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Original Research Article

Antibiotic Resistance in Extended-Spectrum Beta-Lactamase-Producing Escherichia Coli Isolated from Effluents of Tertiary Care Hospitals

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ABSTRACT

The emergence and spread of antimicrobial resistance has become a global public health problem and the indiscriminate use of antibiotics contributes to the dissemination of resistant pathogens in the environment. The objective of the present study is isolation and detection of ESBL (Extended Spectrum β-lactamase) - producing *E coli* present in the untreated effluents of two tertiary care hospitals (H1& H2) located in Mangalore. A total of 55 untreated hospital effluents were collected and analysed in the laboratory. *E coli* isolates were isolated and identified by conventional methods as well confirmed by Polymerase Chain Reaction (PCR). An antimicrobial susceptibility test was performed for isolated *E coli* using Kirby Bauer method as phenotypic method. ESL detection was carried by combined disc methods and confirmed by triple ESBL detection strip. The MICs for selected antibiotics were determined using Ezy MIC^{TM} strip method. Total of 142 *E coli* isolates isolated from 45 effluent samples and 39 isolates were showed ESBL positive. Among ESBL positive *E coli*, high level of resistance was found towards ampicillin 92% followed by nalidixic acid (85%), carbapenem (77%), ciprofloxacin (72%), co-trimoxazole (62%), tetracycline (49%), gentamicin (33%), piperacillin- tazobactam (28%), nitrofurantoin (33%) and chloramphenicol (18%). ESBL positive *E coli* isolates from effluents of hospitals had a high MIC values (≥ 256 µg/ml) for nalidixic acid and tetracycline. Our study showed that waste water coming from these tertiary care hospitals contains high number of multi drug resistant *E coli*. Hospitals should adopt proper waste water treatment procedure before releasing into the sewage system, hence can minimize the spread of antibiotic resistant bacteria into the environment.

Key-words: Hospital effluents, ESBL, tertiary, susceptibility, Mangalore.

INTRODUCTION

Wastewater coming from hospitals may contain several toxic substances and pathogenic microbes, hormones, drugs, radioisotopes, heavy metals and antimicrobial resistant bacteria. [1] Resistant bacteria were isolated from clinical and environmental sources, there are only limited studies were observed in the detection of ESBL producing bacteria, this widespread occurrence of ESBL-producing isolates suggests that the spread of these strains may be through food chain and the possibility that community act as a reservoir. [2] The continuous exposure of bacterial strains to β-lactam antibiotics

induces mutation and produce β-lactamases enzymes and show resistance against the third and fourth generation cephalosporins such as ceftazidime, cefotaxime and cefepime hence these new β – lactases are called extended spectrum β-lactamases (ESBLs), which are plasmid mediated enzymes. [3]

ESBL producing multidrug resistant *Citrobacter* species were isolated from a tertiary care health center. Bla_{CTX-M} gene was detected in most of the isolates and is important for epidemiological purpose because of its tendency for rapid dissemination to other unrelated bacterial species. [4] Beta-lactams are the largest group of antibiotics used to treat bacterial infections caused by gram negative bacteria in humans. *E coli* most common inhabitant in the gut of human, represents a large part of bacterial communities colonize in effluents of hospitals, these opportunistic human pathogens cause various infectious diseases, in particular nosocomial infections such as septicemia, urinary tract infection and meningitis. [5-6] The β-lactamases like AmpC β-lactamases (AmpC), ESBLs and metallo-β-lactamases (MBLs), have emerged worldwide as a cause of antimicrobial resistance in gram negative bacteria (GNB). Genes responsible for their resistance were carried in the plasmids, facilitating rapid spread between the microorganisms. [7] Since hospital effluents are found to be major reservoir for antibiotic resistant bacteria, we assessed the presence of ESBL producing *E coli* isolates in untreated hospital effluents and phenotypic resistance pattern of isolated bacteria.

