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Antimicrobial Susceptibility Profile of Extended Spectrum Beta- lactamases Producing *Enterobacteriaceae* isolated from clinical samples refereed to the National Bacteriology and Mycology Reference Laboratory, Ethiopia

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Research

Keywords: Enterobacteriaceae, antimicrobial resistance, extended spectrum beta-lactamases

Posted Date: January 7th, 2020

DOI: https://doi.org/10.21203/rs.2.20224/v1

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Abstract

Background Extended-spectrum beta-lactamases (ESBL) producing Enterobacteriaceae are prevalent worldwide and they are unique challenges for treatment and control of bacterial infectious diseases. ESBL genes not only confer resistance to oximino-cephalosporins and aztreonum but also, they are multidrug-resistant to other commonly available antimicrobial agents used in clinical practice.Objective To determine the prevalence and antimicrobial susceptibility profile of ESBL producing Enterobacteriaceae isolated from clinical samples referred to the national clinical bacteriology and mycology reference laboratory.

Materials and Methods A cross-sectional study was conducted on Enterobacteriaceae culture- positive clinical samples that were referred to the national bacteriology and mycology reference laboratory from August 2018 to July 2019. Bacterial isolation was performed according to the inoculation and incubation conditions of each clinical specimen and identifications of the isolates were performed using standardized biochemical tests for gram-negative bacteria. Antimicrobial susceptibility profiles of these cultures were determined using the disk diffusion method on Muller Hinton agar according to the recommendation by Clinical and Laboratory Standard Institute (CLSI). ESBL production was detected using CLSI Screening and confirmation test. A double-disk synergy test was used for confirmation.

Results Out of 371 culture positive for Enterobacteriaceae , 240 (64.7%) were positive for ESBL production, and the most prevalent species were Klebsiella sp 131(54.6%) followed by E. coli 79 (32.9%). Of 131 ESBL positive Klebsiella spp, 95 (72.5%) were obtained from blood samples and among 79 E. coli isolates, 51 (64.6%) of the strains were isolated from urine samples. All ESBL positive isolates were resistant to ampicillin and all generation of cephalosporins. In addition, 100% of them were multidrug resistant. There were also high proportions of resistant ESBL positive isolates to other classes of antimicrobial agents. Less resistance rates were documented for carbapenems drugs and amikacin from the class of aminoglycosides.

Conclusion ESBL producing Enterobacteriaceae we reported in this study was not only highly prevalent but also they are multidrug resistant to most clinically available antimicrobial agents including carbapenems. Therefore, public awareness and regular monitoring

Introduction

Pathogenic organisms resistant to antimicrobial agents have become a worldwide public health threat because of their great impact on morbidity and mortality as well as length of hospital stay and on treatment and diagnosis costs. The significant infections are those caused by multidrug resistant organisms showing resistance to various antimicrobial agents commonly used in clinical practices [1–2].

Eneterobacteriaceae producing extended spectrum beta-lactamases (ESBL) are known in developing multidrug resistance which challenges the treatment of infections caused by theses group of organisms. They are the main agents of hospital and community acquired infections throughout the world [3].

Extended spectrum beta-lactamses producing organisms are resistance to the action of extended spectrum cepahlosporins such as third and fourth generation cephalosporins and monobactams such as aztreonam [4]. ESBLs are encoded on plasmids born genes and also located on integrons and transposons which facilitate the dissemination of these resistance genes within or between different genera of bacteria [5]. The genes those encode resistance determinants for other classes of antimicrobial agents are also carried on plasmids encoded ESBL genes. These included resistant genes for trimethoprim, sulphonamides, tetracyclines, aminoglycosides, chloramphenicol and fluoroquinolones [6].

