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Study of Ctx-M Types Esbls Produced by Enterobacteriaceae Collected from Clinical Isolates

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ABSTRACT

The extended-spectrum β-lactamases (ESBLs) have been reported in various clinical isolates obtained from hospitals in India, however, there is no data on the prevalence and antibiotic susceptibility patterns of CTX-M type ESBL produced by Gram-negative bacilli. The aim of this study was to determine the frequency and distribution of the *bla*$_{CTX-M}$ genes and the susceptibility patterns in ESBL producing clinical isolates of Gram-negative bacilli. A total of 200 non-duplicate and pure isolates obtained from clinical isolates and identification of the isolates was performed by MALDI-TOF mass spectrometry. Susceptibility testing and ESBL detection was performed using VITEK® 2, according to EUCAST v4.0 guidelines. Genotypic analysis was performed using Check-MDR CT103 Microarrays. Out of total 112 isolates screen positive for ESBLs, 71 tested positive for ESBL encoding genes by Check-MDR array. Among the CTX-M types, *bla*$_{CTX-M}$ carrying *Enterobacteriaceae* (n = 64) isolates showed no resistance against imipenem and meropenem and a moderate resistance rate against tigecycline, fosfomycin and amikacin. On the other hand, all the *bla*$_{CTX-M}$ positive *Enterobacteriaceae* showed a multidrug resistant (MDR) phenotype with remarkable co-resistances (non-susceptibility rates) to aminoglycosides, fluoroquinolones and trimethoprim/sulfamethoxazol. This study demonstrates a remarkably high prevalence of *bla*$_{CTX-M}$ genes among ESBL-producing isolates. The high level of resistance to β-lactam and non-β-lactam antibiotics as well as the trend to a MDR profile associated with the *bla*$_{CTX-M}$ genes are alarming and poses the requirement for appropriate choice of antimicrobial therapy.

Keywords: Enterobacteriaceae, Extended spectrum beta lactamase, CTX-M, antibiotic resistance

INTRODUCTION

Extended-spectrum β-lactamases (ESBLs) are a predominant cause of β-lactam resistance in Gram-negative bacilli (GNB)[1,2]. Incidences of infections caused by ESBLs producing GNB are increasing in prevalence worldwide, both in the healthcare as well as community settings, posing significant therapeutic challenges[3]. ESBLs are most often a plasmid mediated heterogeneous group of β -lactamase enzymes, that confer resistance to a wide range of commonly used β -lactam antibiotics including third generation cephalosporins (e.g., ceftriaxone, cefotaxime and ceftazidime) as well as monobactams (aztreonam)[4]. TEM and SHV type ESBLs used to be the dominant ESBL genotypes[5]. However, in the past decade, the CTX-M type ESBLs has become the most widely distributed and globally dominant genotypes[6].

The CTX-M type enzymes are a group of class A ESBLs that in general exhibit much higher levels of activity against cefotaxime and ceftriaxone than ceftazidime[7]. The presence of CTX-M type

ESBLs is often associated with co-resistance phenotypes in particular to fluoroquinolones and aminoglycosides, in addition to tetracycline, and trimethoprim/sulfamethoxazole co-resistance, which is commonly observed among TEM and SHV type ESBLs [8,9]. The group of CTX-M type ESBLs currently constitutes more than 170 allelic variants, which cluster into five major groups based on sequence homologies. The five CTX-M groups are: CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25[9]. Each group consists of a number of particular variants with dominant variants being restricted in distribution to specific geographic areas, while few others are globally distributed. CTX-M-14 and CTX-M-15 were the most commonly isolated variants worldwide[10]. CTX-M-15 is the most frequently reported variant, although some other variants were also detected in the region. CTX-M type ESBLs have now spread and could be detected among many different bacterial strains of clinical importance[11]. This is particularly true for *Enterobacteriaceae* revealing an ESBL phenotype such as *Escherichia coli* and *Klebsiella pneumoniae*, which often cause potentially serious infections in the hospital as well as community setting [10].