MATERIALS AND METHODS

Bacterial isolates: *E coli* isolates were isolated from the untreated sewage outflow of two different tertiary care hospitals located in Mangalore. The effluents comes from these hospitals were collected and isolation of bacteria carried out using tryptic soya agar (TSA) as a nonselective medium by spread plate method. The plates were incubated at 37 ^{0} C aerobically for 24 hrs and checked for the growth of bacteria. Colonies differing in their morphology were selected and Gram stained. Only gram negative bacteria were selected and identified for *E coli* by conventional method following biochemical tests. [8] *E coli* isolates were confirmed by targeting species specific genes by polymerase chain reaction (PCR).

Polymerase chain reaction (PCR): Biochemically identified *E coli* isolates were further confirmed by PCR. *uidA* PCR primer was used for E coli detection by targeting a 146bp fragment of the gene; UAL-754 forward:5'-AAAACGGCAAGAAAAAGCAG-3' and UAR-900 reverse: 5'-ACGCGTGGTTACAGTCTTGCG-3'
PCR was carried out in a programmable thermo cycler (MJ Research, USA) using 30 µI reaction mixture containing 10X buffer (100mM of Tris- HCl, pH 8.3, 20mM 0f $MgCl_2$, 500mM of KCL and 0.1% gelatin) 200mM of deoxyribonucleotide triphosphate (dATP, dT TP, dGTP and dCTP), 10 picomoles of each primer and 1 U of *Taq* polymerase (Bangalore Genei, Bangalore), with 2.0 µl of DNA as template.

The optimized PCR programme consisted of an initial denaturation at 94^0 C for 5 min followed by 30 cycles with 94^0 C for 30 sec, Tm (annealing temperature) 60^0 C for 30 sec and extension for 72^0 C for 30 sec with final extension at 72^0 C for 10 min. The amplified products were resolved by 1.5 % (w/v) agarose gel electrophoresis.

Antimicrobial Susceptibility Test: All *E coli* isolates were tested for their susceptibility against 12 different commercially available antibiotic discs (HiMedia, India) on Muller Hinton Agar (MHA) as recommended by the Clinical and Laboratory Standards Institute (CLSI, 2012). *E coli* culture were grown in

Mueller-Hinton broth for 4-6 h and the turbidity was adjusted to 0.5 McFarland's standard and plated onto Mueller-Hinton agar (MHA). The respective antibiotic discs were placed on the plates and incubated for 24 hours at 37°C. The antibiotic discs included in the tests were nalidixic acid (30mcg), tetracycline (30mcg), co-trimoxazole (25mcg), ciprofloxacin (5mcg), chloramphenicol (30mcg), ampicillin (10mcg), gentamicin (10mcg), nitrofurantoin (300mcg), imipenem (10mcg), meropenem (10mcg), cefotaxime (30mcg), piperacillin-tazobactam (100/10mcg) and reading of sensitivity and resistance interpreted according to the CLSI (2012) guidelines. [9] (Figure 1). *E.coli* ATCC 25922 was used as control.

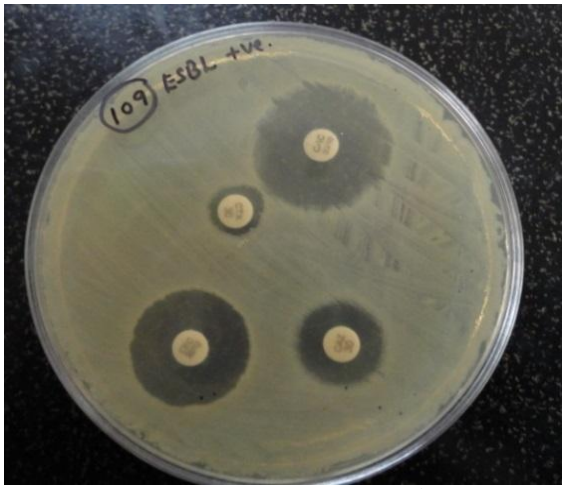

Figure 1: ESBL detection by DDDT [CAZ: Ceftazidime; CAC: ceftazidime + clavulanate; CTX: Cefotaxime; CEC: Cefotaxime + clavulanate]