ESBLs producing bacteria may also hydrolyze carbapenems drugs including impenem, meropenem and ertapenem. This is commonly true in ESBL positive organisms producing Klebsiella pneumoniae carbapenemase (KPC) when ESBL production is associated with chromosomal AmpC-type beta-lactamases [7]. Thus, Enterobacteriaceae harboring ESBLs are multidrug resistant for multiple antimicrobial classes with very limited treatment options available which significantly impact patient outcomes [8].

In Ethiopia, several studies have been done that revealed alarming level of bacterial diversity in various clinical specimens and the majority of those isolates were multidrug resistant (MDR) [10–11]. However, the studies that show the impact of ESBL production on the susceptibility patterns of commonly used antimicrobial agents in clinical practice are limited. Therefore, this study was designed to investigate the occurrence and antimicrobial resistance profile of ESBL producing Enterobacteriaceae from clinical specimens referred to the National clinical Bacteriology and Mycology reference laboratory.

Materials And Methods

A cross-sectional study was done on culture positive routine clinical specimens for *Enterobacteriaceae* from August 2018 to July 2019 at Clinical Bacteriology and Mycology National Reference Laboratory (NRL), Ethiopian Public Health Institute. NRL is a biosafety level two laboratory that serve as a reference laboratory for hospitals. The laboratory is accredited for identification and antimicrobial susceptibility tests on bacteria isolated from various specimens by Ethiopian National Accreditation Office (ENAO). The laboratory also performs fungal culture on different clinical specimens. Ethical clearance for this study was obtained from the Ethiopian Public Health institute scientific and ethical review committee

Bacterial culture and identification

Enterobacteriaceae included in this study were obtained from five clinical specimen types namely, blood, body fluids, pus, sputum and urine. They were routinely cultured according to the standardized laboratory protocol for each sample.

The identification of *Enterobacteriaceae* from positive samples was performed using colony morphology on culture plates and conventional biochemical techniques according to the laboratory guide line used for the identification of Gram negative bacterial pathogens.

Antimicrobial Susceptibility tests

Antimicrobial susceptibility test was performed using standardized Kirby Bauer disk diffusion method on Mueller Hinton agar (Oxoid LTD, Basingstoke, Hampshire, England) as recommended by the Clinical and Laboratory Standard Institute [11]. Antibiotics agents that were tested in this study include; ampicillin (10 µg), piperacillin (100 µg), gentamicin (10 µg), tobramycin (10 µg), amikacin (30 µg), pipracillin-tazobactam (100/10 µg), amoxicillin-clavulanic acid (20/10 µg), doripenem (10 µg), ertapenem (10 µg), imipenem (10 µg), meropenem (10 µg), cefazolin (30 µg), cefuroxime (30 µg), cefotaxime (30 µg), ceftriaxone (30 µg), ceftazidime (30 µg), cefepime(30 µg), ciprofloxacin(5 µg), nalidixic acid (30 µg), norfloxacin (10 µg) and Sulphamethoxazole-Trimethoprim(23.75/1.25 µg). Antibiotics susceptibility results were interpreted according to the CLSI zone size interpretive standards. *E. coli* ATCC 25922 was used as control organism for antibiotic susceptibility testing. Multidrug resistant was defined according to the guideline combined by the European Center for Disease prevention and Control (ECDC) and the Center for Disease Control and Prevention (CDC). Accordingly, bacterial isolates that were non susceptible to at least one antimicrobial agent in three different classes of drugs were considered as MDR [12].

Phenotypic Detection of ESBL producing *Enterobacteriaceae*

All *Enterobacteriaceae* resistances to third generation cephalosporins (cefotaxim, and ceftazidim) and fourth generation cephalosporin (cefepim) were classified as ESBL producer and further detected for ESBL production according to the Clinical and Laboratory Standards institute recommended double disk synergy test (CLISI 2018). Briefly, ESBL producer were detected by using a disc containing cefotaxime (CTX,30 µg) or ceftazidime (CAZ, 30 µg) in combination with and without clavulanate (CLA, 10 µg) (Oxoid; UK). The increase in zone size diameter by a 5 ≥ mm for CTX/CLA and CAZ/CLA when compared with those CTX and CAZ alone was determined as the presence of ESBL. *E. coli* ATCC 25922 ESBL negative and *K. pneumoniae* ESBL positive were used as reference strains.

