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Antibiotic resistance patterns and frequency of ESBL producing isolates of *Escherichia coli* in Hospital sewage water of South Delhi, India.

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Abstract
Extended spectrum β - lactamase (ESBL) producing strains of *E. coli* have caused paramount therapeutic problems globally as majority of them are resistant to variety of antibiotics. In this study, the antibiotic susceptibility testing was carried out to assess the resistance patterns offered by the 70 *E. coli* isolates to seven different antibiotics and to conduct phenotypic screening for ESBL producing *E. coli* strains cultured from the hospital sewage water of South East Delhi during 2017-2018. The Phenotypic screening for ESBL production using preliminary and PDCT revealed 35 (50%) out of 70 *E. coli* isolates as ESBL producers and remaining samples were considered as non-ESBL producers. Further, the presence of genes responsible for ESBL in *E. coli* was confirmed by PCR based techniques. Based on our results, it is recommended that prescription of oxyimino-cephalosporins should be restricted to susceptible isolates and that the use of other effective antibiotics must be considered. There is also a necessity of constant and regular antimicrobial sensitivity surveillance for the presence of ESBL genes. Preliminary disinfection of the hospital sewage before it exits the premises will assist in minimizing the spread of antibiotic resistant bacteria to the environment.

Keywords: Extended spectrum β-lactamase, E coli, Sewage, Oxyimmino- cephalosporins

1. Introduction

It is paradoxical that the, consequences of using drugs once pronounced as "wonder drugs", i.e. antibiotics, has created a life threatening situations mostly among developing and under developed countries nearly twenty years after they were formulated and marketed. Today we stand at the threshold of the catastrophe that confronts medical practitioners which means resistance is fast developing against almost all of the antimicrobials and no more new molecules are being introduced by the pharmaceutical giants. Antibiotic resistance and associated genes are ubiquitous creating a call of dare throughout the world and this monster now staring at the humanity posing a serious challenge. Exploitative use of antimicrobials on a large scale led to appearance of organisms so called antibiotic resistant bacteria (ARB). Emergence of Antibiotic resistance is a natural phenomenon but the pace of this process gets accentuated in the presence of strong selective pressure exerted by these compounds left in the environment by their callous use. Antimicrobial resistance has been identified as a critical issue by the WHO, U.S. Centre for Disease control (CDC), European Centre for Disease prevention and Control (ECDC) and many other global authorities in public health (I. Roca, M. Akova, *et al*., 2015) [16]. Aquatic environment is known to harbour diverse range of microbes and the extensive accumulation of antibiotics from various sources such as sewage treatment plants, hospital effluents, run off from farm areas exert a selection pressure that leads to evolution of bacteria that are resistant to antibiotics, which then grow in numbers and spread in the environment. Resistance thus developed makes them potent to compete with the sensitive ones, specifically in selective environments. The situation could pose a danger to human health as infection with such kind of resistant bacteria could become untreatable. *E. coli*, a versatile Gram - negative,

Facultative ESBL (extended spectrum β-lactamase) producing bacterium existing both as pathogen as well as commensal. Earlier only pathogens were considered as problems for human beings but selective pressure exerted by antimicrobial drug use has led to commensal *E. coli* harboring a number of antibiotic resistance genes *viz.* quinolone, aminoglycosides, tetracycline resistance genes etc. and its ability to transfer antimicrobial drug resistance is well known (Richmond M H 1972). Genetically, the population of *E. coli* strains can be subdivided into one of the four major phylogroups *viz.* A, B1, B2 and D (Herzer *et al*. 1990) [14]. In general the strains of bacteria belonging to phylogroups A and B1 mainly consist of commensals, while virulent extraintestinal strains belong mainly to phylogroup B2 and D (Clermont *et al*. 2000) [9]. A hallmark of such virulent *E. coli* clones is their possession of specialized virulence factors, traits that confers pathogenic potential and characteristically are infrequent among commensal strains. Recognized virulent factors of extraintestinal *E. coli* include diverse adhesions, toxins, and polysaccharide coatings. β-lactam class of antibiotics has been widely used for the therapeutic treatment of the *E. coli* pathogenic strain (Henriques *et al*., 2006) [15]. However, the massive and indiscriminate use of antimicrobials has led to the emergence and widespread dissemination of β-lactamases like extended spectrum β-lactamases (ESBL), carbapenemases including mettalo β-lactamases, and also resistance to the last resort antibiotics for treating Gram negative organisms. The mechanism of resistance involves drug target site alteration, change in the permeability of cell membrane, efflux pumps, inactivation of the drug by target modification. These genes are associated with the mobile

genetic elements like transposons, integrons, and plasmids and hence can be easily transmitted to other species causing a serious threat to the public health domain.

