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Isolation, ABST Pattern and Molecular characterization of ESBL E.coli from UTI samples

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Abstract: Escherichia coli strains were isolated from the patients suffering from urinary tract infection and further characterized for extended spectrum ß-lactamase. These strains are extensively cross studied with multiple of drugs and classified according to their sensitivity. Resistance pattern of ESBL, MBL and Amp C producing isolates also identified and differentiated with high and low resistant such as Ampicillin 91.7%, 80% and 100% to Cefepime 75.0%, 53.3% and 0% respectively. The resistant is due to the TEM and SHV genes present in the isolates carrying the virulence factor.PCR based finger printing techniques and RAPD analysis enhanced the molecular characterization of these isolates. Almost available drugs for UTI against E.coli were considered for the deeper understanding.The collaboration of various drug resistant pattern studies along with their fit in ß-lactum sub-classification and PCR techniques were the core part of this study.

Key words: ß-lactamase, ESBL, MBL and Amp C, TEM and SHV genes, RAPD analysis and resistant pattern.

Introduction

Extended spectrum beta lactamase producing bacteria were first identified in 1983. Since the time, they have been found worldwide in a number of organisms, including Escherichia coli (E. coli),Klebsiellapneumoniae (K. pneumoniae), Klebsiellaoxytoca, Proteus mirabilis, Enterobacter cloacae, Morganellamorganii, Serratiamarcescens, Shigelladysenteriae, Pseudomonas aeruginosa, Burkholderiacepacia,Capnocytophagaochracea, Citrobacter species, and Salmonella species.[6-12] The emergence of ESBL producing bacteria, particularly E. coli and K. pneumoniae is now a critical concern for the development of therapies against bacterial infection. The major ESBL producer was K. pneumoniae before 2000, but now E. coli has become an important ESBL carrier in Western countries.[13-16]Since the ESBL genes are usually found in large plasmids, they also contain other antimicrobial resistant genes. Escherichia coli are one of the main bacterial pathogenes responsible for nosocomial infections especially in immunocompromisedpatients[1]E. coli is generally accepted as the predominant vehicle for the dissemination of resistance genes and

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vectors due to its abundance in such environments (Tauxe, 1997). Extended-spectrum β-lactamases (ESBLs) are enzymes produced by Gram-negative bacilli that mediate resistance to penicillin, cephalosporins, and monobactams and are commonly recognized in Enterobacteriaceae[2]. Although most ESBLs are mutants of TEM and SHV enzymes, the CTX-M type β-lactamases which have become important, originated from β-lactamases found in environmental species of the genus Kluyvera, and this enzyme hydrolyzes cefotaxime and cefriaxone but is weakly active against ceftazidime[3,4] More than 50 variants are found worldwide.[4]The widespread uses of antibiotics coupled with the transmissibility of resistance determinants mediated by plasmids, transposons, and gene cassettes in integrons are factors that contribute to the increase in antibiotic resistance in the isolates[1]

Generally available subtyping methods for E. coli include PFGE, plasmid profiling, ribotyping and PCR-based typing methods such as arbitrary primed PCR, repetitive extragenic palindromes (REPs), and enterobacterial repetitive intergenic consensus (ERIC).[5]However, the genotypic characterization of other resistant isolates has not been reported so far. The objectives of this study were to determine the antimicrobial resistance and ESBL profiles of E. coli and to determine their genetic diversity using PCR based finger printing techniques and RAPD analysis. The presence of resistance genes and integrons was also determined via PCR and their transferability was determined by conjugation and transformation.

