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Prevalence of plasmid mediated bla_{TEM-1} and $bla_{CTX-M-15}$ type extended spectrum beta-lactamases in patients with sepsis

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ABSTRACT

Objective: To characterize the bacterial pathogens in patients having gram negative septicaemia. Further, to evaluate the antimicrobial resistance and underlying molecular mechanisms in these strains. **Methods:** A total number of 70 cases of gram negative sepsis were included in this prospective, open labeled, observational study. Standard methods for isolation and identification of bacteria were used. Antimicrobial susceptibility and ESBL testing was performed by the standard disc diffusion method. PCR amplification was performed to identify bla_{CTX-M}, bla_{SHV} and bla_{TEM} type ESBLs. Conjugation experiments were performed to show resistant marker transfer. **Results:** The most prevalent isolates *Escherichia coli (E. coli)* 58.6%, *Klebsiella* Spp. 32.9% and *Pseudomonas* 8.6%, were resistant to most of the antimicrobials including cefazolin, ceftriaxone, cefuroxime, ampicillin and co-trimoxazole but sensitive to imipenem and meropenem. ESBL and MBL production was seen 7.3% and 12.2% of *E. coli* isolates respectively. Three isoaltes were found to have $bla_{CTX-M-15}$ and two of them also showed bla_{TEM-1} type enxyme. Whereas, none of them showed bla_{SHV}. Conjugation experiments using J-53 cells confirmed these resistant markers as plasmid mediated. **Conclusions:** This work highlights the molecular epidemiology of escalating antimicrobial resistance and likely switch over of $bla_{CTX-M-15}$ type extended spectrum beta-lactamases by bla_{TEM} type ESBLs in India. Further, the antimicrobial resistance by horizontal gene transfer was predominant among Enterobacteraceae in the community setting.

1. Introduction

Multidrug resistant bacteria causing therapeutic problems have become a matter of serious concern in both in hospital and community settings[1]. Extended spectrum β-lactamases (ESBLs) are the bacterial enzymes that impart resistance against advanced-generation cephalosporins. There is a widespread occurrence of ESBLs, particularly in the hospital environment[2,3]. ESBLs are dangerous because they are plasmid-associated and there can be cross-species dissemination of these plasmids. Moreover, these plasmids can carry genes for co-resistance to other antibiotics such as aminoglycosides, fluoroquinolones, tetracyclines, chloramphenicol and sulfamethoxazole-trimethoprim. Antibiotic selection for such isolates thus becomes a therapeutic challenge. The widespread clinical use of broad spectrum β-lactam antibiotics has led to a marked increase in the incidence of ESBL-producing gram-negative microbes[4,5].

The majority of ESBLs are derived from the widespread broad-spectrum β-lactamases TEM-1 and SHV-1[6,7]. However, the prevalence of occurrence of these organisms varies significantly in different geographical regions. CTX-Ms are a class of ESBLs that are named after the antibiotic 'cefotaxime'. CTX-Ms have become the most prevalent ESBLs worldwide[8] but have most often been associated with focal outbreaks in Eastern Europe, South America, Southeast Asia-Japan and recently in India with many variants described[9,10]. CTX-M type ESBLs are mainly found in *Escherichia coli (E. coli)* strains and CTX-M producers are extremely common in UTIs[11]. The CTX-M-15 enzyme in particular is increasingly being reported among *E. coli* isolates from northern india. In view of this background we have initiated this study to characterize the bacterial pathogens in patients having gram negative septicaemia. Further, to evaluate the antimicrobial resistance and underlying molecular mechanisms in these strains. The

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present study was undertaken; to assess the prevalence of plasmid mediated ESBL in Medical ICU of a teaching hospital in North India.

2. Material and methods

The present study was conducted in the Department of Medicine, and Interdisciplinary Biotechnology Unit of AMU, India.

2.1. Inclusion criteria

Patients having clinical features and bacteriological evidence of sepsis, septic shock, multi organ dysfunction and/or systemic manifestations of gram negative septicaemia.

2.2. Exclusion criteria

Immunocompromised, patients with disseminated TB, HIV and patients on steroids were excluded from the study. Informed written consent was given by all study subjects and the study was approved by Institutional Ethics committee.

