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Phenotypic and Genotypic Characterization of Extended Spectrum b-Lactamase Producing *Escherichia coli* Clinical Isolates from Semiurban Area

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ABSTRACT

Background: A constant rise in multidrug resistant *Escherichia coli*, producing extended spectrum ß- lactamases, poses a new problem to health care professionals worldwide, which complicates and limits therapeutic options. **Materials and methods:** Ninety *Escherichia coli* isolates were obtained from various clinical specimens from different wards of Rajah Muthiah Medical College and Hospital, Annamalainagar, India. Isolation and identification of isolates were performed using standard procedures. Detection of virulence factors such as hemolysin, slime production and cell surface hydrophobicity were investigated for thirty representative *Escherichia coli* isolates. Antibiotic resistances profile by Kirby-Bauer disc diffusion method, ESBL detection by standard phenotypic methods and serogrouping were performed for all ninety isolates. Molecular confirmation of ESBL types were performed for thirty representative *Escherichia coli* using multiplex PCR**. Results:** Among the thirty representative *Escherichia coli* isolates, 27(90%) were hydrophobic, 23(76.6%) were slime producers, 4(13.3%) were hemolysin producers, and 22(73.3%) had multiple virulence factors. Of the ninety *Escherichia coli* isolates 75.5%, 73.3% and 68.8% were resistance to cephataxime, ceftazidime and ceftriaxone respectively. Double disk approximation test and combined disc diffusion method showed 68(75.5%) isolates to be ESBL producers. Predictor disc approximation test revealed 10(11.1%) isolates to be ESBL producers, 58(64.4%) isolates to be both ESBL and AmpC producers. Molecular analysis of thirty representative isolates revealed CTX-M gene present in 30(100%) isolates followed by TEM in 28(93.3%), SHV in 9(30%) and OXA in 8(26.6%) respectively. **Conclusion:** This present investigation revealed, a high prevalence of plasmid mediated multidrug resistance, which indicates a dire need for effective ESBL surveillance and control and need for urgent search for newer cost effective antimicrobials, especially in rural and semiurban areas of developing nations.

Key words: ESBL, *Escherichia coli*, hemolysin, surface hydrophobicity, slime production, serotypes.

INTRODUCTION

Escherichia coli, a common intestinal microflora has been associated with diverse disease syndromes ranging from UTI to diarrhea, including pyelonephritis, meningitis, septicemia, endocarditis, which can be fatal in immunocompromised children and adults. [1,2]

The ability of pathogenic *Escherichia coli* to cause infection and to survive in the host depends on the virulence factors it possesses and its pathogenicity is even more complicated by the presence of resistance genes.[1,2] The enzymatic inactivation of ß-lactam antibiotics by extended spectrum ß-lactamases, hydrolyzing oximino-cephalosporins and aztreonam, has been detected as the major mechanism of resistance in gram negative bacteria, especially in *Escherichia coli*.[3] The emergence of plasmids encoding genes for extended spectrum ß- lactamases (ESBL) and their rapid dissemination confers resistance to both ß-lactam and non-ß lactam antibiotics. This limits therapeutic options, leading to difficult to treat infections which have become a serious global threat and challenge to health care professionals.[4]

The alarming rise in multidrug resistant *Escherichia coli* poses a new problem in developing countries that could become as serious as MRSA.[5] Disc diffusion method for antimicrobial susceptibility testing is the method of choice in most laboratories in developing countries.[6] However this phenotypic method has the disadvantage of missing out ESBL producing bacteria, leading to treatment failures and economic loss.[6,7,8] There is very limited local data available on the most prevalent ESBL types. Besides, the existing data on ESBL prevalence shows hospital to hospital variation, within the countries itself.[9]

This study aims at isolating *Escherichia coli* from various clinical specimens and characterizing the different serotypes using phenotypic as well as molecular methods in effective diagnosis of ESBL from semiurban area.

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MATERIALS AND METHODS

Clinical isolates

A total of 90 *Escherichia coli* clinical isolates from 50 urine, 20 stool, 12 pus, 4 high vaginal swab, 3 blood and 1 sputum specimens were obtained from various wards, from patients between 0-90 years of age attending the Rajah Muthiah Medical College and Hospital, Chidambaram, South India. This study was approved by Institutional Review Board. Clinical specimens were collected under sterile procedures and immediately processed using standard protocols. Identification of the organisms was based on colony morphology on Macconkey Agar, Eosin Methylene Blue Agar, Blood agar, Chromogenic agar and standard biochemical reactions. [10]

Detection of virulence factors

Thirty representative clinical isolates were taken and analyzed for the presence of various virulence factors.

