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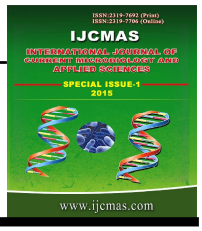

Original Research Article

Carriage of ESBL and AmpC-Positive Enterobacteriaceae in Gastrointestinal Tract of Healthy Community Subjects and Hospitalized Patients and Detection of *bla*$_{CTX-M}$ Gene in ESBL Positive Isolates

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Keywords

AmpC, bla$_{CTXM}$, *Escherichia coli*, *ESBL*, *Klebsiella pneumoniae*

A B S T R A C T

The study aimed at analyzing ESBL and AmpC positive *Enterobacteriaceae* in the gastrointestinal tracts of healthy community subjects and hospitalized patients and detection of *bla*$_{CTX-M}$ type gene in ESBL positive isolates. Bacteria were isolated from stool samples of the study population. Production of ESBL-type beta-lactamases was screened by double-disk synergy test as well as automated system, and AmpC enzyme production was detected by the AmpC disk test. ESBL positive isolates were subjected to detection of the *bla*$_{CTX-M}$ gene. A total of 792 stool samples (50% from healthy subjects and 50% from hospitalized patients) were studied. The prevalence rates of ESBL-positive *Enterobacteriaceae* were 9.3% in hospitalized patients and 4.4% in healthy community subjects. Production of the AmpC enzyme was detected in 0.5% of bacterial isolates from the community and 1.7% in hospital isolates. 51.2% of the ESBL producers were positive for *bla*$_{CTX-M}$ gene. This study revealed a high prevalence of faecal carriage of ESBLs in hospitalized patients whereas healthy community subjects showed moderate values. Although the existence of AmpC enzyme in bacterial isolates from community and hospital samples was less, their existence showed that they may emerge as a threat in future.

Introduction

Multidrug resistance is increasingly seen in many Gram-negative bacteria as a result of widespread use of various antibiotics (Livermore, 2003; Waterer and Wunderink, 2001) which is causing a major threat to public health. Despite intense efforts to limit their spread, the number of multidrug resistant Gram-negative bacteria continues to increase globally. The

Enterobacteriaceae producing broad-spectrum beta-lactamases are amongst the bacterial pathogens that are feared the most. Of particular clinical importance are ESBL (extended-spectrum beta-lactamase) and AmpC enzymes capable of hydrolyzing penicillins, monobactams and 3rd generation cephalosporins with a broad spectrum of activity (Moland *et al.,* 2002). ESBLs are most commonly found in *Escherichia coli* and *Klebsiella pneumoniae* (Waterer and Wunderink, 2001). Extended-spectrum beta-lactamases (ESBLs), which hydrolyse expanded-spectrum cephalosporins and monobactams but not cephamycins and carbapenems, are being increasingly found among Enterobacteriaceae (Waterer and Wunderink, 2001). ESBL enzymes are encoded by transferable conjugative plasmids, which often code resistance determinants to other classes of antimicrobial agents and are also responsible for the dissemination of resistance to other Gram-negative bacteria in the community and in hospitals (De Champs *et al.,* 1989). This resistance has been associated with efficient dispersion of specific clones and plasmids harboring bla_{ESBL} genes (Coque *et al.,* 2008, 2002; Novais *et al.,* 2006). From a clinical point of view, the danger of ESBL- and AmpC-positive Enterobacteriaceae is that they increase the risk of antibiotic treatment failure, mortality and economic costs of treatment of infections (Tumbarello *et al.,* 2007; Schwaber *et al.,* 2006).

Studies have shown that ESBL producing uropathogens have their reservoir in the digestive tract (Bradford *et al.,* 1995). Gastrointestinal colonization by multidrug resistant bacteria is associated with subsequent clinical infection. A recent study emphasized the importance of identifying individuals carrying antimicrobial-resistant bacteria in both patient and healthy populations (Smith *et al.,* 2004). An increase in the proportion of carriers in the community increases the risk that other individuals will also become carriers via human-to-human transmission (Levin, 2001). In addition, the admission into hospital of patients harbouring resistant bacteria increases the risk of other hospitalized patients contracting an infection (Tschudin-Sutter *et al.,* 2010).