2. Materials and methods

2.1 Bacterial isolates

In total, 150 bacterial isolates were collected from sewage of the Holy Family Hospital and Al- Shifa hospital located in South East Delhi, using conventional sampling methods. Samples were collected in sterilized autoclaved capped vials (10ml), brought to the lab, and were processed the same day. Spread plate method was employed to grow microbes present in the samples. Samples were serially diluted and 100µl of each dilution was spread on Luria agar plates and incubated overnight at 37°C. Morphologically distinct bacteria were picked up from the Luria agar plates, streaked on MacConkey Agar and incubated at 37°C. Pink colonies on MacConkey agar plates were considered as lactose fermenting colonies and selected for transfer onto Eosine Methylene Blue (EMB) agar, incubated over night at 37°C. Metallic green sheen colonies on EMB plates were selected as presumptive *E. coli* strain. Then isolates were recognized by biochemical tests like: Indole test, Simon citrate, MR-VP.

2.2 Molecular confirmation of *E. coli* by 16S rRNA analysis

The identity of all the *E. coli* strains positive for the biochemical tests were confirmed by performing 16S ribosomal RNA (rRNA) analysis. PCR amplification of 16S rRNA gene was carried out by using specific primers. PCR conditions for this gene comprised a thermal temperature of 94°C for 5 min, followed by 35 cycles of 94°C for 2 min, annealing at 70°C for 30 sec, and extension at 72°C for 2 min, followed by final extension for 10 min at 72°C.

2.3 Antimicrobial susceptibility testing

The tested antimicrobial agents were: Ampicillin (AMP 10µg), Nalidixic acid (NA 30µg), Streptomycin (S 10µg), Ceftazidime (CAZ 10µg), Cefotaxime (CTX 10µg), Ceftriaxone (CTR 10µg) and Imipenem (IPM 10µg).

2.3.1 Screening of ESBL producers by disk diffusion method

a. Preliminary test

The screening for ESBL producers was done by the disc diffusion test as recommended by the CLSI (Clinical and Laboratory Standard Institute). Ceftazidime (30µg), Cefotaxime (30µg), Ceftriaxone (30µg) were used as indicator drugs. Mueller Hinton Agar (MHA) plates were inoculated with test isolates and antibiotic discs were placed at appropriate distance. Parafilm sealed plates were incubated at 37°C for 12-14 hours and analysed for zone of inhibition.

b. Phenotypic Disc Confirmatory Test (PDCT)

The test inoculum was spread onto the MHA by using sterile glassbeads;

c. a ceftazidime (CAZ) disc and a ceftazidime + clavulanic acid (CAC) disc were placed at distance of 30mm from each other,

d. a cefotaxime (CTX) disc and a cefotaxime + clavulanic acid (CEC) disc were placed at adistance of 30 mm from each other. The plates were incubated overnight at 37°C and the results were read. A $\geq$ 5 mm increase in the zone diameter for CAC versus its zone diameter whentested alone by CAZ and/or a>5 mm increase in the zone diameter for CEC versus its zone diameter when it is tested alone by CTX, confirmed an ESBL-producing organism.

2.4 Screening for blaCTX-M gene

The key to the PCR lies in the designing of oligonucleotide primers. A search for the nucleotide sequences of CTX-M gene was carried out in the NCBI nucleotide database. CTX-M gene of different bacterial species were chosen for designing of forward and reverse primers.

3. Results

A total of 76 (50%) among 150 bacterial isolates were confirmed by biochemical tests and identified as *E. coli*. Among the screened isolates, 35 (50%) strains of *E. coli* were designated as ESBL positive isolates by preliminary and PDCT test.

3.1 16S rRNA gene analysis

16S rRNA gene analysis of the *E. coli* genome confirmed 70 isolates to be *E. coli*. Following homology determination between conserved complete DNA sequences (CDS) regions of 16S rRNA gene, following primer pairs were chosen for amplifications of desired genes (Table 1). Fig.1 shows the agarose gel electrophoresis of the 702 bp amplicon of the 16S rDNA gene of *E. coli.*

Table 1: Primers and amplification conditions used for detection of 16S rRNA gene.