Hemolysis*:*

Detection of α-hemolysin, a major virulence factor in some pathogenic *Escherichia coli*, was carried out by culturing the isolates on 5% sheep blood agar at 37ºC for 24 hours. Hemolysin production was detected by the presence of a zone of lysis around the colonies.[11]

Slime production*:*

The adherence potential of the pathogenic *Escherichia coli* was determined using slime production test. The isolates were plated on Congo red agar plates and incubated at 37ºC for 24 hours followed by an overnight incubation at room temperature. Positive slime production was detected by the appearance of crystalline black pigmented colonies, whereas non-pigmented colonies were considered as non- slime producers.[12]

Cell surface hydrophocity:

Hydrophobic interactions are involved in the adherence mechanisms of microorganisms and hence an important virulence factor. Cell surface hydrophobicity was determined by "salt aggregation test", using 1M, 1.4 M and 2M concentration of ammonium sulphate, employing the method described by Raksha *et al* 2003.[11]

Serogrouping*:*

Serogrouping of various *Escherichia coli* clinical isolates were done at "National Salmonella and Escherichia Center", Kausauli, Himachal Pradesh, India.

Determination of antibiotic resistance profile and extended spectrum ß-lactamases production.

Detection of antibiotic susceptibility and extended spectrum ß-lactamase production was performed for all 90 clinical isolates using conventional phenotypic tests and confirmed using molecular methods in 30 representative isolates.

Antibiotic susceptibility testing:

Antibiotic susceptibility testing was performed for various classes of antibiotics on Muller Hinton agar plates by Kirby Bauer disc diffusion methods.[7] Four different classes of antimicrobials were used (Himedia, Mumbai).

Susceptibility to *Cephalosporins*: Ceftazidime (30µg), Cephotaxime (30µg), Ceftriaxone (30µg), Cephoxitin (30µg); *Quinolones:* Ciprofloxacin (10µg), Nalidixic acid (30µg), Norfloxacin (10µg), Ofloxacin (5µg), Pefloxacin (5µg); *Aminoglycosides:* Gentamicin (10µg), Kanamycin (30µg), Amikacin (30µg); *other* ß-*lactum drugs:* Imipenem (10µg), Aztreonam (30µg), Ampicillin (10µg) were tested. After 18 hours incubation at 37ºC the results were recorded and interpreted as per CLSI criteria[7] . *Escherichia coli* ATCC 25922 was used as the control.

Double disk approximation test:

The test is based on synergy between a cephalosporin and clavulanic acid as described by Jarlier *et al* 1988 [13] for ESBL detection. Briefly, the test organisms were swabbed onto Muller Hinton agar plates with amoxicillin /clavulanic acid(20/10µg) and ceftazidime (30µg) discs placed 15mm apart and incubated at 37ºC for 18 hours. ESBL production was identified by clear ceftazidime zone towards the disc containing clavulanate.

Predictor disc approximation test:

This test is based on specific displacement of various antimicrobials as inducers and indicators of induction, for detection of both ESBL and AmpC. Positive producers were detected according to the criteria formulated by Rodrigues *et al* 2004.[14] Imipenem (10µg) was placed in the center and 15mm apart on either side were placed ceftazidime (30µg) and cefotaxime (30µg); ceftazidime/clavulanic acid (30/10µg) was placed 15mm apart from ceftazidime; cefoxtin (30µg) was placed 15mm from cefotaxime, opposite to ceftazidime/clavulanic acid; ceftriaxone (30µg) and aztreonam (30µg) was also placed as shown in the Figure 1.

Phenotypic confirmatory test: Combined disc diffusion method:

Confirmation of ESBL production was accomplished using the ESBL combination disc confirmatory test.[3] The antibiotic combination discs used were ceftazidime (30µg), ceftazidime/clavulanic acid(30/10µg), and cefotaxime (30µg), cefotaxime /clavulanic acid (30/10µg). The discs were placed on Muller Hinton agar plates, swabbed with test organism of 0.5 McFarland turbidity and incubated. Positive ESBL producers were confirmed by–>5mm increase in zone diameter of ceftazidime/clavulanic acid and, cefotaxime/clavulanic acid than ceftazidime, cefotaxime alone respectively.