Resistance to broad spectrum β-lactams, mediated by extended spectrum β-lactamases (ESBL), metallo β-lactamases (MBL) and AmpC β-lactamases (AmpC) enzymes is an increasing problem worldwide[1] .Presence of the latter two enzymes in clinical infections can result in treatment failure if one of the second- or third-generation cephalosporin is used. The scenario worsens in cases of MBL production where the drugs of last resort the carbapenems are rendered inactive.[1]ESBLs are the enzymes produced by Gram-negative bacilli that have the ability to hydrolyze β-lactam antibiotics containing an oxyimino group (third generation cephalosporins and aztreonam) and are inhibited by β-lactamase inhibitors such as clavulanic acid, sulbactam and tazobactam.[3]Multi-drug resistance (MDR) in Gram-negative organisms is an alarming problem in the world. MDR and extensively-drug resistance (XDR) is in increasing trend due to the production of different types of beta (β)-lactamases. Thus the aim of this study was to document the incidence of MDR and XDR which are keen similar to ESBL E.coli in clinical isolates of E.coli. The RAPD technique is based on the polymerase chain reaction (PCR). A target DNA sequence is exponentially amplified with the help of arbitrary primers, a thermostable DNA polymerase, dideoxy nucleotide tri - phosphates, magnesium and reaction buffer. The reaction involves repeated cycles, each consisting of a denaturation, a primer annealing

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and an elongation step is also used.Further epidemiological classification can be done for further understanding for external influencing factors along the study.

Antimicrobial susceptibility testing

Antibiograms of the isolates were determined by modified Kirby-Bauer disk diffusion method on Mueller-Hinton agar standard media using commercially prepared disks (HiMedia Laboratories Pvt. Limited, India) in compliance with Clinical and Laboratory Standards Institute (CLSI) guidelines.[17]Antimicrobials used were: penicillin [ampicillin (10 μg)], penicillin with β-lactamase inhibitors ampicillin-sulbactam (10/10 μg), amoxicillin-clavulanic acid (10 μg)], narrow spectrum cephalosporin [cefazolin (30 μg)], extended spectrum cephalosporins [ceftazidime (30 μg), ceftriaxone (30 μg), cefepime (30 μg)], cephamycin [cefoxitin (30 μg)], ticarcillin-clavulanic acid (75/10 μg)], monobactam [aztreonam (30 μg)], carbapenems [imipenem (10 μg), meropenem (10 μg)], aminoglycosides [gentamicin (10 μg), amikacin (30 μg), tobramycin (10 μg)], fluoroquinolones [ciprofloxacin (5 μg), ofloxacin (5 μg)], folate pathway inhibitor [co-trimoxazole (25 μg)], phenicol [chloramphenicol (30 μg)] and polymyxin [colistin (10 μg)]. Interpretation of susceptibility was made according to the tables for interpretative zone diameters of CLSI.[17] E. coli 25922 was used as a control organism for antibiotic sensitivity testing.

a) Identification of MDR, XDR and PDR isolates

MDR, XDR and PDR (Multi Drug Resistance, Extensively Drug Resistance and Pandrug Resistance) isolates were identified according to the guidelines recommended by joint initiative of the European Centre for Disease Prevention and Control (ECDC) and the Centers for Disease Control and Prevention (CDC).[18] According to the guidelines, the isolates showing non-susceptibility to at least one agent in three or more antimicrobial categories were identified as MDR, non-susceptibility to at least one agent in all but two or fewer antimicrobial categories (i.e. bacterial isolates remained susceptible to only one or two categories) were identified as XDR and non-susceptibility to all agents in all antimicrobial categories were identified as PDR. To ensure correct application of these definitions, bacterial isolates were tested against all or nearly all of the commercially available antimicrobial agents within the antimicrobial categories (recommended by ECDC and CDC) and selective reporting and suppression of results were avoided.[18]

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b) Phenotypic detection of ESBL

Isolates of E. coli were examined for their susceptibility to third generation cephalosporins by using ceftazidime (30 μg) and cefotaxime (30 μg) disks. The isolates showing diameter of ≤22 mm zone of inhibition for ceftazidime and/or 27 mm for cefotaxime were considered as ESBLs suspects as per National Committee for Clinical Laboratory Standards (NCCLS) guidelines.[19]All suspected isolates for ESBLs production were confirmed by the combination disk method on Mueller Hinton agar plates that were inoculated with standardized inoculums (comparable to 0.5 McFarland standards) of the isolates to form a lawn culture. Separate commercial disks containing cefotaxime (30 μg) and ceftazidime (30 μg) with and without clavulanic acid (10 μg) were placed over the lawn culture. An increase in zone size of more than or equal to 5 mm for cefotaxime and ceftazidime with and without clavulanic acid indicated ESBL production as described by Carter et al. [20]