Phenotypic method for ESBL detection: The isolates showing resistance to cephalosporins (3rd generation drug) were tested for the presence of ESBL by double disc diffusion test (DDDT) and were confirmed by Triple ESBL detection Ezy MIC ™ strip. A young culture was grown in MHB and the turbidity was adjusted to 0.5 McFarland standards and swabbed on Muller Hinton Agar plates. Four cephalosporin discs namely Ceftazidime (CAZ-30mcg) and Ceftazidime + Clavulanic acid (CAC 30/10 mcg), Cefotaxime (CTX-30mcg) and Cefotaxime + Clavulanic acid (CEC-30/10 mcg) were placed on Muller Hinton Agar plates containing the inoculum. The plates were incubated for 24hrs at 37^0 C, a > 5mm increase in the diameter of the zone around the disc with clavulanic acid compared to disc without clavulanic acid was considered positive for ESBL production. (Figure -1). Isolates showing inhibition zones ≤ 22 mm for cefotaxime, ≤ 17 mm for ceftazidime were identified as potential ESBL producers and again confirmed using triple ESBL detection strip. (Figure 2). *E coli* ATCC 25922 strains were used as control strain.

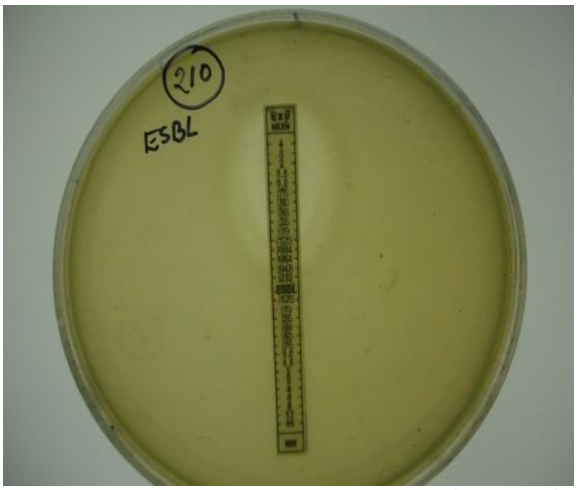

Figure 2: Triple ESBL detection E strip showing deformation of the inhibition ellipse indicative of ESBL

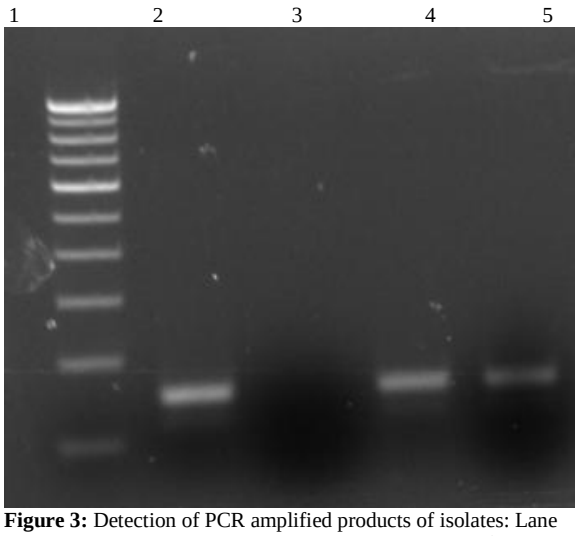

Figure 3: Detection of PCR amplified products of isolates: Lane 1: 100bp DNA ladder, Lane 2-*uid A* gene (146bp) of *E coli isolate*. Lane 2- negative control, lane 4 & 5 positive isolates.

Determination of Minimum inhibitory concentration (MIC): The MIC was

considered as the lowest concentration of antibiotic which produced no significant growth. The MICs for selected antibiotics were determined using Ezy MIC^{TM} strips (HiMedia Laboratories Pvt. Ltd., India) method on MH medium following the guidelines of the Clinical and Laboratory Standards Institute (2011). [9] Briefly, a young culture of *E coli* grown for 4-6 h in 5ml MH broth (HiMedia, Laboratories Pvt. Ltd., India) was poured on well dried MH agar (HiMedia Laboratories Pvt. Ltd., India) to prepare a lawn. After gently air drying in a laminar flow, the MIC strips were placed on the surface of the medium with the help of provided applicator and incubated for 18-24 h at 37 °C. After sufficient bacterial growth the MIC was read where the ellipse intersects the MIC scale on the strip. *E. coli* ATCC 25922 was used as the quality control strain.