Preparation of plasmid DNA template:

Single bacterial colony from freshly streaked plate was inoculated into 5ml Luria Bertani broth and incubated at 37ºC for 16 hours. Plasmid DNA extraction was performed using Genie pure plasmid purification Kit (Genie, Bangalore).

Molecular detection of ESBL:

Genotypic identification of ESBL type was done using multiplex PCR assay, for 30 representative isolates, that were ESBL positive by phenotypic methods.

Detection of ß -lactamase genes by multiplex PCR:

All 30 clinical isolates were screened for the 4 major resistance gene SHV, TEM, CTX-M and OXA by multiplex PCR using universal primer (Sigma).[15] PCR amplification reactions were performed in a volume of 25µl with 0.25µM concentration of each primers and 2µl of DNA template, dNTPs and Taq DNA polymerase were obtained from Genie, Bangalore. Cycling conditions were: initial denaturation at 95ºC for 15 mins: 30 cycles of 94ºC for 30s, 62ºC for 90s and 72ºC for 60s and a final elongation for 10mins.[15] The amplified PCR products were subjected to electrophoresis in a 1.5% agarose gel in 1xTAE buffer. Amplified DNA fragments were visualized under a UV- transilluminator. A 100bp molecular weight marker was used as a standard for the determining the molecular size of PCR products.

RESULTS

Of the 90 *Escherichia coli* clinical isolates obtained, 50(55.5%) were from urine, 20(22.2%) were from stool, 12(13.3%) from pus, 3(3.3%) from blood, 4(4.4%) from high vaginal swab and 1(1.1%) from sputum samples. The male to female sex distribution of various ESBL positive clinical isolates showed male to female

Table 1: Antibiotic resistance profile of clinical isolates *Escherichia coli* for various classes of antibiotics.
Number of Isolates: 90

Sl.No	Antibiotics	Resistance	Sl.No	Antibiotics	Resistance
1	Cephotaxime(30µg)	68(75.5%)	9	Kanamycin(30µg)	68(75.5%)
2	Ceftriaxone(30µg)	62(68.8%)	10	Amikacin(30µg)	32(35.5%)
3	Ceftazidime(30µg)	66(73.3%)	11	Nalidixic acid(30µg)	85(94.4%)
4	Cephoxitin(30µg)	75(83.3%)	12	Norfloxacin(10µg)	78(86.6%)
5	Ampicillin(10µg)	81(90%)	13	Ofloxacin(5µg)	79(87.7%)
6	Aztreonam(30µg)	78(86.6%)	14	Pefloxacin(5µg)	83(92.2%)
7	Imipenem(10µg)	0(0%)	15	Ciprofloxacin(10µg)	79(87.7%)
8	Gentamicin(10µg)	61(67.7%)			

Table 2. Distribution of Virulence factors in representative *Escherichia coli* Clinical isolates.Number of Isolates: 30

Sample type	Hemolysin	Slime p roducers	Cell surface hydrophobicity	No. of isolates with one VF*	No. of isolates with two VF*	No. of isolates with three VF*
Stool no=8	0	5	9	2	5	0
Urine no=15	3	13	11	4	8	3
HVS* no=1	0	0	1	1	0	0
Blood no=1	0	1	1	0	1	0
Pus no=5	1	4	5	0	5	0
Total no= 3	4(13.3%)	23(76.6%)	27(90%)	7(23.3%)	19(63.3%)	3(10%)
					Total % of multiple VF* 22(73.3%)	

***VF- virulence factor**
***HVS- High Vaginal Swab**

Table 3: Distribution of various serotypes in *Escherichia coli* in Clinical isolates.