Data analysis

The data were entered and cleaned using Microsoft excels and imported to SPSS version 20.0. The frequency of total culture positive, ESBL producers and corresponding clinical samples were calculated. Cross-tabulation was used to present the different relation between data. Proportions and the actual number of *Enterobacteriaceae* and the susceptibility patterns of the isolates were used to describe frequency of categorical variables. The data were presented in table and graphs.

Results

Bacterial Strains

A total of 371 clinical specimens that were culture positive for Enterobacteriaceae were considered for this study. Among these, 240 (64.7%) of the bacterial isolates had a positive screening tests for ESBL

production. The most prevalent species was Klebsiella sp 131 (54. 5%) followed by E .coli 79 (32.9%), Eneterobacter sp 16 (6.7%), Proteus spp 11 (4.6%) and Citrobacter spp 3 (1.3%) (Table 1).

Table 1
Distribution of ESBL positive Enterobacteriaceae according to sex and age of study participants

Variables		Organisms (n = 240)				
		Citrobacter sp n = 3 (%)	E. coli n = 79 (%)	Enterobacter sp n = 16 (%)	Klebsiella sp n = 131 (%)	Proteus sp n = 11 (%)
Sex	Female 100(41.7)	2 (75.0)	36 (45.6)	4 (25.0)	57 (43.5)	1 (9.1)
	Male 140 (58.3)	1 (25.0)	43 (54.4)	12 (75.0)	74 (56.5)	10 (90.9)
Age group	≤ 1 year 84 (35.0)	0 (0.0)	5 (6.3)	3 (18.8)	76 (58.0)	0 (0.0)
	> 1–20 25 (10.4)	0 (0.0)	12 (15.2)	0 (0.0)	11 (8.4)	2 (18.2)
	21–30 32 (13.3)	1 (25.0)	10(12.6)	4 (25.0)	14 (10.7)	3 (27.3)
	31–40 27 (11.3)	1 (25.0)	11 (13.9)	4 (21.4)	12 (25.0)	3 (27.3)
	41–50 23 (9.6)	1 (25.0)	14(17.7)	2 (12.5)	6 (4.6)	0 (0.0)
	51–60 14 (5.8)	0 (0.0)	8 (10.1)	1 (6.3)	5 (3.8)	0 (0.0)
	> 60 35 (14.4)	0 (0.0)	18 (22.9)	2(12.5)	11 (8.4)	3 (27.3)

With respect to patients' demographic variables among positive culture results, the frequency of males infected with ESBL producing isolates were more than females with 140 (58.3%) and 100 (41.7%), respectively. The mean age of the study participants was 27.1 ± 25.7 years and patients were divided as follows into ages group of ≤ 1, > 1–20, 21–30, 31–40, 41–50, 51–60 and > 60 years. The majority of infected patients ESBL positive Enterobacteriaceae were young children ≤ 1 year of age 85 (35.0%) followed by elderly > 60 years 35 (14.4%) (Table 1).

ESBL producing Enterobacteriaceae strains were mainly found in blood 111 (46.3%), in urine 74 (30.8) and pus 37 (15.4%). Klebsiella sp was the most predominant bacterial strains isolated from blood

samples while E. coli was predominantly recovered from patients with urinary tract infections (Fig. 1 and Fig. 2).

Antimicrobial susceptibility and ESBL production in Enterobacteriaceae

The antimicrobial susceptibility results were analyzed for 240 ESBL positive and 131 ESBL negative isolates. Accordingly, 100% of the ESBL positive isolates were resistant to ampicillin and all generations (first to fourth) of cephalosporins. The non ESBL isolates have also shown very high resistance rate to ampicillin (82.4%). However, the extended spectrum cephalosporin antimicrobials agents were proved to be effective for non-ESBL-producing strains.

