investigated by conventional semi-quantitative culture technique, antibiotic susceptibility test and ESBL detection test.

Results: Out of 98 significant positive cases, 60 cases were due to *E. coli*, 5 were due to *Klebsiella oxytoca* and 4 were due to *Klebsiella pneumoniae*. Multidrug resistance (MDR, resistant to two or more than two classes of antibiotics) was observed in 61.7% (37/60) of *Escherichia coli*, 40% (2/5) of *Klebsiella oxytoca* and 75% (3/4) of *Klebsiella pneumoniae*. Out of total 69 cases of *E. coli* and *Klebsiella* species that were subjected to preliminary Ceftazidime ESBL screening test, 22 isolates were suspected of ESBL production. Finally 27.5% (19/69) were confirmed for production of ESBL by Double Disc Synergy Test (DDST). Out of 19 ESBL producing strains, 16 were found out to be *E. coli*, one was *K. oxytoca* and 2 were *K. pneumoniae*. All ESBL producing strains were found to be multidrug resistant.

Conclusion: There is a great need to conduct this type of study throughout the year to determine their seasonal variation and also for the study of increasing trends of MDR and ESBL. This type of study should also be conducted in other hospital as well.

Keywords: UTI, Extended Spectrum Beta Lactamase (ESBL), *Escherichia coli*, *Klebsiella* species, DDST

Introduction

ESBLs are group of enzymes occasionally present in *Klebsiella* species and *Escherichia coli* that confer upon the bacteria the additional ability to hydrolyze the β -lactam rings of oxyimino- third and second generation cephalosporins (ceftaxime, ceftazidime and ceftriaxone) and/or aztreonam¹. These β -lactamases generally belong to Ambler's molecular class A² and Bush's functional class

2b³. They are susceptible to β -lactamase inhibitors such as clavulanic acid⁴ but do not effect cefamycins as cefoxitin, cefotetan and cefmetazole and carbapenems as imipenem and meropenem⁵.

ESBLs include: Cephalosporin-hydrolysing mutants of TEM and SHV - the common plasmid-mediated penicillinases of Enterobacteriaceae (over 100 such variants are known)⁶; CTX-M types that evolved separately, at least some of them

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via the escape and mutation of chromosomal β -lactamases of *Kluyvera* spp. (over 30 variants are known) ⁷ and Obscure types, e.g. VEB, PER and OXA (Class D) ESBLs from *Pseudomonas aeruginosa*.

ESBLs are not the sole β -lactamases to confer resistance to 2nd and 3rd generation cephalosporins, but are the most important. They should be distinguished from other important modes of resistance to 2nd and 3rd generation cephalosporins ⁸ as hyperproduction of chromosomal AmpC β -lactamases, especially in *Enterobacter* spp. β -lactamases, in *Klebsiella* spp. and *E. coli* (rare); hyperproduction of K1 or KOXY chromosomal β -lactamases in *K. oxytoca* (not *K. pneumoniae*); efflux-mediated resistance in *P. aeruginosa* and various ill-defined mechanisms in *Acinetobacter* spp.

ESBLs are clinically important because they destroy cephalosporins given as first-line agents to many severely ill patients, and delayed recognition and inappropriate treatment of severe infections caused by ESBL producers with cephalosporins has been associated with increased mortality ⁹. Many ESBL producers are multi-resistant to non- β -lactam antibiotics such as quinolones, aminoglycosides and trimethoprim, narrowing treatment options.

The National Committee for Clinical Laboratory Standards (NCCLS) has developed broth microdilution and disk diffusion screening tests using selected antimicrobial agents ¹⁰. Each *Klebsiella pneumoniae*, *K. oxytoca*, or *Escherichia coli* isolate should be considered a potential ESBL-producer if the test results are as follows:

Disc diffusion	MICs
cefpodoxime ≤ 22 mm	cefpodoxime ≥ 2 g/ml
ceftazidime ≤ 22 mm	ceftazidime ≥ 2 g/ml
aztreonam ≤ 27 mm	aztreonam ≥ 2 g/ml
cefotaxime ≤ 27 mm	cefotaxime ≥ 2 g/ml
ceftriaxone ≤ 25 mm	ceftriaxone ≥ 2 g/ml

Enterobacteriaceae isolates resistant to any indicator cephalosporin in the screening tests outlined above should be subjected to confirmatory tests. Confirmation of ESBL production depends on demonstrating synergy between clavulanate and those indicator cephalosporin(s) to which the isolate was initially found resistant. Methods that can be used for confirmation of ESBL production include: Double Disc Synergy Tests (DDST), Combination disc methods and Etest ESBL strips methods. *K. pneumoniae* ATCC 700603 (positive control) and *E. coli* ATCC 25922 (negative control) are supposed to be used for quality control of ESBL tests ^{10,11}.