c) Phenotypic detection of MBL

Isolates that were found resistant to imipenem, meropenem or third generation cephalosporins (ceftazidime) in Kirby Bauer disk diffusion method were presumptively considered MBL(Metallo Beta Lactamase) producers and were confirmed by the imipenem disk with EDTA methods. Briefly, the test inoculums (comparable to 0.5 McFarland standards) were prepared and transferred on to Mueller Hinton agar plates. Two imipenem (10ug) disks were placed on the surface of agar plate and 10 μl EDTA solutions was added to one of them to obtain a desired concentration of 750 μg. Plates were incubated for 16 to 18 hours at 35°C. An increase in zone size of more than or equal to 7 mm for imipenem-EDTA disk compared to imipenem disk alone indicated MBL producer strain.[21]

d)Phenotypic detection of AmpC

All E. coli isolates resistant to cefoxitin in Kirby-Bauer disk diffusion method were confirmed for AmpC β-lactamase production by modified Hodge test. In the test, a cefoxitin susceptible E. coli indicator strain (ATCC 25922) was plated on Muller Hinton agar medium and the cefoxitin disk was placed. Test organism was streaked toward the cefoxitin disk. If the test organism expressed AmpC, it hydrolyzed the cefoxitin and showed growth along the intersection of the streak and the zone of inhibition from the cefoxitin disk.[22]

Criteria for defining MDR, XDR and PDR in Enterobacteriaceae
MDR: non-susceptible to ≥ 1 agent in ≥ 3 antimicrobial categories
XDR: non-susceptible to ≥ 1 agent in all but ≤ 2 categories

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PDR: non-susceptible to all antimicrobial agents listed.
DNA extraction for PCR is processed as on the kit instruction.

Polymerase chain reaction

Two sets of primers were used to amplify TEM and SHV genes. PCR amplification of TEM and SHV was carried out on plasmid DNA and chromosomal DNA of E. coli.

PCR reactions were performed in a total volume of 25 μl, including 1.5mM MgCl

2, 50mM KCl, 10mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 200 μm of each dNTP (Fermentas), 1 μm primers, 1 IU of Taq DNA polymerase (Fermentas), and 5 μl (40–260 ng/μl) of DNA. Amplification reactions were carried out using a DNA thermo-cycler (ABI Multi 96 well PCR, USA) as follows: Three min at 95 °C, 35 cycles each consisting of 1 min at 94 °C, 90 s at ~55 °C (show in Table 1) and 1 min at 72 °C, followed by a final extension step of 10 min at 72 °C. Amplified samples were analyzed by electrophoresis in 1.5% agarose gel and stained by ethidium bromide. A molecular weight marker with 100 bp increments (100 bp DNA ladder,Biolab) was used as a size standard.

Primer = (F) TGGCCAGAACTGACAGGCAAA

(R) TTTCTCCTGAACGTGGCTGGC

Isolation of TEM and SHV gene

Reference strains

Three strains, E. coli J53 R1, E. coli C_{600}PUD16 were used as standard ESBL-positive strains. E. coli J53 R1 harboured TEM-ESBL and the remaining strains carried SHV-ESBL. A non ESBL-producing organism (E. coli ATCC 25922) was used as negative control.

Preparation of plasmid and genomic DNA

Plasmid DNA was isolated from bacterial cells by alkaline lysismethod.[13]Genomic DNA was purified by phenol extraction and ethanol precipitation method.[13] The DNA was stored at -20°C. The samples were run on 0.8 per cent agarose gel and stained with ethidium bromide. The stained gel was examined under UV light to look for the presence of plasmid bands of particular size using a molecular weight marker; λ DNA hind III double digest (Roche, USA).