The high percentage of ESBL producing isolates also showed resistance to other antimicrobial agents particularly for piperacillin (95.4%), sulphamethoxazole-trimethoprim (92.1%), and tetracycline (88.3%). The non-ESBL isolates have also shown significant rate of resistance to these antimicrobial agents with 66.4% for piperacillin, 64.9% for sulphamethoxazole-trimethoprim and tetracycline, and 53.4 for amoxicillin-clavulanic acid.

Reduction in susceptibility for ciprofloxacin in ESBL positive isolates were 75% which is quite higher than that of resistance rate exhibited by ESBL negative isolates (47.4%).

Norfloxacin and nitorofurantoin were only tested for urine samples isolates and a very high percentage (92.5%) of ESBL positive strains showed reduced susceptibility to norfloxacin while only 50.4% of the non-ESBL producing isolates were resistant to this agent. The isolates showed relatively lower resistance patterns to nitorofurantoin. However, the resistance rate of the isolates to the antibiotic is still higher in ESBL positive organisms.

The susceptibility rate to carbapenems antibiotic agents such as imipenem and morepenem and amikacin in the class of aminoglycoside of antibiotic were more than 80%. Yet, in comparison with non-ESBL positive isolates, ESBL positive isolates were highly resistant to these classes of antibiotics. In general, ESBL positive Enterobacteriaceae were highly resistant to all classes of antimicrobial agents tested as compare with non-ESBL isolates in which the association is statistically significant with $P < 0.001$. Moreover, 100% of ESBL positive Enterobacteriaceae analyzed in this particular study were MDR (Table 2).

Table 2
Antimicrobial susceptibility patterns of ESBL positive Enterobacteriaceae in comparison with non ESBL Enterobacteriaceae

Antimicrobial agents	**Number of Entetrobacteriaceae (n = 371)**		
ESBL positive (n = 240)	Non ESBL (n = 131)	P-value	
N (%)	N (%)		
AMP	240 (100)	108 (82.4)	< 0.001
PIP	229 (95.4)	87 (66.4)	< 0.001
AMC	213 (88.8)	70 (53.4)	< 0.001
TZP	147 (61.3)	44 (33.6)	< 0.001
CZO	240 (100)	45 (34.4)	< 0.001
CXM	240 (100)	45 (34.4)	< 0.001
CRO	240 (100)	0 (0.0)	< 0.001
CAZ	240 (100)	0 (0.0)	< 0.001
CTX	240 (100)	0 (0.0)	< 0.001
CEF	240 (100)	0 (0.0)	< 0.001
IMP	28 (11.7)	2 (1.5)	< 0.001
MEM	23 (9.8)	2 (1.5)	< 0.001
CIP	180 (75.0)	62 (47.3)	< 0.001
NOR	222 (92.5)	66 (50.4)	< 0.001
TTC	212 (88.3)	85 (64.9)	< 0.001
NIT	78 (32.5)	25 (19.1)	< 0.001
GEN	140 (58.3)	11 (8.4)	< 0.001
TOB	111 (46.3)	23 (17.6)	< 0.001
AMK	19 (7.9)	2 (1.5)	< 0.001
SXT	221 (92.1)	85 (64.9)	< 0.001

AMP: ampicillin; PIP: piperacillin; AMC: amoxicillin-clavulanate; TZP: piperacillin-tazobactam; CZO: cefazolin; CXM: cefuroxime; CRO: ceftriaxone; CAZ: ceftazidime; CTX: cefotaxime; CEF: cefepime; MER: meropenem; CIP: ciprofloxacin; NOR: norfloxacin; TTC: tetracycline; NIT: Nitrofurantoin; GEN: gentamicin; TOB: tobramycin; AMK: amikacin; SXT: trimethoprim-sulfamethoxazole