Target	Primers for 16S rRNA gene	Amplicon Size (bp)	Annealing Gene Temp.
16S rRNA	F-acatgcaagtcgaacggtaaca	702	54°C
Gene *(E coli)*	R- gcacctgagcgtcagtcttc		

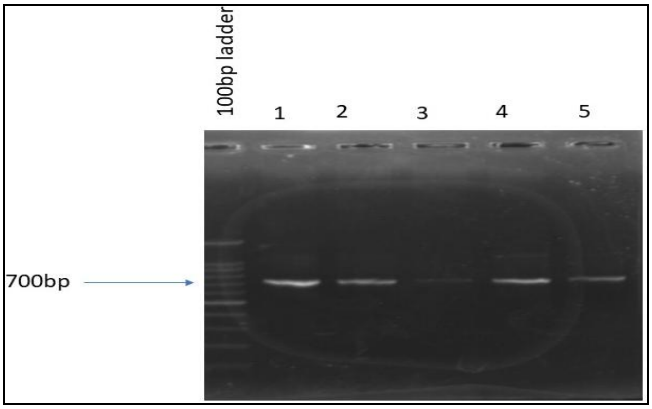

Fig 1: 100bp ladder (Genei), Lane 1-5 is 16S rDNA gene amplicon of *E. coli*

3.2 Antimicrobial susceptibility

Ninety five percent (95%) of the isolates demonstrate most effective antibiotic susceptibility against imipenem followed by streptomycin (63%). Average effectiveness was found for Ceftriaxone (56%), Cefotaxime (50%) and Ceftazidime (50%); and least effective drugs were Ampicillin (4%) and Nalidixic Acid (14%) (Fig. 2).

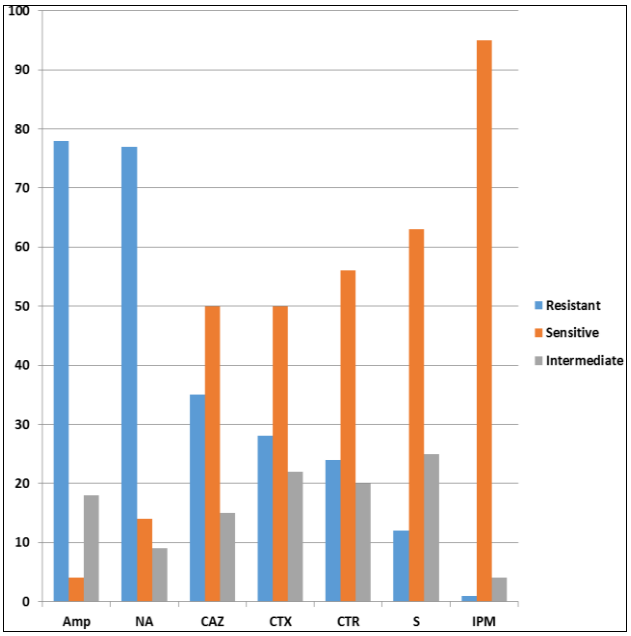

Fig 2: Antibiogram showing antibiotic susceptibility among *E. coli* isolates during AST

The highest resisted antibiotics were Ampicillin (78%) among 55 strains and Nalidixic Acid (77%) in 52 strains. This was followed by Ceftazidime (35%) in 25 strains, Cefotaxime (28%) in 20 strains, Ceftriaxone (24%) in 17 strains, Streptomycin (12%) in 9 strains and Imipenem (1%) in 1 strain.

3.3 Screening of ESBL producers by Preliminary test and PDCT

Those with a zone diameter of <22mm, <27mm,<25mm and <27mm for ceftazidime, cefotaxime, ceftriaxone and aztreonam respectively were suspected to be ESBL producers and it was further confirmed by the phenotypic disc confirmatory test. 35 isolates were tested for the production of ESBL by preliminary tests and phenotypic disc confirmatory tests (PDCT) as per CLSI guidelines (Fig.3a, Fig. 3b).

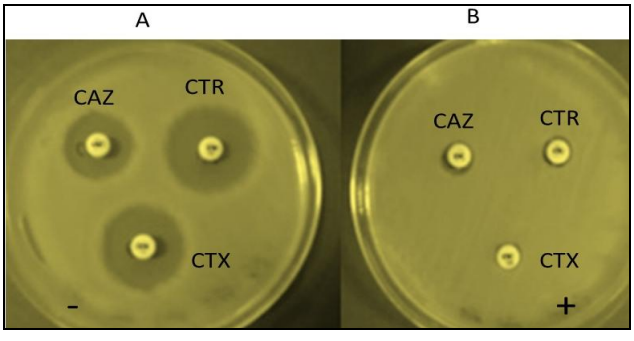

Fig 3: Mueller Hinton agar plates showing A) negative result B) ESBL production via Preliminary test.