As the incidence of antimicrobial resistance rises, the costs associated with consequences also do and hence can be

considered an economic burden to society more so in context of developing country like Nepal. Antibiotic susceptibility profile and reporting of drug resistant strain especially ESBL producing strains would enlighten the appropriate antibiotic therapy and would help in global awareness towards misuse and overuse of antibiotics. Therefore this study was conducted with the objective of studying the aetiological agents causing UTI and their antibiotic susceptibility profile with the special reference to Extended Spectrum Beta Lactamases producing strains.

Material and Methods

Study Population: The present research work was conducted in the well-equipped laboratory of Bacteriology section of Tribhuvan University Teaching Hospital, one of the major hospitals of Kathmandu valley, from March 2005 to May 2005. During the research period, 371 midstream urine specimens, collected from patients that were suspected of Urinary Tract Infection (UTI), was enrolled. The age of these patients ranged from 8 to 86 years.

Culture, Identification and Antibiotic susceptibility test: Semi quantitative culture technique was used to culture urine specimens and to detect the presence of significant bacteriuria. An inoculating loop of standard dimension was used to take up approximately fixed ($\pm 10\%$ error is accepted) and known volume of mixed uncentrifuged urine was inoculated on the surface of 5% Blood Agar (Oxoid, England) and MacConkey Agar (Oxoid, England). The inoculated MA and BA were incubated at 37°C for 24 hours. After incubation the number of colonies was counted on each plate and organisms per ml was estimated. The bacterial isolates in the plates was identified if significant growth occurs ($\geq 10^5$ CFU/ml). Blood agar was observed for haemolysis and MacConkey agar for lactose fermentation.

Identification of significant isolates was done by using standard microbiological techniques ¹² that included study of colony morphology, Gram staining and biochemical tests (catalase test, oxidase test, TSI test, SIM test, Citrate Utilization test, Urea hydrolysis test and others as required).

Antibiotic susceptibility test of all the isolates was performed by Kirby Bauer disc diffusion method ¹³ as recommended by National Committee for Clinical Laboratory Standards (NCCLS). In this method, the broth culture of test organism (comparable to McFarland tube no.0.5; inoculum density 1.5×10^8 organisms per ml) was uniformly carpeted on the surface of Mueller Hinton agar (Oxoid). Then, antibiotic discs were seeded over the lawn culture of test organism. The inoculated and seeded MHA plate was then incubated at 37°C for 18 hours (or overnight). After incubation the zone diameter of each antibiotic was interpreted using the

interpretative chart and the organism was reported “resistant”, “moderately/intermediate susceptible” or “susceptible. Control strains (ATCC) were used in parallel as a part of quality control test system.

Antibiotics used for gram negative bacteria were Ampicillin (10 µg per disc, Oxoid), Ciprofloxacin (5 µg per disc, Oxoid), Cephalexin (30 µg per disc, Oxoid), Norfloxacin (10 µg per disc, Oxoid), Nitrofurantoin (300 µg per disc, Oxoid) and Ceftazidime (30 µg per disc, Oxoid). Multi-drug Resistant isolates were defined as those, which showed resistance to two or more than two classes of antibiotics of the first line used for the sensitivity testing.

Laboratory Detection of Extended Spectrum Beta Lactamase Producing Strain:

Screening test for ESBL detection: The indicator drug (Ceftazidime, 30 µg per disc, Oxoid) was included in primary susceptibility testing. As directed by NCCLS^{10,11}, the organisms that gave ≤ 22 mm diameter of zone of inhibition, were suspected of ESBL production i.e., regarded as ESBL screening test positive.

Confirmatory test for ESBL detection (Double Disc Synergy Test or DDST): *E. coli* and *Klebsiella* species that were suspected as ESBL producing strains in the screening test were subjected to the phenotypic confirmatory test. The Double Disc Synergy Test or DDST was performed by standard disc diffusion assay on Mueller Hinton Agar.