RESULTS

In our study we collected 55 untreated hospital effluents before releasing into the corresponding effluent treatment plant. Total 142 *E coli* isolates were isolated from effluents of two hospitals. All *E coli* isolates were identified by conventional methods and confirmed by polymerase chain reaction. PCR amplification yielded expected product size for *E coli* by agarose gel electrophoresis (Figure 3). Confirmed pure cultures were preserved in 30 % glycerol broth at – 80 ˚ C for further studies.

Table 1: Antibiotic resistance pattern of ESBL positive *E coli* isolates showing resistance more than 6 different classes of antibiotics NA-Nalidixic acid, TE-Tetracycline, COT-Cotrimoxazole , CIP-Ciprofloxacin , C-Chloramphenicol , AMP-Ampicillin , GEN-Gentamicin, AMP-Ampicillin, GEN- Gentamicin, NIT-Nitrofurantoin, IPM-Imipenem, MRP-Meropenem, CTX-Cefotaxime, PIT - Piperacillin/Tazobactam.

Type	Antibiotic resistance pattern	No. of isolates showing resistance
1	NA,CIP,AMP,MRP,CTX,PIT	1
2	NA,CIP,AMP,GEN,MRP,CTX,PIT	1
3	NA,TE,COT,CIP,AMP,GEN,MRP,CTX,PIT	1
4	NA,TE,COT,CIP,AMP,GEN,NIT,IPM,MRP,CTX,PIT	1
5	NA,C,AMP,NIT,IPM,CTX,PIT	1
6	NA,CIP,C,GEN,MRP,PIT	1
7	NA,TE,COT,CIP,C,AMP,GEN,NIT,MRP,CTX,PIT	1
8	NA,TE,COT,CIP,AMP,GEN,MRP,CTX	4
9	NA,TE,COT,AMP,GEN,MRP,CTX	1
10	NA,CIP,AMP,GEN,IPM,MRP,CTX,PIT	1
11	NA,TE,COT,CIP,AMP,MRP,CTX,PIT	2
12	TE,CIP,GEN,NIT,MRP,CTX,PIT	1
13	NA,TE,COT,CIP,C,AMP,MRP,CTX	1
14	NA,COT,CIP,AMP,MRP,CTX	4
15	NA,TE,COT,C,AMP,NIT,MRP,CTX	1
16	NA,TE,COT,CIP,AMP,MRP,CTX	3
17	TE,CIP,GEN,NIT,MRP,CTX,PIT	1
18	NA,TE,COT,CIP,C,AMP,MRP,CTX	1
19	NA,TE,CIP,AMP,MRP,CTX	1
20	NA,COT,CIP,C,AMP,GEN,MRP,CTX	1
21	NA,CIP,AMP,GEN,NIT,MRP,CTX	1
22	NA,TE,COT,CIP,AMP,GEN,NIT,MRP,CTX	1
23	NA,TE,CIP,AMP,MRP,PIT,CTX	1
24	NA,TE,COT,CIP,AMP,MRP,PIT,CTX	1
25	TE,COT,AMP,CTX	1
26	COT,AMP,CTX	2
27	CIP,AMP,MRP,CTX	1
28	NA,COT,AMP,CTX	1
30	COT,AMP,CTX	1
Total		39

Out of 142, thirty nine isolates (27%) were found to be positive for ESBL by the ceftazidime-clavulanate and cefotaxime- clavulanate combined disc test respectively. Strains that expressed a phenotype of resistance to two or more classes of antibiotics were considered as multidrug resistant. In this way, all ESBL producing *E coli* strains were multidrug resistant. *E coli* isolated from hospital

effluents showed higher resistance to antibiotics tested and most of them were multidrug resistance. Among all group of antibiotics tested, highest resistance was observed towards ampicillin 92% followed by nalidixic acid (85%), carbapenem (77%), ciprofloxacin (72%), co-trimoxazole (62%), tetracycline (49%), gentamicin (33%), piperacillin-tazobactam (28%), nitrofurantoin (33%), chloramphenicol (18%). The resistance pattern exhibited by the bacterial isolates in the study is presented in Table 1. All ESBL positive *E coli* isolates from effluents had a high MIC values (≥ 256 µg/ml) for nalidixic acid and tetracycline. The MICs range for other antibiotics is represented in a Table 2.