2.3. Study design

Prospective, observational, non-interventional studies of a two tertiary care teaching hospital.

2.4. Isolation and identification of organism

On admission appropriate culture: urine, sputum, pus, urinary catheter tip, tracheal aspirate was sent in vials using standard techniques. Standard methods for isolation and identification of bacteria were used throughout the study[12]. The identified bacteria were also confirmed by the selective media of the 'Hi-crome series' (Hi-media, Mumbai, India).

2.5. Antimicrobial susceptibility and ESBL detection

Antimicrobial susceptibility and ESBL testing was performed was performed by the standard disc diffusion method as recommended by the Clinical and Laboratory Standards Institute[13]. *E. coli* ATCC 25922 was used as ESBL negative and *K. pneumoniae* 700603 was used as ESBL-positive reference strain.

2.6. DNA preparation and PCR amplification

Plasmid DNA was prepared by using methods of Birnboim and Doly[14], and genomic DNA by the method of Boom *et al*[15]. PCR amplification of ESBLs markers gene were performed on 0.5 mg of genomic DNA as described earlier[16]. Primers for the detection of *bla*CTXM (5′-GCT GTT GTT AGG AAG TGT GC-3′ and 5′-CCA TTG CCC GAG GTG AAG-3′), *bla*TEM (5′-GAC AGT TAC CAA TGC TTA ATC-3′ and 5′-GAC AGT TAC CAA TGC TTA ATC-3′) and *bla*SHV(5′-TGG TTA TGG GTT ATA TTC GCC-3′ and 5′-GGT TAG CGT TGC CAG TGCT-3′) were used. PCR conditions for these genes comprised a thermal temperature to 94 ℃ for 5 min, followed by 35 cycles of 94 ℃ for 30 s, annealing at 54 ℃ for 30 s, and extension at 72 ℃ for 1 min, followed by final extension for 7 min at 72 ℃.

2.7. Marker transfer experiments

Marker transfer experiments were performed to check the transmissibility of the bla_{CTX-M} resistance markers and to ask for co-transferred markers. *E. coli* strains that were PCR-positive for any of the tested genes were used as donors in transconjugation experiments as described elsewhere[17]. Azide-resistant *E. coli* J53 was used as the recipient in all marker transfer experiments. Transconjugants/transformants were selected on Luria-Bertani agar plates containing azide (250 mg/L) and either cefotaxime (2 mg/L) or gentamicin (50 mg/L).

3. Results

3.1. Clinical background of isolates

Total 70 patients with mean age of (55.94±10.77) years, predominantly men (70%) and women (30%) were studied. On clinical evaluation, urinary tract (61.4%) was the most common site of infection, followed by respiratory tract (28.6%) and skin and soft tissue (10.0%). The most common organism encountered for sepsis was *E. coli* 41(58.6%) followed by *Klebsiella* Spp. 23(32.9%) and *Pseudomonas* 6(8.6%). In patients having urosepsis most common organism responsible was *E. coli* 35(81.4%) followed by *Klebsiella* Spp. 6(14.0%) and *Pseudomonas* 2(4.6%). In patients having pneumonia with sepsis the most common organism encountered was *Klebsiella* Spp. 15(75.0%) followed by *Pseudomonas* 4(20.0%) and *E. coli* 1(5.0%). In patients having diabetic foot with sepsis in the study group most common organism encountered was *E. coli* 5(71.0%) followed by *Klebsiella* Spp. 2(28.6%). The most common co-morbidity was diabetes mellitus 35(50.0%), followed by benign prostatic hypertrophy 10(14.3%) and chronic obstructive airway disease 3(4.3%) (Table 1).

3.2. Antimicrobial resistance

In the study group, *E. coli*, *Klebsiella* Spp. and *Pseudomonas* found to be resistant to gatifloxacin in 31.7%, 17.4% and 66.7% patients respectively. *E. coli, Klebsiella* Spp. and *Pseudomonas* were found to be resistant to the amikacin in 43.9%, 69.6% and 16.7% patients respectively. *E. coli* showed 7.3% intermediate resistance to Amikacin. All the three organisms were 100.0% resistant to ampicillin and co-trimoxazole. *E. coli* and *Pseudomonas* showed 0% resistance to Imipenem on the other hand *Klebsiella* Spp. showed 4.3% resistance. *E. coli, Klebsiella* Spp. and *Pseudomonas* were found to be resistant to meropenem in 9.7%, 17.4% and 50.0% patients respectively. *E. coli* and *Klebsiella* Spp. showed intermediate resistance to meropenem in 4.8% and 47.8% patients respectively. Most of the gram negative organisms are sensitive to imipenem and meropenem. Almost all gram negative organisms (*E. coli*, *Klebsiella* Spp. and *Pseudomonas*) were resistant to co-trimoxazole (Table 2).