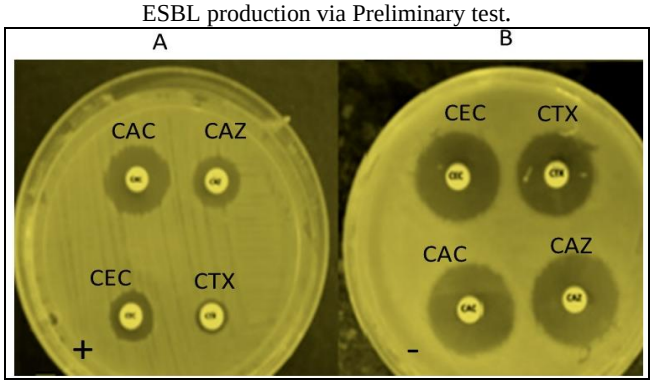

Fig 4: Mueller Hinton agar plates showing A) ESBL production by PDCT B) negative result.

3.4 Screening for CTX-M gene

The CTX-M gene was detected in 23/35 (65%) of ESBL resistant *E. coli* by PCR (Fig. 4) showing that the *bla*$_{CTXM}$ gene was the predominant group of the plasmid-coded ESBLs.

Primer and amplification conditions used for detection of *bla*CTX-M are given below:

Target	Primers for CTX-M gene	Amplicon Size (bp	Annealing Gene Temp.
*bla*CTX-M	F-AAAAATCACTGCGTCAGTTCAC	823	60^0C
(*E. coli*)	R-TAGCCGCCGACGCTAATAC		

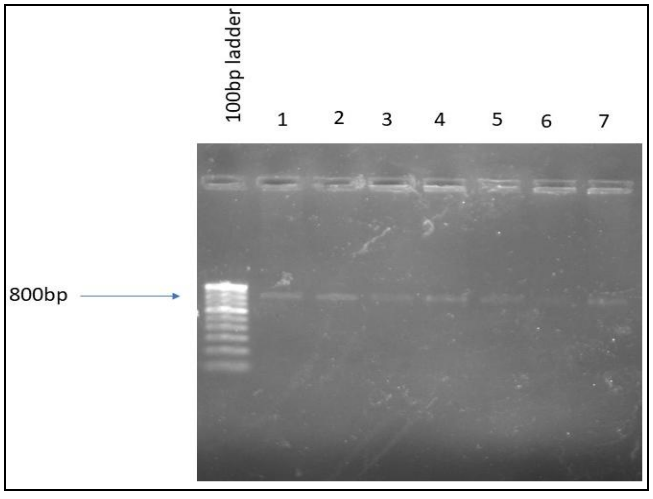

Fig 4: Agarose gel electrophoresis of PCR amplification of *blaCTX-M* gene. 100 bp DNA ladder a) Lane 1-7 amplified *blaCTX-M* gene product of *E. coli* (823bp) from different samples.

4. Discussion

Hospitals are hotspots for antibiotic resistant bacteria and play a major role in both their emergence and dissemination. Large numbers of these ARB will be ejected from hospitals via wastewater systems (D. Hocquet, A. Muller, *et al.*, 2016) [10]. The World Health Organisation (WHO, 2014) [26].recommend that hospitals should have onsite facilities for the pre-treatment of hospital effluent prior to its release into the general wastewater stream in order to remove the presence of harmful components including microbiological

pathogens, toxins and antimicrobials. In recent years, some countries including Germany and Denmark, have started treatment of hospital effluent onsite for the elimination of all harmful contaminants prior to release into the general wastewater stream (Krarup *et al*., 2015 [19]; Microdyn-Nadir, 2012) [20]. Unfortunately, due to high-cost and operational challenges associated with onsite treatment of hospital effluent, progress on this issue has been slow in many other countries (Pauwels and Verstraete, 2006) [24].

Antibiotic resistance is a major public health concern globally owing to its ability to hamper the efficiency of the antibacterial treatment. It also poses an immense challenge in the path of development of novel and effective antibiotics. The advent of novel resistance patterns is increasingly intensifying the menace of drug resistance in bacteria. Thus this inefficacy of the antibacterial treatment is leading to the past of "pre- antibiotic" era. Therefore, the necessity for innovative methodologies to confront antibacterial resistance is the need of the hour now.