The *CTX-M* family is a rapidly growing group of ESBLs that have disseminated globally. In Asia, it is now the predominant ESBL type among Enterobacteriaceae, and similar epidemiological changes are taking place in Europe (Cantón and Coque, 2006; Ho *et al.,* 2005). Most *CTX-M* β-lactamases confer a higher level of resistance to cefotaxime than ceftazidime. Several factors like plasmids, insertion sequence, transposons and integrons are involved in the mobilization of *CTX-M* gene although multiclonal spread has also been recognized (Cantón and Coque, 2006; Woodford *et al.,* 2004). Unlike TEM- and SHV-derived ESBLs, the prevalence of *CTX-M* enzymes has been increasing to a greater extent in the community than in the hospital setting. It has been suggested that the rise of *CTX-M* in health care settings might be a consequence of the influx of these enzymes from the community (Woodford *et al.,* 2004). The emergence of *CTX-M* type enzyme as the predominant ESBL in *Escherichia coli* in faecal carriers is not an isolated phenomenon. *Escherichia coli* producing *CTX-M* β-lactamases seem to be true community ESBL-producers and the current emergence and spread of these bacteria is intriguing but worrying (Kumar and Babu, 2013).

The presented study aimed at determining the prevalence of ESBL- and AmpC-positive Enterobacteriaceae in the GIT of subjects in both the hospital and community

settings and detection of bla_{CTX-M} gene in ESBL positive isolates. In this study, we describe the bla_{CTX-M} genes in commensal *Escherichia coli* and *Klebsiella pneumonia* from hospitalized as well as non-hospitalized healthy individuals.

Materials and Methods

Study group

The study was performed at a tertiary care hospital in Western UP, India. A total of 792 faecal samples were included in this study: 396 from healthy individuals and 396 from hospitalized patients not suffering from GIT infection. A single sample was taken from each member of the study group.

Sample processing

Each stool sample was spread on two MacConkey agar plates, one supplemented with 1 μg/mL of cefotaxime and another with 1 μg/mL of ceftazidime, and incubated at 37°C for a minimum of 24 h before initial examination. Plates demonstrating no growth in a primary examination were incubated for other 24 h. Organisms were identified by standard techniques mentioned in the book Mackie and McCartney and later were confirmed by automated system (Vitek2) (BioMérieux, France).

Double disk synergy test

Samples yielding bacteria that grew on MacConkey agar were initially identified as suspicious for ESBL. Isolates were screened for ESBL production by the double-disk synergy test (DDST) in which an amoxicillin–clavulanate (AMC) (20 μg/10 μg) disk was placed in the centre with ceftazidime (CAZ) (30μg) and cefotaxime (CTX) (30μg) disks at a 15 mm distance from AMC. Strains producing ESBL were defined as those showing synergism between AMC and any one of CTX and CAZ (Jarlier *et al.*, 1988).

Combined disk method

The standard CLSI combined disk method involving CAZ and CTX with and without the inhibitor clavulanic acid (30 μg) (Himedia) was used to confirm the presence of ESBL. ESBL production was indicated by an increase in zone size of more than 5 mm with and without clavulanic acid. Quality control testing was done using *K. pneumoniae* ATCC 700603 (positive control) and *Escherichia coli* ATCC 25922 (negative control).

AmpC disk test

AmpC disk test was performed for the detection of AmpC enzymes. AmpC disk was prepared in-house by applying 20 μl of a 1:1 mixture of saline and 100x tris EDTA to sterile filter paper disks, allowing the disks to dry, and storing them at 2–8^{0}C.

The surface of a Mueller Hinton agar plate was inoculated with a lawn of *Escherichia coli* ATCC 25922 as for standard disk diffusion method. Immediately prior to use, AmpC disks were rehydrated with 20 μl of saline and several colonies of each test organism was applied to a disk.