3.3. Molecular analysis of ESBLs

In the present study 7.3% *E. coli* isolates showed ESBL

production while 12.2% showed MBL production on the other hand *Klebsiella* Spp. and *Pseudomonas* showed no ESBL/MBL production. In the present study majority of the isolates encountered for gram negative sepsis was *E. coli* (58.6%), and 81.4% *E. coli* were isolated from patients having urosepsis. In the present study only 3 (7.3%) *E. coli* strains had a phenotype consistent with production of an ESBL. These 3 *E. coli* isolates were screened for the presence of ceftrixone hydrolyzing β-lactamases (CTX-M) and bla_{TEM} and bla_{SHV} types by PCR using universal primers. Three isolates (UE-59, UE-61, and UE-64) showed the presence of bla_{TEM}, and bla_{CTX-M} type enzyme, while all the three were found negative for SHV (Figure 1 & 2). Furthermore, conjugation experimenst using J-53 as recipient susceptible cells confirmed that these markers are present on plasmid.

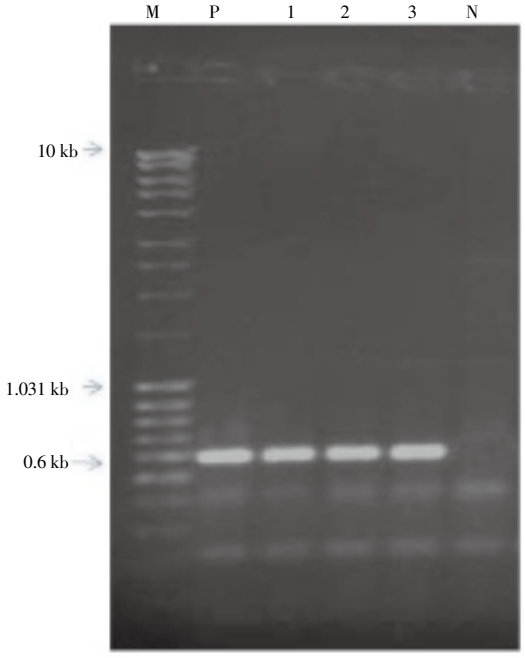

Figure 1. PCR amplification of CTX-M gene.
Lane M: DNA ladder; Lane P: Positive control; Lane 1-3: *E. coli* clinical isolates (UE-59, UE-61, and UE-64); Lane N: Negative control.

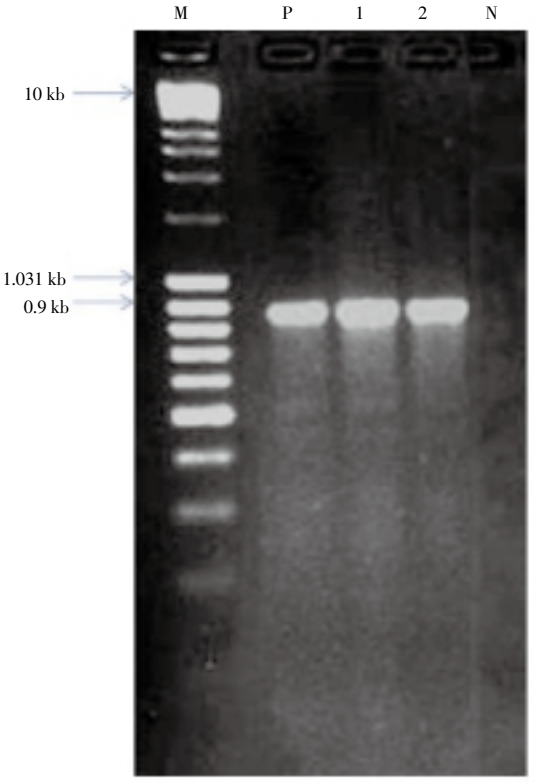

Figure 2. PCR amplification of TEM gene.
Lane M: DNA ladder; Lane P: Positive control; Lane 1-3: *E. coli* clinical isolates (UE-59, and UE-64); Lane N: Negative control.