Antimicrobial agents	Number of Entetrobacteriaceae (n = 371)		
MDR	240 (100)	64 (48.9)	< 0.001

AMP: ampicillin; PIP: piperacillin; AMC: amoxicillin-clavulanate; TZP: piperacillin-tazobactam; CZO: cefazolin; CXM: cefuroxime; CRO: ceftriaxone; CAZ: ceftazidime; CTX: cefotaxime; CEF: cefepime; MER: meropenem; CIP: ciprofloxacin; NOR: norfloxacin; TTC: tetracycline; NIT: Nitrofurantoin; GEN: gentamicin; TOB: tobramycin; AMK: amikacin; SXT: trimethoprim-sulfamethoxazole

Discussion

Phenotypic screening of ESBL producing Enterobacteriaceae is essential for understanding and proper management of the development of resistance mechanisms as well as very important for epidemiology purposes [13]. In Ethiopia various fragmented studies have been conducted on ESBL producing Enterobacteriaceae in different parts of the country [10–11]. The present study was conducted at National Clinical Bacteriology and Mycology reference laboratory which is the only laboratory that gives reference service for the country. The data we obtained from the study may indicate the level of ESBL burden and its impact on antimicrobial resistance management in the country.

In current findings, ESBL production was detected in different genera of Enterobacteriaceae with overall prevalence of 64.7% in the strains tested and Klebsiella sp and E. coli were among the majority in the strains. This finding is comparable to that was reported in Addis Ababa (57.7%) [14], in Pakistan (60%) [15] and reported from Nigeria (65%) [16].

However, the current result was higher than of the study findings reported from Jima Ethiopia (51%) [17] and of study conducted in Algeria (47.6%) [18] and lower than reported from a tertiary care hospital in Riyadh capital Saudi Arabia (72%) [19]. These indicate that the distribution of ESBL producing organisms vary from region to region and may be higher in certain geographic areas [20].

Regarding the clinical samples analyzed in this study, higher rate of ESBL producers were identified from blood samples with most common isolates being klebsiella sp followed by E. coli and Enterobacter sp. Similar findings have been reported from Bahirdar Ethiopia [21], Pakistan [22] and Iran [23] and showed these organisms as the major Gram negative bacteria responsible for bloodstream infections. The incidence of these organisms was particularly very high among pediatrics population with age group of $\leq$ 1 year. This finding was in agreement with studies in Ethiopia [24] and other counties [25]. This suggests that the risk of children in acquiring bloodstream infections is greater due to different factors. These included undeveloped in immune system in the young children, poor skin integrity, frequent visit to health care facility, and the parents' socioeconomic status, poor hygiene practices and high incidence of delivery at home particularly in developing country[25]. High rate of the ESBL producing Eneterobacteriaceae were identified from elderly patients greater than 60-year-old group. This may be explained by the longer exposure of these individuals to extended spectrum cephalosporins drugs which has been well described as the age of the patients are one of the factors for antimicrobial resistance [26].

Bacteria characterized in present study showed varied degree of susceptibility with high level of resistance to all tested antimicrobial agents. These are especially very high level in ESBL positive organisms. In addition to their ability to hydrolyze the activity of common beta-lactams antimicrobial ages such as penicillins, cephalosporins and aztreonam, ESBLs producing organisms are also associated with resistance to other antimicrobial classes and as a result they manifest a multidrug resistance trait [27].

Based on the present findings, ESBL producing Enetrobacteriaceae showed high resistance rate to amoxicillin-clavulanate, the drug that are proved to inhibit the action of ESBL. This may be explained by production of chromosomal beta-lactamases (AmpC) with serine active sites which have the ability to hydrolyze cphalosporins and also resistant to beta-lactamases inhibitors including clavulanate, sulbactam and tazobactam [28].