A 30 μg cefoxitin disk was placed on the inoculated surface of the MHA plate. The inoculated AmpC disk was placed almost touching the antibiotic disk with the inoculated disk face in contact with the agar surface. The plate was incubated at 35°C for 16-18 h. An indentation or a flattening of the zone of inhibition indicates enzymatic inactivation of cefoxitin and the strain is identified as AmpC producer. The absence of distortion indicates no significant inactivation of cefoxitin and the test strain is negative for AmpC β-lactamase production.

Gene identification

The *bla*$_{CTX-M}$ genes were identified by PCR. DNA samples at a concentration of 0.1 ng/mL were used as PCR templates and *bla*$_{CTX-M}$ genes were amplified using the universal primers *CTX-M*-U1 (5′-ATGTGC AGY ACC AGT AAR GTK ATG GC-3′) and *CTX-M*-U2 (5′-TGG GTR AARTAR GTS ACC AGA AYC AGC GG-3) obtained from Operon Biotechnologies Germany. DNA from a reference *Escherichia coli* bla$_{CTX-M}$-positive strain was used as a positive control (Monstein *et al.,* 2007).

Results and Discussion

Of the 792 samples analyzed in this study, growth on MacConkey agar was observed in 61 samples. Subsequent phenotypic detection confirmed 54 ESBL (Fig. 1 and 2) and 9 AmpC (Fig. 3) positive isolates of the family Enterobacteriaceae. The most prevalent ESBL and AmpC producing isolates in healthy community and hospitalized patients were *Escherichia coli.* Among all the strains, *Escherichia coli* accounted for 82.35% and 54.05% production of ESBL in healthy and hospitalized patients respectively whereas for AmpC, prevalence was 100% and 42.86% respectively. Two strains of *Escherichia coli*, both from hospitalized patients, produced both ESBL and AmpC. Comparison of the prevalence of ESBL and AmpC positive isolates in hospital and community healthy subjects showed significant difference between the two groups (Table 1). The data obtained revealed 9.34% prevalence of ESBL positive isolates in admitted patients. In healthy volunteer from the community, a total of 17 (4.29%) ESBL positive strains were isolated. Out of 9 AmpC positive isolates, 7 were detected in hospitalized patients; a prevalence of 1.77% and 2 were in community subjects, a prevalence of 0.51%.

Genetic analysis of 54 bacterial isolates with the ESBL phenotype revealed the presence of the *bla*$_{CTX-M}$ gene in 25 isolates (46.3%). Distribution of *bla*$_{CTX-M}$ gene in *Escherichia coli* and *Klebsiella pneumonia* is shown in Table 2 and Figure 4. Rest of the bacterial isolates did not show *bla*$_{CTX-M}$ gene.

Colonization with multi-resistant bacteria, including ESBL-producing isolates, is a prerequisite for infection. The importance of detection of carriers harboring antimicrobial-resistant bacteria has been emphasized not only in patient populations but also in healthy people (Smith *et al.*, 2001). Resistant bacteria may be transmitted from human-to-human or through the environment resulting in an increase in the proportion of carriers in the community (Levin, 2001). The admission of carriers harboring resistant bacteria to hospitals increases the risk of infection in other hospitalized patients (Harris *et al.,* 2004; Bonten *et al.,* 1998). Antibiotic selective pressure in hospitals may amplify the number of carriers harboring resistant bacteria and enhance the opportunity for these bacteria to cause infections (Bonten *et al.,* 1998).