A MHA plate was inoculated with Nutrient broth culture of organism (comparable to McFarland nephelometer tube no. 0.5) as for a routine susceptibility tests. Discs containing cefotaxime (30 µg per disc, Oxoid) and ceftazidime (30 µg per disc, Oxoid) were applied either side of one with Co-amoxycylav (20+10 µg per disc, Oxoid). The distance between Co-amoxycylav and either third generation cephalosporin was adjusted 20 to 30 mm (center to center) or 15 mm (edge to edge) depending on the species^{14, 15}.

Enhancement of the inhibition zone towards the disc of Co-amoxycylav (clavulanic acid effect with either screening agent) was considered and confirmed suggestive of ESBL producing strain.

Results

Out of 371 MSU samples 98 (26.4%) samples showed monomicrobial significant growth (i.e. $\geq 10^5$ CFU/ml), 218

Table 1: Pattern of bacterial Isolates and Multidrug Resistance among urinary isolates

SN	Organism	Number of cases	Total% N=98	Total MDR	Total MDR%
1	<i>Escherichia coli</i>	60	61.2	37	61.7
2	<i>Klebsiella oxytoca</i>	5	5.1	2	40.0
3	<i>Klebsiella pneumoniae</i>	4	4.1	3	75.0

Table 2: Antibiotic susceptibility profile of MDR strains in urinary tract infection

S.N	Organism	Antibiotics used	Antibiotic Susceptibility Profile ^a				
			R	R %	I	I%	S %
1	<i>Escherichia coli</i> (N=37)	Ampicillin	37	100	0	0	0
		Ciprofloxacin	30	81.1	0	0	18.9
		Cephalexin	25	67.6	7	18.9	13.5
		Norfloxacin	31	83.8	2	5.4	10.8
		Nitrofurantoin	8	21.6	4	10.8	67.6
		Ceftazidime	17	45.9	0	0	54.1
2	<i>Klebsiella pneumoniae</i> (N=3)	Ampicillin	3	100	0	0	0
		Ciprofloxacin	3	100	0	0	0
		Cephalexin	2	66.7	0	0	33.3
		Norfloxacin	3	100	0	0	0
		Nitrofurantoin	2	66.7	0	0	33.3
		Ceftazidime	1	33.3	1	33.3	33.3
3	<i>Klebsiella oxytoca</i> N=2	Ampicillin	2	100	0	0	0
		Ciprofloxacin	2	100	0	0	0
		Cephalexin	2	100	0	0	0
		Norfloxacin	2	100	0	0	0
		Nitrofurantoin	0	0	0	0	100
		Ceftazidime	1	50	0	0	50

^a Abbreviations: R, Resistant; I, Intermediate or Moderately Sensitive; S, Sensitive

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(58.7%) samples were sterile, 34 (9.2%) samples showed insignificant growth (i.e. $<10^5$ CFU/ml) and 21 (5.7%) samples showed polymicrobial insignificant growth (i.e. more than two types of isolates).

Out of 98 significant culture positive cases, 83 (84.7%) were due to gram negative bacteria and 15 (15.3%) were due to gram positive bacteria. Eleven different species of bacteria were isolated among which *Escherichia coli* (61.2%) was found to be the most common one followed by *Klebsiella* species (9.2%), *Pseudomonas aeruginosa* (7.1%), *Enterococcus faecalis* (6.1%), *Staphylococcus aureus* (6.1%) and *Proteus mirabilis* (4.1%). The other organisms are *Citrobacter freundii* (2.0%), *CONS* (2.0%), *Morganella morganii* (1.0%) and *Staphylococcus saprophyticus* (1.0%).

Among the multidrug resistant *Escherichia coli*, 100% isolates were found to be resistant to Ampicillin, 81.08% resistant to Ciprofloxacin and 21.62% resistant to Nitrofurantoin.

The phenotypic profile for ESBL detection test result is expressed in figure 1. Out of 69 potential isolates (excluding non- *Escherichia* and non *Klebsiella* species), 22 isolates were suspected of ESBL production i.e. Ceftazidime screen positive. And out of 22 only 19 isolates were confirmed for ESBL production in DDST.