In India, multiple studies have reported prevalence of ESBLs ranging from 25 to 38.5% among *Enterobacteriaceae* in clinical samples. However, there is no data on the prevalence and antibiotic susceptibility patterns of CTX-M type ESBLs produced by GNB[12]. Therefore, the aim of the present study was to determine the relative frequency and distribution of the *bla*$_{\text{CTX-M}}$ genes, as well as the overall susceptibility patterns in ESBL producing clinical isolates of GNB obtained from various inpatients at Chhattisgarh Institute of Medical Sciences, Bilaspur, between January 2023 to December 2023.

MATERIALS AND METHOD

A total of 200 randomly selected, non-duplicate, pure and clinically relevant Gram-negative bacilli isolates had been recovered from clinical specimens submitted to the bacteriology laboratory for routine culture and antimicrobial susceptibility testing Chhattisgarh Institute of Medical Sciences (CIMS), Bilaspur, between January 2023 to December 2023. The specimens included wound swabs, urine, biopsies, sputum and others. All inpatient clinical specimens were obtained after more than 48hours of hospitalization of the patient. Along with the specimens, basic demographic and medical data were recorded using standard clinical and laboratory record forms.

Isolation and identification of the bacterial isolates was performed using standard microbiological techniques in use at the bacteriology laboratory in Department of microbiology, CIMS, Bilaspur[13]. All isolates were identified to the species level by MALDI-TOF mass spectrometry (MALDI Biotyper, Bruker Daltonik, Bremen, Germany, Biotyper software package, version 3.0)[14], and then retested for antibiotic susceptibilities using VITEK® 2 compact automated system (N215 and N248, bioMérieux, France) accordingly. Software supplied by the manufacturer in compliance with the EUCAST v4.0 guidelines was used. The system included an Advanced Expert System (AES) that analyzed growth patterns and detected the phenotype of organisms. Calculated MICs of piperacillin, piperacillin-tazobactam, cefotaxime, ceftazidime, cefepime, aztreonam, imipenem, meropenem, amikacin, gentamicin, ciprofloxacin, tobramycin, moxifloxacin, fosfomycin, tigecycline, colistin and trimethoprim/sulfamethoxazole were determined and interpreted according to EUCAST v4.0 guidelines[15]. ESBL screening and phenotypic tests All *Enterobacteriaceae* isolates with reduced susceptibility or resistance to ceftazidime and/or cefotaxime and/or aztreonam and

all non-fermenting GNB with multi-resistant phenotype[16] were considered as ESBL-screen positive and subjected to phenotypic and genotypic analysis. Phenotypic detection of ESBL production was performed with the VITEK® 2 compact automated systems (bioMérieux, France).

RESULTS

Among clinical bacterial isolates and specimens of 200 Gram-negative bacterial strains, 112 (50%) isolates were considered as screen positive for ESBLs. These isolates consisted of 73 *Enterobacteriaceae* (31 *Klebsiella pneumoniae,* 2 *Klebsiella oxytoca*,14 *Enterobacter cloacae,* 13 *Escherichia coli, 5 Providencia stuartii, 4 Proteus mirabilis, 3 Morganella morganii,* and 1 *Escherichia hermanii)* and 39 non-fermenting Gram-negative bacilli (14 *Acinetobacter baumanii*, 2 *Acinetobacter pittii,* 1 *Acinetobacter haemolyticus*, 14 *Pseudomonas aeruginosa*, 3 *Alcaligenes faecalis*, 4 *Stenotrophomonas maltophilia* and 1 *Bordetella bronchiseptica*). The majority of these isolates was recovered from inpatients (83.9%, *n* = 94) mainly from surgical wards (60.6%, *n* = 57) followed by medical wards (21.3%, *n* =20) and from two types of specimens; wound (54.5%,*n* = 61) and urine samples (26.8%, *n* = 30), which together account for 81.3% (*n* = 91) of the total. The total 112 screen positive isolates were collected from 100 patients out of which 90 (90%)of them yielded one isolate for inclusion whereas 10 (10%) patients yielded multiple species. Phenotypic detection of ESBLs was observed in 62.5% (*n* = 70) of the total screen positive isolates (*n* = 112) using VITEK® 2 compact automated system (bioMérieux,).