Number of isolates serotyped: 74(82.2%)
Number of isolates not assigned to any serotype: 16(17.7%)

Sl.No	No. of Serotypes	No. of isolates	Total No. of isolates serotyped
1	O25	12	1 x 12=12
2	O60	10	1 x 10=10
3	O68	7	1 x 7 = 7
4	O1	6	1 x 6 = 6
5	O20	5	1 x 5 = 5
6	O46	4	1 x 4 = 4
7	O153,O141,O8,O138	3	4 x 3= 12
8	O6,O4,O9,O56	2	4 x 2 = 8
9	O93,O12,O53,O170,O101, O2,O41, O5,O89,O17	1	10x 1 = 10
	Total number of isolates serotyped		74

Table 4: Detection of ESBL positive *Escherichia coli* in Clinical isolates by phenotypic methods.
Number of isolates : 90

Serial No	Specimen Type	DoubleDiscApproximationmethod		PredictorDisc Approximation method				Phenotypic confirmatory test. Combinationdisc diffusion method
		+ve ESBL Isolate	-ve ESBL Isolate	+ve ESBL Isolate	+ve ESBL + AmpC Isolate	+ve AmpC Isolate	-ve ESBL Isolate	+ve ESBL Isolate
1	Urineno=50(55.5%)	40	10	5	35	0	10	40 (44.4%)
2	Stool no=20(22.2%)	12	8	2	10	1	7	12(13.3%)
3	Pus no=12(13.3%)	10	2	1	9	0	2	10(11.1%)
4	Blood no=3(3.3%)	3	0	2	1	0	0	3(3.3%)
5	HVS* no=4(4.4%)	2	2	0	2	0	2	2(2.2%)
6	Sputum no=1(1.1%)	1	0	0	1	0	0	1(1.1%)
	Total	68 (75.5%)	22(24.4%	10(11.1%)	58(64.4%)	1(1.1%)	21(23.3%)	68(75.5%)

+ve - positive -ve - negative
***HVS – High Vaginal Swab**

Table 5a: Distribution of different genotypes of ESBL genes in representative *Escherichia coli* Clinical isolates [Multiplex PCR analysis].

CTX-M genes	TEM genes	SHV genes	OXA genes
30/30	28/30	9/30	8/30
100%	93.3%	30%	26.6%

Number of isolates: 30

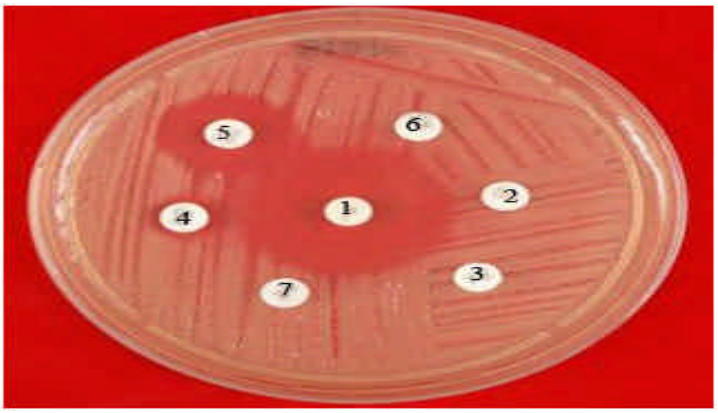
Table 5b: Detection of ESBL genes in representative *Escherichia coli* from Clinical isolates by genotypic method [Multiplex PCR analysis].

Number of isolates: 30

S.no	Distribution of serogroup in ESBL +ve isolated	Combination of ESBL genes detected	No. of ESBL +ve isolateS	Total % of multiple ESBL genes
1	O141,O46,O25,O2,0153	CTX-M,TEM,SHV,OXA	6(20%)	Six isolates with 4 genes (20%)
2	O101,O1,O25	CTX-M,TEM,SHV	3(10%)	Four isolates with 3 genes
3	O68	CTX-M,TEM,OXA	1(3.3%)	
4	O1,O153,O56,O25,O60, UT,O8,O138,O20,O6	CTX-M,TEM	18(60%)	Nineteen isolates with 2 genes
5	O60	CTX-M,OXA	1(3.3%)	
6	UT*	CTX-M	1(3.3%)	One isolates with 1gene (3.3%)
		Total	30	

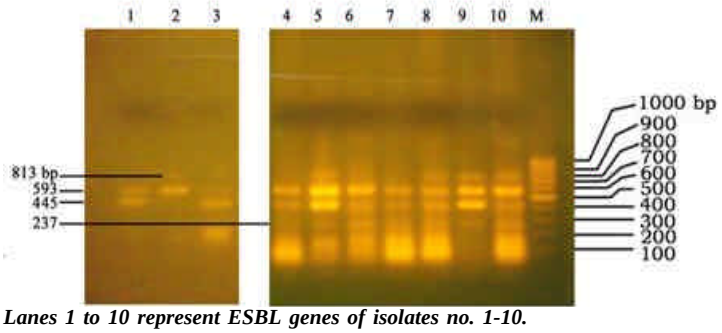
*** UT-untypable**

Figure 1: Antibiotic disc placement for predictor disc approximation test.