Table 2: ESBL positive *E coli* isolates showing different MIC values for different antibiotics tested

Antibiotics	MIC(µg/mL)
Ampicillin	8 - ≥256
Piperacillin-Tazobactam combination	4 - ≥256
Cefotaxime	0.063 - >32
Ceftazidime	≥256
Meropenem	0.75 - > 32
Nalidixic acid	≥256
Ciprofloxacin	≥32
Cotrimoxazole	0.025 - ≥32
Chloramphenicol	2 – 256
Tetracycline	≥256
Nitrofurantoin	8 – 192

DISCUSSION

Prevalence of ESBL producing organisms in the environment pose unique challenges to scientists, researchers, infection control professionals in finding of new antibacterial molecules. ESBL producing strains are found frequently in hospitals where they use excess of antibiotics and the patients are in critical condition. In India, several studies have reported the prevalence of ESBLs in tertiary care hospitals. [10-12] Extended spectrum β-lactam antimicrobial drugs are commonly included in empirical antibiotic regimens for the treatment of gram negative sepsis but the emergence of these bacteria poses a serious threat to humans. [13]

In our study, of the 142 *E coli* isolated from hospital effluents, 39 were found to produce ESBLs (27%). In a study carried by George *et al*, 2014, [14] out of 363 *E coli* isolates 122 isolates were showed ESBL positive (34%) when tested using same double disc diffusion method. The MDR present study showed multi drug resistant (MDR) pattern for all ESBL positive *E coli* isolates, similar findings were observed in the study were percentage of MDR bacteria for hospital samples ranged widely from 0.58-40% and the number of MDR bacteria was alarmingly high for the hospital effluent samples; resistance for ampicillin, piperacillin - tazobactam, second and third generation cephalosporins, cotrimoxazole, gentamicin, ciprofloxacin formed the common multidrug pattern. [15] The antibiotic resistant patterns in this study revealed that among the ESBL producers, 92 % of *E coli* isolates were resistant to ampicillin, 62% to cotrimoxazole, 49% to tetracycline and 33 % to gentamicin. Such wide spread resistance ability of ESBL producers, even resistance to some other drugs such as sulfonamides, aminoglycosides have been observed by others. [16,17]

Our study results correlates with the study carried in Madrid, Spain Hospitals and community health on *E coli* isolates where high level of resistance to cefotaxime and ceftazidime was observed. [18] Study reported that ciprofloxacin resistance and ESBL production in *Klebsiella pneumonia* are closely associated [3] and found that, globally 18 % of ESBL producing isolates were resistant to ciprofloxacin. Our results also showed that a greater percentage of resistant towards ciprofloxacin that is 72%. *E coli* isolated from the sewage of hospitals contained greater percentage of antibiotic resistance than *E coli* isolated from municipal sewage. Among the antibiotics tested, the highest resistance was found to ampicillin (AMP) (up to 18%) and piperacillin-tazobactam (PIT) (up to 12%). [19] But our isolates showed high level of

resistant to ampicillin (92%) and PIT resistant was found to be 28%. Gentamicin was found to be most effective antibiotic against *Enterobacteriaceae*; only 2.6% of these isolates were resistant to 10µg/ml, while 2.1% were resistant up to 256µg/ml. [20] In the present study 67% of the ESBL positive isolates were sensitive to gentamicin of concentration 10mcg.

In our study greater MIC values were observed for ceftazidime and tetracyclines (≥256 µg/mL) for ESBL producers. MIC values were varied to different antibiotics ampicillin ≥32 µg/ml, chloramphenicol ≥32µg/ml, ciprofloxacin ≥2 µg/ml when tested *Acinetobacter* spp isolated from sewers receiving waste effluents from a hospital. [21]

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