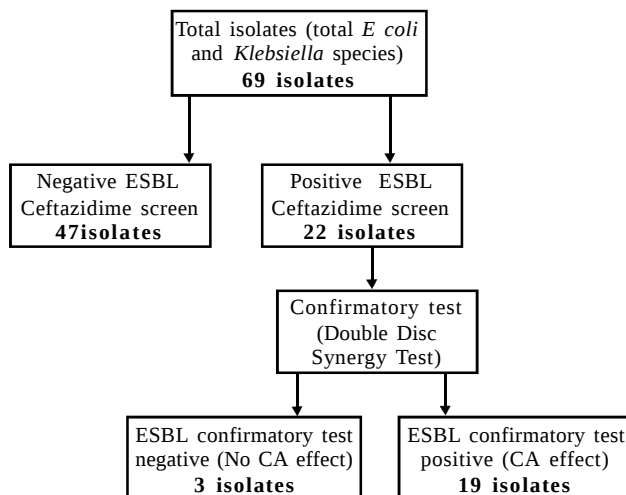


Fig. 1: Summary of phenotypic profiles of study isolates

The percentage of ESBL producing strains in *Escherichia coli* was found out to be 26.7 (16/60), and that in *K. pneumoniae* and *K. oxytoca* was found out to be 50 (2/4) and 20 (1/5) respectively. No ESBL phenotypic confirmatory test was performed for non-*Escherichia coli* and non-*Klebsiella* gram negative bacteria although they were suspected to be ESBL by preliminary ESBL screening test. Gram positive bacteria were not screened for ESBL detection.

Discussion

The increasing prevalence of antibiotic resistance is a cause of serious concern and multidrug resistance among some of the most important human pathogens is increasing. In our study Multidrug resistance was found out to be 61.7% (37/60) in *Escherichia coli* and that in *K. oxytoca* and *K. pneumoniae* was 40% (2/5) and 75% (3/4) respectively. Multidrug resistance (MDR) in our study is higher than in other studies that have same MDR criterion¹⁶. Outcome of prevalence of MDR depends on various factors, MDR criterion being the chief one followed by the types of antibiotics used in antibiogram, study isolates and study population. The emergence of MDR is clearly related to the quantity of antibiotics and how they are being used¹⁷.

First described in 1983¹⁸, ESBLs have contributed to dramatic increase in resistance to β -lactam agents among gram negative bacteria in recent years¹⁹. However laboratory detection of ESBL can be problematic, because in many cases conventional breakpoints of resistance are not reached²⁰. All *E. coli* and *Klebsiella* species isolated were subjected to phenotypic laboratory detection of ESBL production. The NCCLS has issued guidelines for ESBL screening and confirmatory tests that apply only to *E. coli* and *Klebsiella* species^{10,11}. Hospital based study of in USA²¹ demonstrated that ESBLs are detected infrequently in non *Klebsiella* and non *E. coli* isolates of Enterobacteriaceae. Other isolates of Enterobacteriaceae, such as *Salmonella* spp., *Proteus mirabilis*, *Enterobacter* spp. and *Citrobacter freundii* and isolates of *Pseudomonas aeruginosa*, *Acinetobacter* spp. and *Stenotrophomonas maltophilia* also produce ESBLs. However, at this time, methods for screening and phenotypic confirmatory testing of these isolates have not been determined by NCCLS²². NCCLS detection methods are based on a phenotypic profile that has potential to yield false positive and false negative results. In some of the isolates, additional mechanisms of resistance, such as AmpC- β -lactamases, porin changes, and inhibitor resistant TEMs (IRTs) and SHV β -lactamases with reduced affinities for β -lactamase inhibitors can mask CA inhibition²³. In addition hyperproduction of Class A 'K1' chromosomal protease by *K. oxytoca* can give positive clavulanate synergy test with cefotaxime and cefepime (never ceftazidime), so the producers are confused with ESBL producers.

Organisms inferred to have ESBLs should be reported resistant to ALL penicillins (except temocillin), cephalosporins (except cefoxitin), and to aztreonam, irrespective of routine susceptibility results. Treatment failures and death have occurred when cephalosporins were used against ESBL producers that appeared susceptible in vitro^{24,25}.

Despite the introduction of very promising molecular methods (e.g. DNA probes, PCR, nucleotide sequencing, isoelectric focusing and chip technology), the phenotypic detection tests are considered by many the simplest and most cost effective strategies for detection of ESBLs among gram negative bacteria.