In comparison to amoxicillin-clavulanate, piperacillin-tazobactam was relatively active against ESBL producing organisms in in present study. This was justified in study conducted by Drawz and Bonomo in that they indicated piperacillin in combination with beta-lactamases inhibitors was resistant to the hydrolysis of some plasmid mediated beta-lactamses as compared with amoxicillin or ampicillin combination with bata-lactamases inhibitors [29].

Fluoroquinolones such as ciprofloxacin and norfloxacin are used for treatment of various bacterial infections and are among the therapeutic options for infections caused by ESBL positive organisms [30]. The resistance rate of ESBL producing Enterobacteriaceae to fluoroquinolones is reported in different studies [31].These support our finding in which 75% and 92.5% of ESBL positive isolates were resistant to ciprofloxacin and norfloxacin, respectively as compared with 47.3% and 50.4% of resistance rates for non ESBL producers. This is described in that, plasmids encode ESBL genes also carry plasmid mediated quinolone resistance genes. As resistance plasmids with ESBLs encoding genes are transferred among different species of Enterobacteriaceae by conjugation, this helps for dissemination of plasmid mediated quinolones resistance genes in these group of organisms [32]. Moreover, plasmid mediated quinolones resistance genes facilitate the chromosome-encoded quinolones resistance. The chromosome-encoded quinolones resistance is the most known mechanisms of quinolones resistance due to chromosomal mutations in the quinolone resistance-determining region of genes encoding DNA gyrase (gyrA and gyrB) and topoisomerase IV (parC and parE) genes [33].

Plasmid-mediated quinolone resistance genes play also an important role in resistance to aminoglycosides [34]. This justify our study results in that the resistance rate of the ESBL positive isolates to aminoglycoside (gentamicin and tobramycin) was very high when comparing with non ESBL strains. There were also studies those identified the co-existence of as extended-spectrum beta-lactamase with qnr, aac(6')-Ibcr genes and genes encoding 16S rRNA methylases in the same ESBLs-producing strain to mediate multidrug resistance [35–36].

Similarly, the resistance rates to trimethoprim-sulfamethoxazole in ESBLs-producing strains in this study were very high. This is not surprising among ESBL positive organisms and it was well described in

various studies [37]. This indicates the coexistence of trimethoprim-sulfamethoxazole associated resistant genes such as sul1 and sul2 encoded on ESBL plasmids which facilitate the transmissions of the determinants [38].

ESBL positive isolates tested in this study showed relatively lower resistance to Nitrofurantoin, a relatively active agent against both ESBL positive and ESBL negative. Similar study results were reported from Kenya [39] and Brazil [40]. This may be due to low prescription of this drug by physicians and therefore, nitrofurantoin remains active for treatment of non-life-threatening urinary tract infections [39].

Carbapenems, such as imipenem, meropenem and ertapenem, are the most effective antibiotics for the treatment of infections caused by ESBL producing Enterobacteriaceae [41]. The rising in the prevalence of ESBL producing organisms directly associated with the increase in consumption of carbapenems led to the emergency of resistant organisms to this last generation antimicrobial agents which in turn limit the treatment option for infections caused by ESBL producing organisms [41]. In the current study, we reported high prevalence of carbapenems resistant in ESBL positive isolates. This may indicate that using carbapenems for treatment bacterial infections is widely spread in the country.

Conclusions

The prevalence of ESBL producing *Enterobacteriaceae* were found to be very high in this study. Enterobacteriae ESBL producing *Enterobacteriaceae* are public health threat not only because of the production the enzymes ESBL that hydrolyze penicillin and extended-spectrum cephalosporins, but they are multidrug resistant to most clinically available antimicrobial agents as was evident in this study. Our study also investigated the level of carbapenems resistance isolates. Therefore, the impact of ESBL producing organisms on the treatment of bacterial infection diseases in Ethiopia is potentially very high. Regular monitoring and strengthen national antimicrobial surveillance system and antimicrobial stewardship at health care facilities are very essential. Moreover, national wide studies are needed to determine the overall epidemiology of ESBL related infections and the associated clinical burden.