Several studies have focused on beta-lactam resistance in Enterobacteriaceae isolated from stools in healthy people, although specific detection of ESBLs was neither performed nor reported (Brinas *et al.,* 2002; Osterblad *et al.,* 2000). Mirelis *et al.* (2003) reported that 2.1% of outpatients were fecal carriers of ESBL–producing bacteria in 2001 and this percentage increased to 3.8% one year later. Valverde *et al.* (2004) also reported that rates of fecal carriage of ESBL-producing isolates increased significantly ($P < 0.001$) in both hospitalized patients and outpatients, from 0.3% and 0.7%, respectively, in 1991 to 11.8% and 5.5%, respectively, in 2003. The rate of

ESBL-producing isolates among healthy volunteers was reported to be 3.7%.

In the present study, 37 (9.34%) of 396 hospitalized patients and 17 (4.29%) of 396 healthy volunteer from community harbored ESBL-producing Enterobacteriaceae in the gut (P =0.03). Inpatients were not further categorized into intensive care unit patients or ward patients.

In contrast to ESBL enzymes, carriage of AmpC positive bacterial enzymes have not been studied much. Kaneko *et al.* (2006) reported an *Escherichia coli* isolate producing AmpC beta-lactamase from the CIT group in a healthy medical student. They suggested that both constitutive and inducible AmpC beta-lactamases may extensively spread in the community. In 2008, carriage of AmpC beta-lactamases in the GIT was detected in nearly 4% of healthy Danish army recruits. The same group was also found to carry ESBL-positive Enterobacteriaceae (Hammerum *et al.*, 2011).

In our study, the prevalence of AmpC positive bacteria in the GI tract of healthy community subjects was reported to be 0.51% and in hospitalized patients it was found to be 1.77%. Most prevalent strains were *Escherichia coli* followed by *Klebsiella pneumoniae.* An important result of this study was the detection of both ESBL and AmpC in two isolates of *Escherichia coli* of hospitalized patient.

Table.1 Overview of ESBL- and AmpC-positive isolates and differences in species distribution of these isolates in hospital and community settings

	Total number of isolates		Hospitalized patients				Community subjects			
Species	ESBL+ (count)	AmpC+ (count)	ESBL+ (count)	ESBL+ (%)	AmpC+ (count)	AmpC+ (%)	ESBL+ (count)	ESBL+ (%)	AmpC+ (count)	AmpC+ (%)
Citrobacter freundii	01	00	01	2.7	00	0.0	00	0.0	00	0.0
Enterobacter aerogenes	00	01	00	0.0	01	14.29	00	0.0	00	0.0
Enterobacter cloacae	01	00	01	2.7	00	0.0	00	0.0	00	0.0
Escherichia coli	34	05	20	54.05	03	42.86	14	82.35	02	100
Klebsiella oxytoca	03	01	03	8.11	01	14.29	00	0.0	00	0.0
Klebsiella pneumonia	15	02	12	32.43	02	28.57	03	17.65	00	0.0
Total	54	09	37		07		17		02	

Table.2 Genetic analysis of ESBL-positive Enterobacteriaceae

bla gene	Number of isolates			
	Escherichia coli		*Klebsiella pneumonia*	
	hospital	community	hospital	community
CTX-M	12	06	06	01