1-Imipenem 2-Cefotaxime 3-Cephoxitin 4-Ceftazidime 5-Ceftazidime+clavulanic acid 6-Aztreonam 7-Ceftriaxone.

Figure 2: Identification of Beta lactamase Genes by Multiplex PCR



Lanes 1 to 10 represent ESBL genes of isolates no. 1-10.

Figure 3: Lanes 1 to 18 represent ESBL genes of isolates no. 11-28.

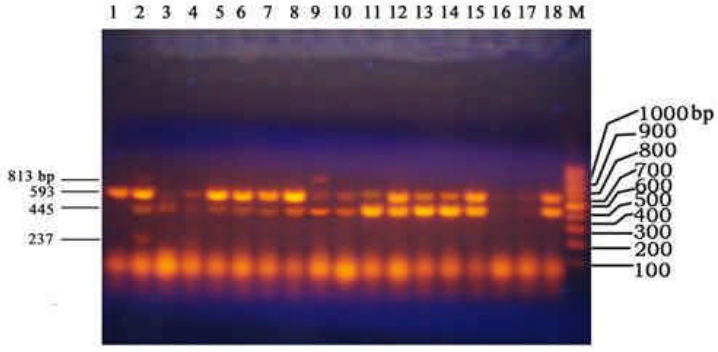
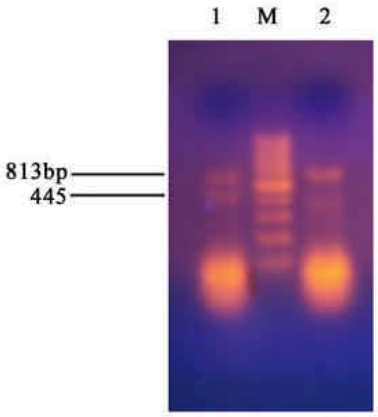


Figure 4: Lanes 1 and 2 represent ESBL genes of isolates no. 29 & 30.



The antibiotic resistance profile was studied for all 90 clinical isolates to various classes of antibiotics, 75.5% were resistance to cephataxime, 73.3% to ceftazidime, 68.8% to ceftriaxone which are third generation cephalosporins. A high degree of resistance (83.3%) was seen to the second generation cephalosporin, cephoxitin. However, imipenem showed 100% susceptibility. Co-resistance to other classes of antibiotics is shown in the Table 1.

The most commonly identified virulence factor was cell surface hydrophibicity in 27(90%) isolates followed by slime production in 23(76.6%) isolates and the least common was hemolysin production in 4 (13.3%) isolates. Multiple virulence factors were seen in 22(73.3%) of the isolates [Table 2].

.Serogrouping was performed for various clinical isolates, 74(82.2%) of the clinical isolates were serotyped by O antisera, whereas only 16(17.7%) could not be assigned to any serogroups. Twenty four different serogroups were represented, the predominant serotype was O25 followed by O60, O68 and O1 [Table 3].

.Detection of ESBL producers was performed by predictor double disk approximation method, 58(64.4%) isolates were found to be both ESBL and AmpC producers, whereas 10(11.1%) isolates, produced only ESBL, and 1(1.1%) isolate produced only AmpC. Double disk approximation method and phenotypic double disk confirmatory test confirmed 68(75.5%) isolates as ESBL producers [Table 4]. Molecular analysis of 30 representative isolates by multiplex PCR on plasmid DNA confirmed that all 30 (100%) isolates, were ESBL producers, with CTX-M gene being present in all 30 (100%) isolates, followed by TEM gene in 28 (93.3%) isolates, SHV gene in 9 (30%) isolates and OXA gene in 8(26.6%) isolates [Table 5a]. Multiple ESBL genes were present in most isolates [Fig 2, 3, 4]. Six isolates (20%) showed the presence of four ESBL genes, followed by four isolates (13.3%) possessed 3 ESBL genes, whereas nineteen isolates (63.3%) possessed 2 ESBL genes and one isolate (3.3%) had only one ESBL gene [Table 5b].The multiplex PCR amplified product size of ESBL genes CTX-M, TEM, SHV and OXA were 593 bp, 445bp, 237bp and 813bp respectively [Fig 2, 3, 4].