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PCR for b-lactamase encoding genes: PCR analysis for b-lactamase genes of the family TEM and SHV was carried out. Primers obtained from Sigma, USA used for bla TEM were 5'AAAATTCTTGAAGACG 3' and 5' TTACCAATGCTTAATCA 3' and for bla SHV were 5' TTAACTCCCTGTTAGCCA 3' and 5' GATTTGCTGATTTCGCCC 3'.

RAPD analysis

By the use of PCR, RAPD profiles were generated by using single decamerprimers in polymerase chain reaction. The reaction mixture ware prepared such as each reaction mixture contained 0.8µl of template DNA, 25µl of d NTPs, 32µl of tris buffer (50 µmkcl, 10 µmTris-HCl, 1.5 µm MgCl2,pH – 9.0), Nuclear free water 151 µl, 14 µl of TaqDNApolymerase (Bangalore Genie Pvt. Ltd., Bangalore, India) and mineral Oil 142 µl in final cocktail solution of 280 µl.

Each 10µl of reaction mix contains

Component	Volume	Final Concentration
Genomic DNA	3.0µl	15ng
Buffer	1.0µl	1X
dNTPs	1.0µl	0.2mM
Primer	0.6µl	0.6mM
Taq DNA pol	0.2µl	1 unit
MgCl2	1.0µl	2.5mM
Water / Mineral oil	to 10µl	

It is divided into seven tubes, where six tubes with different template DNAs and one tubes without template DNA with mineral oil was used as marker. In cocktail solution the 18 µl of each, two different types of primers were added having the sequence 5' GTTTCGCTCC3' and 5' AAGAGCCCGT3'. The reaction was carried out in (GeneAmp PCR System 2400 thermal cycler). The initial phase consisted of denaturation 95°C for 5 minutes. Then the cycle starts with denaturation phase at 95°C for 1 minute, primer annealing phase at 36°C for 1 minutes and DNA extension phase at 72°C for 2 minutes and repeated for 45 cycles. At the end of cycles, a final extension period of 5 minutes was given at 72°C. The amplified product were stored at 4°C and separated by electrophoresis on 1% agarose gel in 1% TAE buffer EtBr (ethidium bromide) was added to it the gel was run for 3 hours at 50 V. The gel was finally visualized by UV- transilluminator.

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Data analysis

The amplified bands were scored as present (1) or absent (0). Faintly stained bands that were not clearly resolved were not considered in the data collection. Bands with the same migration distance were considered homologous. The genetic similarity between all accessions was calculated according to the Nei (1972). The program PopGene was used for the construction of an unweighted pair group method with arithmetic mean (UPGMA) dendrogram and bootstrap analysis. The method used to find the percentage of diversity occurs from given samples.

Result

Resistance pattern of E. coli

All of the E. coli isolates tested exhibited resistance to amoxicillin-clavulanic acid and 77 % of them remained resistant to ciprofloxacin whereas all the isolates were susceptible to colistin and few isolates (7%) were resistant to imipenem.

MDR and XDR isolate

Of total E. coliisolates tested, 156 (78%) isolates were MDR and 14 (7%) isolates were XDR whereas no PDR isolate was identified (Table 1).

All MDR isolates were resistant to amoxicillin-clavulanic acid and 91% isolates were resistant to ciprofloxacin whereas amikacin, imipenem and colistin were found as the most effective antibiotics for the MDR isolates. All XDR isolates were resistant to most of the antibiotics tested whereas colistin was found as the effective regimen against all XDR isolates (Table 1).

ESBL, MBL and AmpC producing isolates and their resistance profile

Among total tested isolates, the synthesis of ESBL, MBL and AmpC was detected in 48 (24%), 30 (15%) and 18 (9%) isolates respectively (Tables 2 and 3). Most of the antibiotics tested were non-effective against ESBL, MBL and AmpC producers whereas imipenem, amikacin, chloramphenicol and colistin were found effective regimens against ESBL producers and only colistin was effective against MBL and Amp C producing isolates (Table 1).