Conclusion

Based on invitro susceptibility test and phenotypic Double Disc Synergy Test (DDST), it was concluded that a significant number of urinary isolates are MDR and often co-produce ESBL, which can result in unavoidable treatment failures. No doubt the reporting of MDR and ESBLs and other beta lactamases should continue to challenge treatment strategy for years to come.

Recommendations

All the hospitals and health institutes should have the provision for detection of ESBL producing strains as these are associated with treatment failure in many cases.

Even if ceftazidime and/or cefotaxime appear to be sensitive in vitro, but the zone of inhibition is ≤ 22 mm and ≤ 27 mm respectively, the organism should be suspected for ESBL production. If the organism is confirmed to do so, it should be reported to be resistant to all penicillins (except temocillin), cephalosporins (except cephamycins) and aztreonam. If the patient is not responding to a particular third generation cephalosporins, the clinicians should look for ESBL producing organisms.

Techniques for detection of enzymes with marginal ESBL activity, those expressed weakly, those produced alongside other enzymes (as AmpC, K1 protease) and techniques for detection of these AmpC, K1 protease should also be applied.

The study should be continued and the MDR and ESBL producing strains should be subjected for genetic study to acquire their detailed genetic makeup and to characterize the mechanism of drug resistance. The phenotypic methods outlined in our study will never be so precise as the best molecular methods.

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References

1. Livermore DM and Woodford N. Laboratory detection and reporting of bacteria with extended spectrum beta lactamases. Health Protection Agency. June 2004 last update. Available from : URL: <http://www.bsac.org.uk>.
2. Ambler RP. The structure of β -lactamases. *Philos Trans R Soc Lond B Biol Sci* 1980; **289**: 321-331.
3. Bush K, Jacoby GA and Medeiros AA. A functional classification scheme for β -lactamases and its correlation with molecular structure. *Antimicrob. Agents Chemother* 1995; **39**:1211-1233.
4. Philippon A, Arlet G and Jacoby GA. Plasmid-determined AmpC-type β -lactamases. *Antimicrob Agents Chemother* 2002; **46**: 1-11.
5. Nagano N, Shibata N, Saito Y, Nagano Y and Arakawa Y. Nosocomial outbreak of infections by *Proteus mirabilis* that produces Extended Spectrum CTX-M-2 type β -lactamase. *J Clin Microbiol* 2003; **41**: 5530-5536.
6. Livermore DM and Brown DF. Detection of β -lactamase-mediated resistance. *J Antimicrob Chemother* 2001; **48**:59-64. Updated version available from: URL: <http://www.bsac.org.uk/uploads>.
7. Bonnet R. Growing group of extended-spectrum β -lactamases: the CTX-M enzymes. *Antimicrob Agents Chemother* 2004; **48**: 1-14.
8. Livermore DM, Threlfall EJ, Reacher, MH, Johnson AP, James D and Cheasty T. Are routine sensitivity test data suitable for the surveillance of resistance. Resistance rates amongst *Escherichia coli* from blood and CSF from 1991–1997, as assessed by routine and centralized testing. *J Antimicrob Chemother*.2000; **45**: 205–11.
9. Paterson DL, Ko WC and Von Gottberg A. International prospective study of *Klebsiella pneumoniae* bacteremia: implications of extended-spectrum β -lactamase production in nosocomial infections. *Ann Intern Med* 2004; **140**: 26-32.
10. National Committee for Clinical Laboratory Standards. Performance standards for antimicrobial susceptibility testing. Ninth informational supplement, M100-S9. National Committee for Clinical Laboratory Standards, Wayne, Pa; 1999.
11. National Committee for Clinical Laboratory Standards. Performance standards for antimicrobial disk susceptibility tests, 6th ed., vol. 17, no. 1. Approved standard M2-A6. National Committee for Clinical Laboratory Standards, Wayne, Pa; 1997.
12. Cheesbrough M. Medical laboratory manual for tropical countries vol-II: Microbiology. 1st ELBS edition.

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Cambridge :University Press, 1984.