4. Discussion

Gram-negative sepsis is a significant problem in hospitalized and community-dwelling patients. Although the percentage of hospital-acquired bloodstream infections associated with gram-negative bacilli has decreased, these organisms now pose serious therapeutic problems because of multidrug resistance[18].

The treatment of gram-negative bacteremia is increasingly complicated by the occurrence of multidrug resistant gram-

Table 1
Percent etiology and sources of sepsis(*n*,%).

Source of sepsis	*E. coli*	*Klebsiella* Spp	*Pseudomonas*	Total
Urosepsis	35(85.36)	6(26.08)	2(33.33)	43(61.40)
Pneumonia	1(2.49)	15(65.21)	4(66.66)	20(28.60)
Diabetic foot	5(12.19)	2(8.69)	0(0.00)	7(10.00)

Table 2
Percentage of resistance to the selected antimicrobial agents among the Gram negative bacteria.

Pathogens	β-Lactam		Aminoglycosides			Flouroquinolne			Cycline		Cloxacillin	Cephalosporin groups				Carbapenem			
(ESBL- producing)	A	P	G	Tb	Ak	Na	Cip	Gat	Te	Cot	Cox	Cz	Cn	Ce	Ci	Cpm	Mrp	Etp	Ipm
E. coli (41 isolates)	100.0	100.0	43.9	51.0	43.9	100.0	100.0	31.7	95.1	100.0	100.0	100.0	100.0	100.0	100.0	100.0	9.7	0.0	0.0
Klebsiella((23 isolates)	100.0	100.0	28.0	100.0	69.6	100.0	100.0	17.4	87.0	100.0	10.0	91.3	91.3	91.4	95.6	95.6	17.4	4.3	4.3
Pseudomonas(6 isolates)	100.0	16.0	12.0	12.0	16.7	100.0	100.0	66.7	83.3	100.0	58.0	100.0	100.0	100.0	83.3	100.0	50.0	33.0	0.0

A=Ampicillin, P=Penicillin, G=Gentamicin, Tb=Tobramycin, Ak=Amikacin, Na=Nalidixic acid, Cip=Ciprofloxacin, Gat=Gatifloxacin, Te=Tetracycline, Cot=Cotrimoxazole, Cox=Cloxacillin, Cz=Cefazolin, Cn=Cephoxitin, Ce=Cephotaxime, Ci=Ceftriaxone, Cpm=Cefepime, Mrp=Meropenem, Etp=Ertapenem, Ipm=Imipenem.

negative bacilli strains. Over-production of chromosomal beta-lactamases by *Enterobacter* Spp. has become one of the most common mechanisms of resistance to third generation cephalosporins[19].

In our study group most common organism encountered for sepsis was *E. coli* 41(58.6%) followed by *Klebsiella* Spp. 23(32.9%) and *Pseudomonas* 6(8.6%). Similar results for the most common isolates were identified in community acquired sepsis in an Italian study: *E. coli* (76.0%); *P. aeruginosa*(7.9%); *K. pneumoniae* (5.4%); *Proteus mirabilis*-4.2 percent; *Enterobacter* species-3.7 percent[20]. UTI remains the commonest type of community acquired gram negative sepsis, especially in developing countries.

Similar type of study done by Geoffrey D. Taylor *et al* in nosocomial urosepsis showed most common organism responsible was *E. coli* (36.8%) and 8.9% *Pseudomonas*[21]. In our study most common organism isolated from patients having pneumonia was *Klebsiella* Spp. 15(75%) followed by *Pseudomonas* 4(20%) and *E. coli* 1(5%). A similar study done by C. Feldman *et al* who found 31.9% isolates were *Klebsiella* Spp and 8.9% were *Pseudomonas*[22].