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Table 1

Resistance pattern of ESBL, MBL and AmpC producing isolates

Antibiotics	ESBL (n = 48)	MBL (n = 30)	Amp C (n = 18)
	No. of resistant isolates (%)	No. of resistant isolates (%)	No. of resistant isolates (%)
Ampicillin	44 (91.7)	24 (80.0)	18 (100)
Ampicillin-sulbactam	44 (91.7)	24 (80.0)	18 (100)
Cefazolin	48 (100)	28 (93.3)	16 (88.9)
Ceftazidime	48 (100)	30 (100)	18 (100)
Ceftriaxone	48 (100)	24 (80.0)	12 (66.7)
Cefepime	36 (75.0)	16 (53.3)	0 (0)
Cefoxitin	34 (70.8)	26 (86.7)	18 (100)
Piperacillin-tazobactam	22 (45.8)	18 (60.0)	12 (66.7)
Ticarcillin-clavulanic acid	44 (91.7)	22 (73.3)	16 (88.9)
Amoxicillin-clavulanic acid	48 (100)	30 (80.0)	18 (100)
Aztreonam	48 (100)	22 (73.3)	12 (66.7)
Imipenem	2 (4.2)	14 (46.7)	4 (22.2)
Meropenem	32 (66.7)	22 (73.3)	12 (66.7)
Gentamicin	14 (29.2)	18 (60.0)	6 (33.3)
Amikacin	2 (4.2)	16 (53.3)	4 (22.2)
Tobramicin	16 (33.3)	16 (53.3)	6 (33.3)
Ciprofloxacin	46 (95.8)	28 (93.3)	18 (100)
Ofloxacin	40 (83.3)	24 (80.0)	14 (77.8)
Cotrimoxazole	40 (83.3)	24 (80.0)	12 (66.7)
Chloramphenicol	6 (12.5)	14 (46.7)	4 (22.2)
Colistin	0 (0)	0 (0)	0 (0)

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Table 2

Type of β-lactamases	No. of producing isolates (%)
ESBL	48 (24)
MBL	30 (15)
Amp C	18 (9)
ESBL + MBL	10 (5)
ESBL + Amp C	8 (4)
MBL + Amp C	6 (3)
ESBL + MBL + Amp C	4 (2)

Type of β-lactamases production among E.coli isolates

Multi-type β-lactamase production

Of the tested isolates, 10 (5%) were producers of both ESBL and MBL, 8 (4%) isolates synthesized both ESBL and AmpC whereas 6 (3%) isolates produced both MBL and Amp C. All the three types of β-lactamases (i.e. ESBL, MBL and AmpC) were detected in 4 (2%) isolates (Table 2).

Resistance rates of antibiotics with different mode of action.

All of the tested isolates were resistant to at least one cell wall inhibiting agents followed by folic acid metabolism inhibiting agent (59%) whereas no isolates were resistant to cytoplasmic membrane damaging agent (Table 3).