In genotypic detection of ESBL encoding genes of the total 112 screen positive isolates, 63.4% (*n* = 71) were positive for ESBL encoding genes by Check-MDR array. This corresponds to 91.8% (67/73) of the total *Enterobacteriaceae* and 10.3% (4/39) of non-fermenting Gram-negative bacilli, namely three *P. aeruginosa* and one *A. faecalis* isolate. No ESBL alleles were detected among *Acinetobacter* spp., *S. maltophilia* and *B. bronchiseptica* (Table 1). Specimen wise, 60.7% (*n* = 37) of isolates from wound samples, 63.3% (*n* = 19) from urine, 66.7% (*n* = 8) from biopsy samples and all the isolates obtained from sputum samples (*n* = 6) as well as eye discharge (*n* = 1) were positive for ESBL encoding genes. Among total inpatient (*n* = 94) and outpatient (*n* = 18) isolates, ESBL genes were detected in 68.1% and 38.9% of the isolates respectively. The comparison of the difference in proportion should be taken with caution as convenient sampling was used and most specimens were obtained from inpatients. Four patients had two different ESBL-positive isolates (*E. cloacae* and *K. pneumoniae* in two cases *E. coli* and *M. morganii*, and *P. aeruginosa* and *A. faecalis* in one case each). One of the four patients had an SHV 238S + 240 K mutation bearing *E. cloacae* and a CTX-M-15 positive *K. pneumoniae* in the specimen, whereas the three other patients each had two different species each positive for CTX-M-15. Ttotal of 71 isolates carrying ESBL encoding genes, 68 (95.8%) carried CTX-M genes either alone or in combination with SHV and/or TEM genes. Sixty-four out of 67 (95.5%) *Enterobacteriaceae* and all non-fermentingGNB (*n* = 4) which carried ESBL encoding genes, were positive for CTX-M genes. The remaining three isolates negative for CTX-M (4.2%) carried SHV-type ESBLs (G238S + E240K) genes and were found to be *E. cloacae* obtained from wound samples. All TEM and SHV β-lactam genes detected were wild type except five G238S + E240K SHV type ESBLs. Three of the five were detected in *E. cloacae* in combination with wild type TEM. The other two were found in one *E. coli* and *K. pneumoniae* isolate along with CTX-M genes.

Multiple β-lactamase genes in a single strain were observed in 83.1% (*n* = 59) of the total isolates carrying ESBL encoding genes. From a total of 68 CTX-M positive isolates, 12 (17.6%) harbored CTX-M alone. The remaining 56 (82.4%) isolates carried CTX-M in combination with wild type TEM and/or SHV (except two SHV E240K + G238S) in different frequencies, which is partly explained due to the general presence of β-lactamases in some strains e.g. in *Klebsiella* spp. CTX-M group 1 was the most dominant group detected in 66 of 68 CTX-M positive isolates (97.1%), either alone (*n* = 63, 92.6%) or in combination with other groups (*n* = 3, 4.5%). All CTX-M-1 genes were sequenced and all were found to be allele CTX-M-15. The remaining two (2.9%) CTX-M positive isolates carried CTX-M group 9 (Table 2) genes which upon sequencing were identified as allele CTX-M-24.