13. Bauer AW, Kirby WMM, Sherris JC and Truck M. Antibiotic susceptibility testing by a standardized single disc method. *American Journal of Pathology*. 1966; **45**: 493-496.
14. Jarlier V, Nicolas MH, Fournier G and Philippon A. Extended broad-spectrum β -lactamases conferring transferable resistance to newer β -lactam agents in Enterobacteriaceae: hospital prevalence and susceptibility patterns. *Rev. Infect. Dis* 1988; **10**: 867-878.
15. Coudron PE, Hanson ND and Climo M. Occurrence of extended spectrum and AmpC beta lactamase in bloodstream of *K. pneumoniae*: Isolates harbour plasmid Fox-5 and ACT-1 AmpC Beta lactamases. *J. Clin. Microbiol* 2003; **41**: 722-727.
16. Shrestha B, Basnet RB, Shrestha P and Shahi P. Prevalence of urinary tract infection in female patients attending Kathmandu Model Hospital. *Journal of Nepal Association for Medical Laboratory sciences* 2005; **7**:10-14.
17. Levy SB. Factors impacting on the problem of antibiotic resistance. *J Antimicrob Chemther* .2002; **49**: 25-30.
18. Knothe H, Shah P, Krcmery V, Antal M and Mitsuhashi S. Transferable resistance to cefotaxime, cefoxitin, cefamandole and cefuroxime in clinical isolates of *Klebsiella pneumoniae* and *Serratia marcescens*. *Infection* 1983;**11**: 315-7.
19. Bradford PA. Extended-spectrum β -lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. *Clin Microbiol Rev* 2001; **14**: 933-951.
20. Katsanis, GP, Spargo J, Ferraro MJ, Sutton L and Jacoby GA. Detection of *Klebsiella pneumoniae* and *Escherichia coli* strains producing extended-spectrum β -lactamases. *J Clin Microbiol* 1994; **32**: 691-696.
21. Schwaber MJ, Raney PM, Rasheed JK, Biddle JW, Williams P, McGowan JE and Tenover FC. Utility of NCCLS guidelines for identifying extended spectrum β -lactamases in non-*E. coli* and non-*Klebsiella* spp. of Enterobacteriaceae. *J Clin Microbiol* 2004; **42**: 294-298
22. Coudron PE, Moland ES and Sanders CC. Occurrence and detection of extended spectrum β -lactamases in members of the family Enterobacteriaceae at a Veterans Medical Center: seek and you may find. *J. Clin. Microbiol* 1997; **35**: 2593-2597.
23. Sanguinetti M, Posteraro B, Spanu S, Ciccaglione D, Romano L and Fiori B.. Characterization of clinical isolates of Enterobacteriaceae from Italy by BD Phoenix extended-spectrum β -lactamase detection method. *J. Clin. Microbiol* 2003; **41**: 1463-1468.
24. Paterson DL, Ko WC and Von Gottberg A. Outcome of cephalosporin treatment for serious infections due to apparently susceptible organisms producing extended spectrum β -lactamases: implications for the clinical microbiology laboratory. *J Clin Microbiol* 2001; **39**:2206-2212.
25. Brun-Buisson C, Legrand P and Philippon AL. Transferable enzymatic resistance to third-generation cephalosporins during nosocomial outbreak of multiresistant *Klebsiella pneumoniae*. *Lancet* 1987; **2**:302-306.
26. Bradford PA. Extended-spectrum β -lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. *Clin Microbiol Rev* 2001; **14**: 933-951
27. Dahl RK, Koirala J, Khadga P, Pokhrel BM, Tuladhar NR. The status of Multidrug resistant and extended spectrum beta lactamase producing *Salmonella* isolated from blood culture. *Journal of Nepal Association for Medical Laboratory sciences* 2005; **7**(7): 24-29.
28. Baron EJ and Tenover FC, Bailey and Scott's Diagnostic Microbiology. 8th edition. St.Louis Missouri Baltimore : CV Mosby – Year book Inc, 1990.
29. Basic Laboratory Manual in Clinical Bacteriology, WHO, 1991, ISBN 9241544252
30. Bradford PA. Extended-spectrum β -lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. *Clin Microbiol Rev* 2001; **14**: 933-951.
31. Collee JG, Fraser AG, Marmion BP, Simmons A, editors. Mackie and McCartney practical microbiology. 14th edition. Newyork: Churchill Livingstone; 1996.
32. Holmes B and Gross JR. Coliform bacteria. In Parker MT, Duerden B, editors. Topley and Wilson's principles of bacteriology, virology and immunity, volume 2, systemic bacteriology. Philadelphia: B.C.Decker Inc; 1990.