In our study *E. coli*, *Klebsiella* Spp. and *Pseudomonas* were found to be resistant to cefazolin & cefuroxime in 100.0%, 95.6% and 100.0% respectively. For ceftazidime, *E. coli* was found to be 100.0% resistsnt, *Klebsiella* Spp. showed 91.4% resistance and *Pseudomonas* showed 83.3% resistance. Our findings are comparable to study conducted by Kumari *et al*[23].

In the present study 7.3% *E. coli* isolates showed ESBL production on the other hand *Klebsiella* Spp. and *Pseudomonas* showed no ESBL/metallo-betalactamase (MBL) production.While MBL production was seen in 12.2% *E. coli*. ESBL and MBL production is a matter of great concern in the field of microbial drug resistance. ESBLs represent a major threat among resistant bacterial isolates of Enterobacteriacea[24]. This renders powerful antibiotics, like expanded spectrum cephalosporins, carbapenems, and monobactams, ineffective. India has significantly high pool of ESBL infections, reflecting poor standards of hygiene and inappropriate use of antibiotics, as compared to other developed societies. The source of ESBL producing Enterobacteriacea differ, while in other countries it is mainly hospital acquired, in India it is mostly community acquired[25]. Different groups of ESBLs, have been described and classified according to their amino-acid sequences, like SHV-1, TEM-2, TEM-3 *etc*. Until the end of the 1990s, most of the ESBLs detected were SHV and TEM types, mostly associated with nosocomial outbreaks[26]. In 1989, almost simultaneously in Germany, France and Italy, a new ESBL family was recognised. It was named CTX-M, because most of the enzymes within this family confer resistance predominantly to cefotaxime rather than ceftazidime[27]. The CTX-M ESBLs have since been detected in many species of Enterobacteriaceae. Currently the CTX-M family includes more than 20 beta-lactamases, which may be grouped on the basis of sequence similarity into 4 distinct subtypes epitomized by CTX-M-1, CTX-M-2, CTX-M-8 and CTX-M-9. The $bla_{\text{CTX-M-15}}$ gene borne on plasmid in Enterobacteriaceae, was first detected in India, which is now the globally dominant ESBL[28]. Unlike in other countries *e.g*. Spain, France, where members of multiple CTX-M lineages have been reported, a virtually absolute prevalence of the members of the CTX-M-1 lineage was observed in this study[26].

Recent reports have shown a rapid and alarming dissemination of Enterobacteriaceae producing ESBLs of the CTX-M type in certain countries including India and have become the most prevalent ESBL-type world-wide[29]. The majority of these isolates are now recovered from community patients, most of them with urinary tract infections[30].Unlike that of TEM or SHV type ESBLs, the population structure of CTX-M-producing isolates is complex and is associated with the spread of specific plasmids and/or other mobile gene -tic elements rather than clonal epidemics[31]. CTX-Ms are one of the means of antibiotic resistant marker transfer among members of Enterobacteriaceae. However, their prevalence in different areas appeared to be highly variable, which could reflect the scenario of a relatively early stage of dissemination of these resistance determinants in the community setting. In the recent past, there are alarming reports about the emergence and spread of resistant strains of Enterobacteriaceae from all around the world.

Bacterial resistance to commonly used antibiotics, in a resource crunched developing country like India, threatens the basic health delivery systems, affecting millions. There are alarming reports about serious consequences of antibiotic resistance. However, there is still a scarcity of data on the magnitude of antibiotic resistance, especially in Asia-Pacific region.

To conclude, this work has provided insights into molecular epidemiology of escalating antimicrobial resistance, especially prevalent in ESBL producing Enterobacteraceae in India. Continuing surveillance will be necessary to monitor the evolution of extended spectrum beta-lactamases and to authenticate whether the CTX-M type ESBLs will eventually prevail over the bla_{TEM}-type ESBLs, which are still widespread, especially in some areas. Our findings uphold the increasing role of the $bla_{\text{CTX-M}}$ β-lactamases in antibiotic resistance and stress upon the significance of appropriate empirical treatment for infections caused by coliforms, especially during septicemia. This study confirms that horizontal gene transfer of antimicrobial resistance markers, is the most prevalent among Enterobacteraceae in the community setting. Further studies are needed, however, to determine whether antibiotic policies or other measures can halt or lower the level of horizontal gene transfer that occurs in a hospital or community setting.

Conflict of interest statement

We declare that we have no conflict of interest.

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