The antibiotic susceptibility testing for CTX-M-positive *Enterobacteriaceae* isolates demonstrated a MIC in the respective susceptible range in < 2% of cases against cephalosporins according to EUCAST guidelines. Susceptibilities to carbapenems and a few other substances were found to be much higher. In terms of non-susceptibility, the highest level of antibiotic resistances was observed as expected against beta-lactams such as piperacillin and cephalosporins, but also against trimethoprim-sulfamethoxazole (92.2%), gentamicin (89.1%), and quinolones (75%). No isolates showed full resistance to imipenem or meropenem, and only 3.1% and 1.6% tested intermediate for these substances, respectively (Table 3). One *E. coli* isolate tested positive for CTX-M-15 but was measured susceptible to third generation cephalosporins using VITEK 2 as well as disc diffusion tests. All the CTX-M-positive *Enterobacteriaceae* (*n* = 64, 100%) and *P. aeruginosa* (*n* = 3, 100%) were non-susceptible to ≥1 agent in ≥3 antimicrobial categories and hence defined as multidrug resistant (MDR) according to the inter- national expert proposal for interim standard definitions for acquired resistance promoted by the European Centre for Disease Prevention and Control (ECDC)[17]. About 92.2%, 78.1% and 92.2% of the total CTX-M-positive *Enterobacteriaceae* were found to be non-susceptible (co-resistant) to aminoglycosides, fluoroquinolones and trimethoprim-sulfamethoxazole, respectively. Non-susceptibility pattern in CTX-M and non-CTX-M carrying isolates. Both CTX-M (*n* = 64) and non-CTX-M producing (*n* = 119) *Enterobacteriaceae* isolates have comparable non-susceptibility patterns to piperacillin/tazobactam, imipenem, meropenem, fosfomycin, and colistin/polymyxin B ($P > 0.05$). However, the non-susceptibility rate to all other antibiotics tested were all significantly higher among CTX-M-positive isolates compared to non-CTX-M ESBL-carrying isolates ($P < 0.001$). All the CTX-M negative isolates were also non-ESBLs except for three isolates expressing SHV type ESBLs. Unlike seen with CTX-M ESBLs, this did not affect the other non-susceptibilities

DISCUSSION

The present study describes the epidemiology status of ESBL encoding genes in India. Here, high level of prevalence of CTX-M-type ESBLs have been demonstrated among all ESBL positive isolates at JUSH. In total, 95.8% of all ESBL genes detected were of CTX-M type, and almost unanimously CTX-M-1 group variant type 15 (97.1% of all CTX-M positive isolates). These findings are in accordance with the fact that the CTX-M type ESBLs is the most widely distributed and globally dominant ESBL genotypes to date[18,19]. Of the groups, CTX-M-1 was found highly prevalent in Italy[20], Switzerland[21], Saudi-Arabia[22], Syria[23], Pakistan[24] and China[25].

Factors and mechanisms which contribute to the emergence and increasing prevalence of CTX-M

ESBLs of all groups are complex and may involve both, plasmid dissemination as well as clonal spread of bacterial strains[26]. In addition, the selective pressure exerted by the frequent use of wide spectrum cephalosporins may promote their epidemiological success. Especially in Ethiopia, the widespread misuse and overuse of cephalosporins may contribute to the selection and spread of CTX-M gene carrying clones[27,28].

Other than *E. coli* (92.3% CTX-M-15) and *K. pneumoniae* (100% CTX-M-15), CTX-M were detected amon members of ESBL producing *Enterobacteriaceae, E. coli* (92.3% CTX-M-15) and *K. pneumoniae* (100% CTX-M-15), *K. oxytoca, M. morganii, P. mirablis, P. stuartii, E. hermannii* and *E. cloacae,* as well as non-fermenting GNB *(P. aeruginosa* and *A. fecalis*[29,30].