Limitations of the study

Phenotypic characterization of AmpC beta lactamases and carbapenemase were not included due to lack of antibiotic disks to perform these experiments. In addition molecular characterization of ESBL encoding genes was not done because our laboratory has no facility for molecular analysis. Based on resource availability we have a plan to do such experiments in the future and this publication may help us in getting collaborators.

Abbreviations

CLSI: Clinical and Laboratory Standards Institute; ECDC: Guideline combined by the European Center for Disease prevention and Control Clinical; ENAO: Ethiopian National Accreditation Office; ESBL: Extended-

spectrum betalactamases; KPC: *Klebsiella pneumoniae* carbapenemase; NRL: National Clinical Bacteriology and Mycology Reference Laboratory

Declarations

Acknowledgements

We thank patients participated in this study as well as Mulushew G/Igzabiher for media preparation and Zelalem Tazu for data entry and management

Funding

This study was done in line with the national Antimicrobial resistance surveillance networking which is financially supported by CDC Ethiopia and technically supported by American Society for Microbiology (ASM).

Availability of data and materials

The materials are available and can be obtained from Abebe Aseffa Negeri through email (abebea84@gmail.com) in case of request

Authors' contributions

AA designed the study and protocol and also wrote the manuscript. ET, DB, ES, DA, ES, SF and SI analyzed the data and wrote the manuscript. AAb, EA, ESe, ZA, TA, YM, YY collected samples and performed laboratory work.

Ethics approval and consent to participate

The study was approved by Ethiopian Public Health institute Institutional Review Board with a protocol number 054/2017.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