E. coli are becoming increasingly resistant to many of the available antibiotics in the pharmaceutical market particularly an important class of antibiotics, members of which contain a β- lactam ring that inhibits peptidoglycan synthesis by covalent binding to the active site serine of penicillin binding protein (PBP's). β-lactam sub-class includes carbapenems, cephalosporin, penicillin, monobactam and clavams. This predicament is further accentuated by the emergence of ESBL producing *E. coli* strains. The earlier known β- lactams that were active against first generation β- lactams, were followed by ESBL's that have the ability to hydrolyse oxyimino-cephalosporins. It is important to note that the genetic determinants of diverse ESBL'S and carbapenemases incuding imipinase, Verona integron encoded metalloβ-lactamase (VIM), K. pneumonia carbapenemase(KPC), oxacillinase (OXA) and NDM enzyme in gram negative bacteria like *K. penumoniae*, *E. coli*, *P. energinosa* and *A. baumannii* has supported the emergence of isolates that demonstrate resistance to all β- lactam antibiotic.

Most of the resistance genes of bacteriaevolve under the influence of antibiotics as a defense mechanism to protect themselves against the action of antibiotics. Much of resistance has also been reported from environmental organisms specifically those originating from soil but had exposure to several different types of drugs during the course of their evolution.

The present study clearly brings forth that there is high prevalence of ESBL producing *E. coli* in sewage water collected from Al- Shifa and Holy family hospitals situated in South east Delhi and therefore, they showed resistance to commonly prescribed antibiotics.

In our study, the highest resisted antibiotics were found to be Ampicillin (78%) among 55 strains and Nalidixic acid (77%) in 52 strains. This was followed by Ceftazidime (35%) in 25 strains, Cefotaxime (28%) in 20 strains, Ceftriaxone (33.8%) in 17 strains, Streptomycin (12%) in 9 strains and Imipenem (1%) in 1 strain.

Imipenem is an antibiotic which is not being used in clinical practice, but still the micro organisms have started to show resistance against it. This is a dangerous trend which could ultimately lead to the gain of complete resistance against imipenem too as the course of evolution takes place.

The CTX-M gene was detected in 23/35 (65%) of ESBL resistant *E. coli* by PCR showing that the *bla* $_{CTX-M}$ gene was the predominant group of the plasmid-coded ESBLs.

Since most of the conventional antibiotics have started showing greater degree of resistance towards most of the pathogen, physicians are compelled to prescribe these last resort drugs to treat infections. Although these drugs are very effective in dealing with the infections, but future is not far away even when these drugs will be rendered ineffective. Interaction of bacteria with these drugs would lead to development of resistance and enhanced resistance of ESBL. In other studies, this is also observed that microorganisms show antibiotic resistance even in the absence of exposure of commercial antibiotic, since these microorganisms have been already exposed to similar naturally occurring antibiotics. Therefore, it is a frightening situation that needs immediate attention of health administration of the world in general and of India in particular. Also antimicrobial resistance should now be seen as an 'environmental pollutant', and new wastewater treatment processes must be assessed for their capability in removing ARB, especially from hospital effluents (D. Hocquet, A. Muller, *et al*., 2016) [10].

Also molecular studies and mechanisms of resistance machinery of the micro organisms need to be monitored regularly. By frequent examination and scrutiny, suitable antimicrobials need to be developed to keep up with the evolutionary trends of the micro organisms.

5. Conclusion

With reference to occurrence of ESBL producing organisms in clinical settings and also in different ecological niches in India is very grim compared to rest of the world because such multidrug resistant microbes are highly stubborn to the commonly prescribed antimicrobials in our clinics. After the introduction of β-lactam drug as antimicrobial in 1940s, the CTX-M enzymes, like TEM and SHV type ESBL emerged in 1980s. Although they were not prevalent until 1955 but in recent times, it has become a hazard all over the world. Their genes are the descendants of chromosomal *bla* genes of *Klebsiella* species and family of *Enterobacteraceae.*

The results in this study are a part of larger study that we have carried out. Since the presence of ESBL type of drug resistance are constantly evolving and are under dynamic flux, hence it poses a global threat to public health concern, there is a a necessity for regular antimicrobial sensitivity surveillance not only for the presence and spread of ESBL genes both in both rural and urban populations but also for more informed treatment. Moreover, the preliminary disinfection of hospital sewage before its inflow into the sewage system might minimize the spreading of antibiotic-resistant bacteria to the environment.

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