The overall resistance pattern of the total CTX-M positive *Enterobacteriaceae* is very high for most antibiotics tested in the present study. The carbapenems (0% resistance) followed by amikacin (3% resistance) were found to have the highest susceptibility rates[31]. However, all CTX-M-positive isolates identified in this study showed a MDR phenotype as well as remarkably high rates of co-resistance to fluoroquinolones, aminoglycosides, and trimethoprim/sulfamethoxazole. Only one *E. coli* isolate positive for an ESBL gene (CTX-M-15) was not resistant against third generation cephalosporins, while still maintaining an MDR phenotype. In this particular case, the CTX-M operon seems to be non-functional perhaps due to mutations. These findings are consistent with studies from Ghana[32] and Lebanon[33] which propose imipenem and amikacin as possible drugs for the management of infection caused by CTX-M-producing isolates. Comparably high rates of co-resistance to non-Beta-lactam antibiotics were also reported from Europe[34].

In the present study, only clinically relevant isolates of in and outpatients were used, a screening upon admission, or screening of healthy controls was not performed. However, the high rates of ESBL positive organisms in outpatients without contact to the health care system within the last 3 months, argues for considerable ESBL carrier rates among the general population. Within the study population, mainly samples from internal medicine, pediatrics and ICU were ESBL positive and MDR, whereas in the surgical patient group many patients were found to harbor non-fermenters with MDR phenotype which are negative for the ESBL and carbapenemase genes tested within this study.

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Table 1 Percentage of Antibiotic resistance showed by CTX-M positive Gram-negative isolates.

	PI	PIT	CTX	CAZ	CPM	AT	IMP	MRP	AK	HLG	TOB	CIP	MOX	FO	TGC	CL	COT
***E. coli* (*n* = 13)**	100	30.8	92.3	92.3	92.3	92.3	0	0	7.7	76.9	76.9	92.3	84.6	7.7	0	7.7	84.6
***K. pneumoniae* (*n* = 30)**	100	60	100	96.7	96.7	96.7	0	0	0	90	96.7	66.7	80	0	0	0	93.3
***E. cloacae* (*n* = 9)**	100	0	100	100	100	100	0	0	0	88.9	88.9	22.2	77.8	0	0	0	100
***M. morganii* (*n* = 3)**	100	0	100	100	100	100	0	0	0	100	100	66.7	100	100	IR	IR	100
***P. mirablis* (*n* = 4)**	100	0	100	100	100	100	0	0	0	100	100	25	25	50	IR	IR	75
***P. stuartii* (*n* = 2)**	100	0	100	100	100	100	0	0	0	IR	IR	50	50	50	IR	IR	100
***K. oxytoca* (*n* = 2)**	100	50	100	100	100	100	0	0	0	100	100	0	50	0	0	0	100
***E. hermanii* (*n* = 1)**	R	R	R	R	R	R	S	S	S	R	R	R	R	S	S	S	R
Total *Enterobacteriaceae* (*n* = 64)	100	35.9	98.4	96.9	96.9	96.9	0	0	1.6	89.1	92.2	59.4	75	10.9	14.1	15.6	92.2
***P. aeruginosa* (*n* = 3)**	66.7	66.7	IR	33.3	66.7	66.7	0	0	33.3	66.7	66.7	100	100	100	IR	0	IR
***A. faecalis* (*n* = 1)**	R	S	IE	IE	IE	R	S	S	S	S	S	IE	S	IE	IE	IE	IE
Total GNB (*n* = 68)	98.5	36.8	97.1	92.6	94.1	95.6	0	0	2.9	88.2	91.2	60.3	76.5	14.7	17.6	14.7	91.2

Key:
PI piperacillin,
PIT piperacillin/tazobactam,
CTX cefotaxime,
CAZ ceftazidime,
CPM cefepime,
AT aztreonam,
IMP imipenem,
MRP meropenem,
AK amikacin,
HLG gentamicin,
TOB tobramycin,
CIP ciprofloxacin,
MOX moxifloxacin,
FO fosfomycine,
TGC tigecycline,
CL colistin,
COT trimethoprim/sulfamethoxazole
n number of isolates,
S sensitive, *R* resistant, *IR* intrinsic resistance, *IE* insufficient evidence