Figure.1 Double disk synergy test

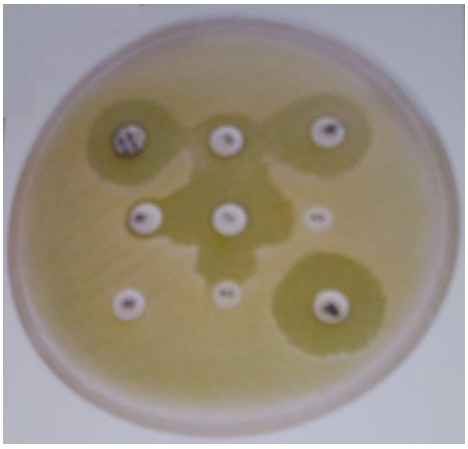


Figure.2 Combined disk test

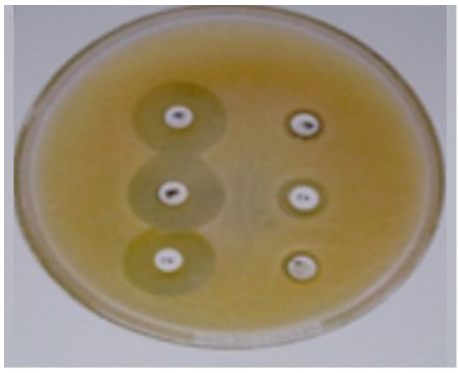


Figure.3 AmpC disk test

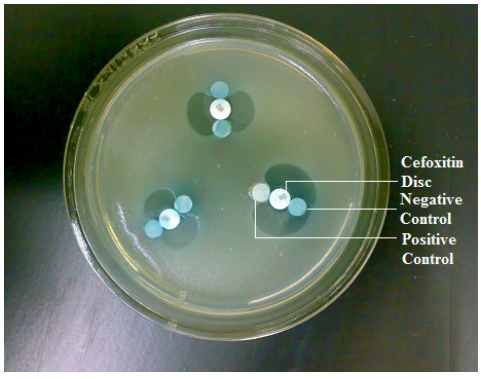
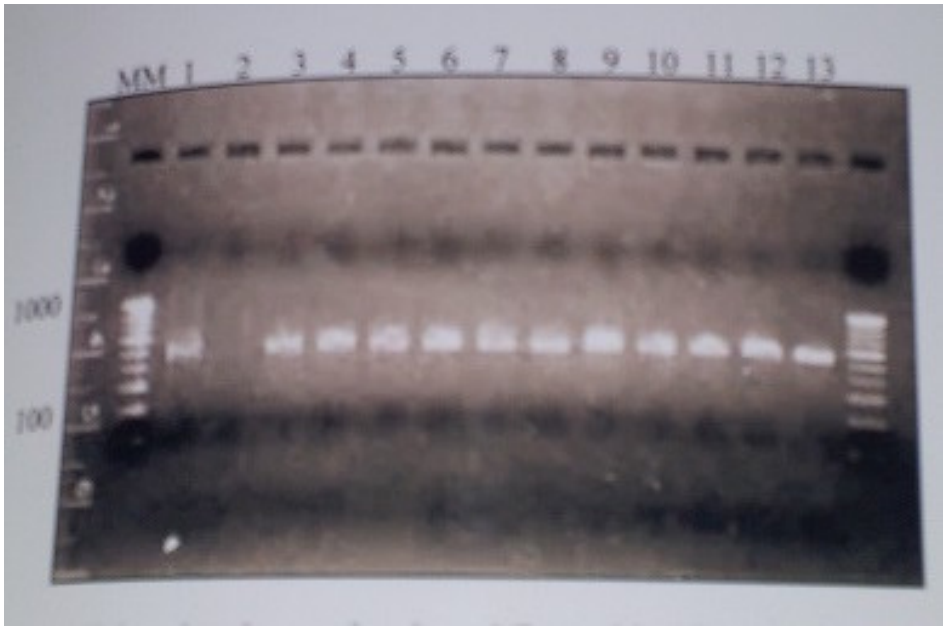


Figure.4 PCR amplification for *blaCTX-M* gene



MM molecular marker lane
Lane 1, 2 positive and negative control for *CTX-M* gene respectively
Lane 3-8, *Escherichia coli* positive for *CTX-M* gene
Lane 9-13 *Klebsiella spp*. positive for *CTX-M* gene

To summarize, asymptomatic colonization of the intestinal compartment with ESBL isolates is considered a prerequisite for infection. This increase is associated with the predominance of ESBLs with *CTX-M*-type enzymes. Travel to countries with high rates of ESBL has also been associated with intestinal colonization with these isolates. The importance of the detection of carriers of antimicrobial resistant bacteria has been highlighted not only in patient populations but also in healthy people.

The increase in the proportion of carriers in the community increases the risk that other individuals will become carriers as a consequence of human-to-human transmission of resistant bacteria or through the environment, enriching the resistance gene pool and thus facilitating the acquisition of resistance mechanisms by susceptible bacteria.

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