DISCUSSION

The present study was undertaken to investigate the prevalence of multidrug resistant *Escherichia coli* in a small semiurban population and characterize them phenotypically, genotypically and to identify the contributing factors. In this study it was found, a few isolates 4(13.3%) were positive for hemolysin production; interestingly all were from extra intestinal specimens just as has been observed by other researchers.[16,17] This suggests the existence of distinctly different virulence determinants in intestinal and extra intestinal *Escherichia coli*.[18]

Adherence property of bacteria is a prerequisite for initiation of early infection and an important colonization factor, even on indwelling medical devices and implants and hence an important virulence determinant.[19] The bacterial biofilm matrix is composed of exopolysaccharide glycocalyx or slime which is highly resistant to environmental stress and is capable of overcoming both humoral and cell mediated immunological mechanisms and even antibiotics.[12] Biofilms initiate colonization and dispersion of pathogenic bacteria inside the host leading to bloodstream infection to UTI.[20] It was found that (76.6%) of *Escherichia coli* isolates in our study were found to be slime producers. A higher percentage (92%)

of bioflim producing *Escherichia coli* has been previously reported in urinary tract infections by some workers.[21,22] The observation that a significant percentage of resistant *Escherichia coli* isolates are slime producers demonstrates[23] that biofilms increase antibacterial resistance and serve as a probable reason for decreasing the efficiency of drugs, even in high concentrations.

The physiochemical property of cell surface hydrophobicity was analyzed using the Salt Aggregation Test for various pathogenic *Escherichia coli* clinical isolates. It was observed that 90% of isolates were hydrophobic and the percentage positivity is much higher than that is reported previously from South India.[11,19,24] Cell surface hydrophobicity facilitates adhesion of pathogenic *Escherichia coli* to cell surface to form biofilms facilitating antibiotic resistance. [25] Ten percent (10%) of the total isolates had all three virulence factors while, 63.3% of isolates possessed two virulence factors and 23.3% of isolates possessed one virulence factor.

Serotyping was performed for both intestinal and extra intestinal *Escherichia coli* isolates. It was found that the most predominant serogroup in this study from various clinical isolates were O25, O60, O68, O1, O20 and O46 similar to the findings of many researchers in India. [26,27,28,29,30] These six serotypes add up to 44(48%) of total isolates and 37(41.1%) were ESBL positive isolates.

The occurrence of ESBL producing *Escherichia coli* among the isolates analyzed in the present study was 75.5%; this was accompanied by AmpC production[14] in 64.4%, this poses a risk of missing out the detection of ESBL by conventional methods. The results of the present study indicates a high percentage of ESBL producing *Escherichia coli* as reported 72% from Mexico, 56.9% from Pakistan and 83% from Saudi Arabia.[31,32,33,34] The percentage of ESBL identified in the present investigation is more, when compared to reports of 41% from Coimbatore, 51.4% from Mangalore, 62.34% from Kochi[9,16,35,36] but the percentage of ESBL producers from previous studies in India has been reported to range from 6%-87%.[8,37] This indicates an increase in the prevalence of ESBL in recent years.[38] The distribution of ESBL positive isolates in male to female showed a sex ratio of 1:1.2 comparable to that observed by other researchers.[35]

In this study 90% of all *Escherichia coli* were resistant to penicillins. The percentage of resistance to second and third generation cephalosporins were 68-83%. Even though all isolates (100%) were susceptible to carbapems similar to the findings of Babypadmini *et al* 2008,[35] resistance to aminoglycoscides were as high as 75.5% for Kanamycin followed by 67.7% for Gentamicin; and a lower percentage of resistance (35.5%) to Amikacin when compared to other antimicrobials. However high percentage of resistance ranging from 86-94% was observed to quinolones and flouroquinolones similar to that observed by other researchers.[16,35] This suggests resistance genes are encoded on ESBL associated plasmids.[31]