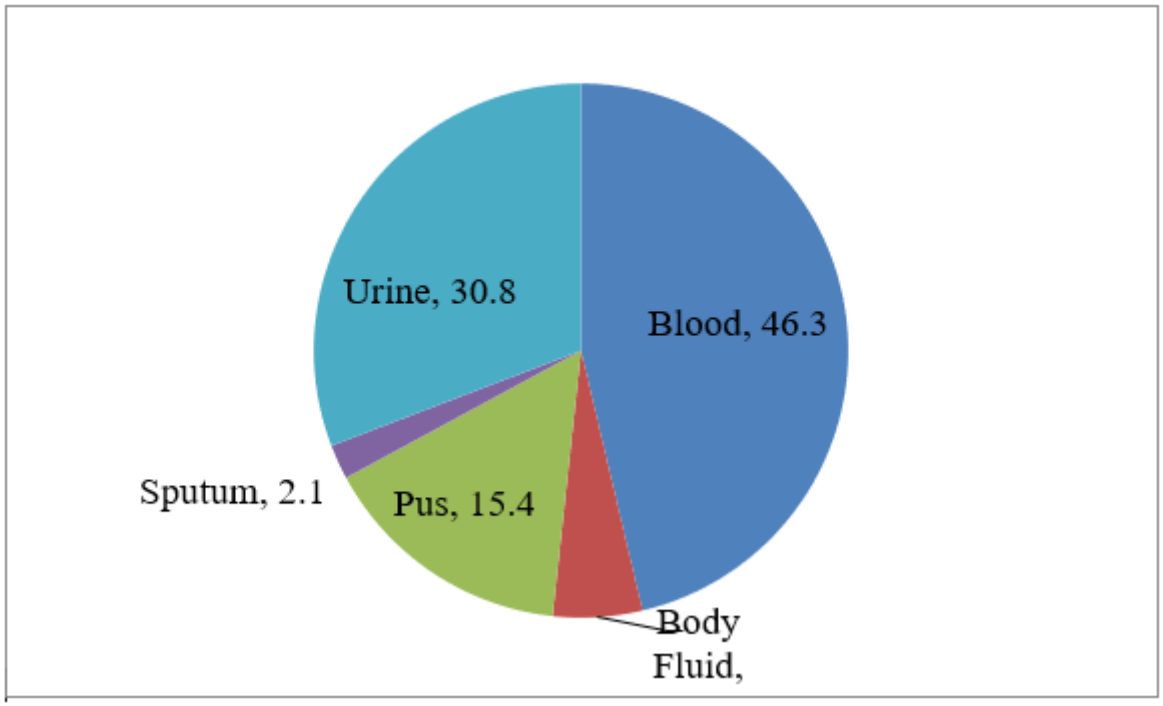

Figure 2

Percentage of specimens positive for ESBL producing Eneterobacteriaceae species

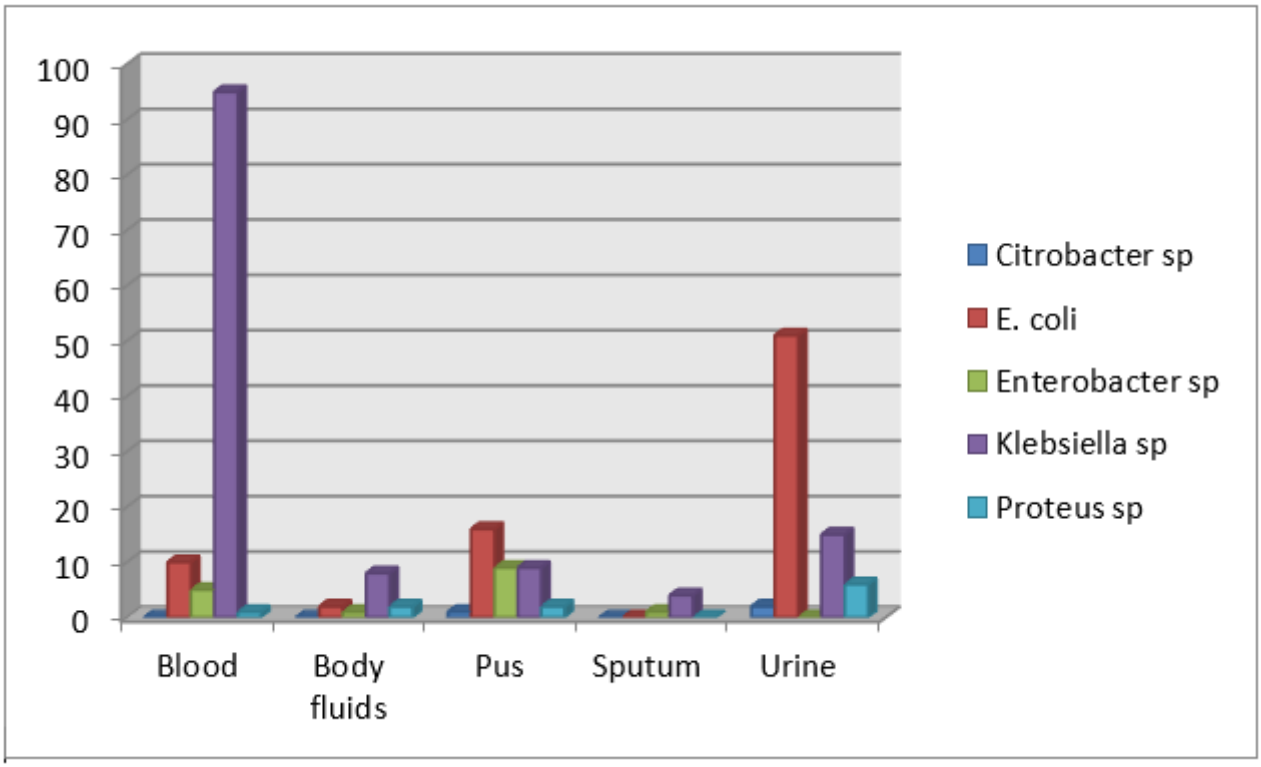

Figure 4

The distribution of ESBL positive Enterobacteriaceae species in term of clinical samples