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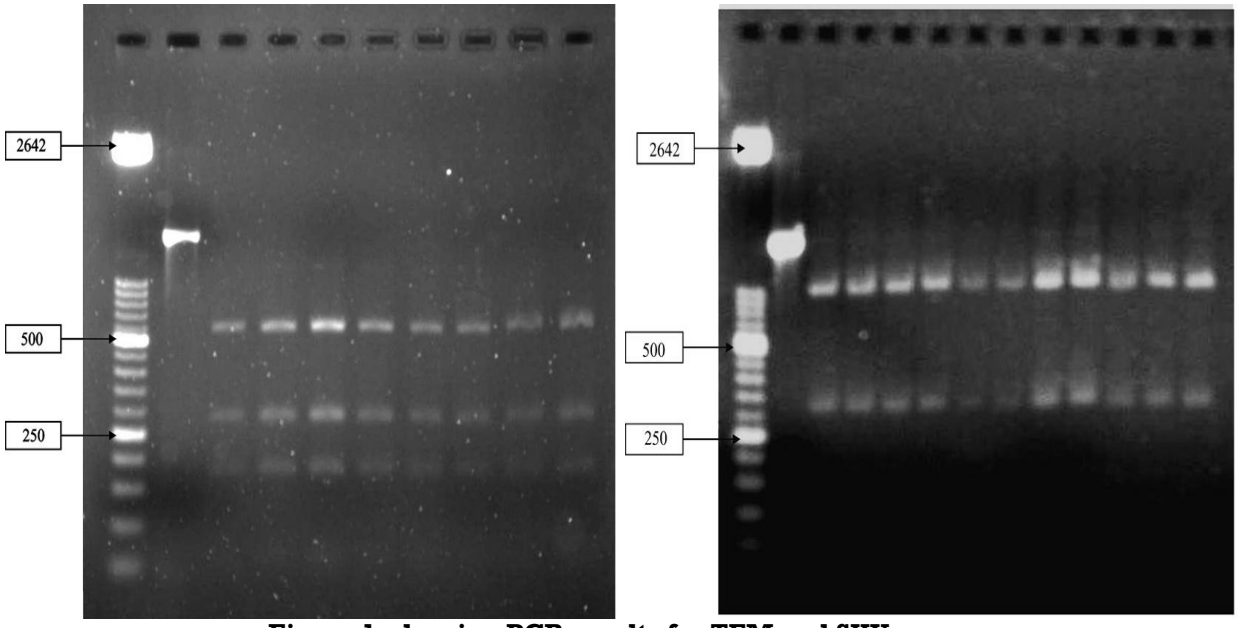

Figure 1 showing PCR results for TEM and SHV genes

Table 3

Resistance profile of isolates tested against 6 major classes of antibiotics with different mode of action

Mode of action	Antibiotic classes	No. of isolates resistant to at least one agent of antibiotic classes (%)
Inhibition of cell wall synthesis	Beta-lactams	200 (100)
Inhibition of protein synthesis	Aminoglycosides	56 (28)
Inhibition of DNA replication	Fluoroquinolones	82 (41)
	Chloramphenicol	34 (17)
Inhibition of folic acid	Cotrimoxazole	118(59)

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metabolism		
Damaging of cytoplasmic membrane	Polymyxins (colistin)	0 (0)

Incidence of resistant isolates

Very minor numbers of isolates (4%) were resistant to only one antibiotic whereas majorities (85%) of isolates were resistant to at least three antibiotics (Table 4).

Table 4

Incidence of isolates resistant to antibiotics

Resistant profile	**No. of isolates (%)**
Resistant to only one antibiotic	5
Resistant to 2 antibiotics	12
Resistant to 3–20 antibiotics	83
Total	100

Conclusion

Total ninety four samples were taken for screening. Among them sixty five were considered for antibiotic resistant gene and carried out for other experiments. These isolates were cultured on nutrient agar and other specialized media along the bio chemical confirmatory tests.The isolates were streaked against commercially available antibiotics which has different mode of action as cell wall lyses and other components destruction activity. The zone of inhibition for many antibiotics show less activity than its normal given inhibition rate.The results show 51(78%) isolates were MDR and 14(22%) isolates were XDR whereas no PDR isolate was identified.Most of the antibiotics tested were non-effective against ESBL, MBL and AmpC producers whereas imipenem, amikacin, chloramphenicol and colistin were found effective regimens against ESBL producers and only colistin was effective against MBL and Amp C producing isolates. The PCR molecular study was carried out none specifically for all genes followed by specific strains (around 20 samples among screened) such

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as TEM and SHV genes which are responsible for their resistant activity as mentioned by the previous study on resistant pattern.

The future aspect of study can be taken over the other nosocomical infection causing E.coli strain and other causative organisms prospect on same manner and available sub typing methods for E. coli include PFGE, plasmid profiling, ribotyping and PCR-based typing methods such as arbitrary primed PCR, repetitive extragenic palindromes (REPs), and enterobacterial repetitive intergenic consensus (ERIC) can be studied for deep understanding. The epidemiological study could able to give further knowledge on the gene transfer mechanism and mode of transformation with elaborate molecular study.

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