The molecular analysis of 30 representative ESBL positive isolates investigated in this study revealed that all 30(100%) isolates encoded the CTX-M gene, while 28(93.3%) encoded TEM, 9(30%) encoded SHV and 8(26.6%) encoded the OXA gene. Hong Fang *et al* 2004, [15] Apisarnthanarak *et al* 2008, [39] reported similar findings, demonstrating the predominance of CTX-M and TEM over the OXA and SHV genes. The dominant expression and dissemination of CTX-M gene involves multiple genetic mechanisms. CTX-M genes are encoded on a number of plasmids, some of them are a part of transposons or constitute a cassette in intergrons and these are responsible for conferring resistance to ß-lactam and other classes of antibiotics.[32,40] More recently there has been an epidemiological shift in the prevalence of ESBL genes. TEM and SHV, which were once the most dominant of ESBL genes, are now being superceded by CTX-M. Asian countries especially India and Pakistan have reports of a high prevalence of ESBL (>60%), of which in an South Indian study carried out by Srikanth *et al* 2004, India,[41] showed 93.3%, CTX-M appears to be the dominant genotype, followed by TEM (80%). However reports of other genotypes are very limited. [42, 43] The findings of our study confirm the predominance of CTX -M in the Indian population as reported by other researchers. [9, 42, 43]

The present study also demonstrates, that the predominant serogroup was O25, [1,29,30] out of twelve O25 serogroup isolates, eleven isolates were positive for ESBL production and had acquired co resistance to other classes of antimicrobials.[44] Genotyping of seven O25 serogroup plasmids revealed the predominant presence of CTX-M and TEM genes, slime production with other virulence factors were also observed. The O25 serogroups detected in this study appear to be the probable clonal lineage of O25 serogroups that were reported by David Livermore, [45] to have spread across the various continents harboring highly adaptative plasmids influencing drug resistance and dissemination.

The fitness of pathogenic *Escherichia coli* have been demonstrated by many workers, and has been attributed to difference in physiology and gene expression and increased rate of gene exchange, especially in biofilms that may contribute to its association with infectious diseases. Ghigo *et al* 2008[1,44,46] had demonstrated conjugation pilius serving as adhesion factors in *Escherichia coli* for transmission of plasmids which serve as reservoirs of multidrug resistance genes, and biofilms may select and promote spread of resistance gene to antimicrobial agents.

This present study shows the dire need of effective and novel control strategies for eradicating bioflim formation on indwelling medical devices by pharmaceutical and healthcare industries for prevention of bioflim formation rather than treatment due to the potent role of bioflims in antimicrobial drug resistance. [19].

This study also indicates judicious treatment of *Escherichia coli* infections with Imipenem treatment would offer better prognosis than patients receiving ß-lactam and monobactum treatment. Co-resistance to other class of non- ß-lactam drugs was observed, however Amikacin proves to be a better alternatives in treating patients with low cost [35].

Implementation of isolation precaution may prevent, person to person transmission of ESBL positive pathogenic *Escherichia coli* from patients to staff, visitors and other patients [47].Moreover control of multidrug resistance pathogenic *Escherichia coli* also include application of molecular technique in microbiology laboratory in simultaneous and rapid detection of more than one ESBL genes, this will ensure reliable and timely reporting of ESBL producing isolates and guide health care professional in more targeted and inexpensive antibiotic prescribing and this may slow down rapid dissemination of ESBL producing *Escherichia coli* [48].

CONCLUSION

In conclusion, this investigation revealed a high prevalence of plasmid mediated multidrug resistance genes in pathogenic *Escherichia coli*, especially CTX-M, that have probably evolved during the past years due to successful dissemination. The detection of multiple virulence factors prove to be an aiding factor of microbial pathogenicity that facilitates colonization and ultimately an important aeitiological agent for chronic and recurrent infections. The factors contributing to the emergence of high antimicrobial resistance in Asian countries are believed to be the direct result of indiscriminate antibiotic use. The scenario is enhanced by drug counterfeiting which still remains a threatening social issue in developing countries, rendering high rate of morbidity and mortality and economic loss.[49] Moreover the existing phenotypic methods have their own limitations, in overlooking ESBL producing isolates and ESBL detection becoming more complex with the presence of AmpC. This necessitates an urgent strategic planning of precise surveillance of drug resistance with newer molecular diagnostic methods. Moreover there is a dire need for extensive research in exploring newer areas in drug discovery and drug delivery which should also be cost effective in combating newer and multiple ESBL enzymes. .Controlling of antimicrobial resistance by effective monitoring and rational usage of existing antimicrobials are of utmost importance to confront ESBL especially in rural and semi urban areas of developing countries.

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