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RESEARCH ARTICLE

PHENOTYPIC SCREENING AND GENOTYPIC CHARACTERIZATION OF EXTENDED SPECTRUM BETA LACTAMASE PRODUCING GENES BLA TEM, BLA SHV, BLA CTX-M AMONG UROPATHOGENIC *ESCHERICHIA COLI*

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ABSTRACT

Background: *E. coli* accounts for up to 80% of UTI. Emergence of MDR and ESBL enzyme producing *E. coli* is increasing which are resistant to commonly used antibiotics. Phenotypic expression of the gene depends on gene regulation. **Aim:** To determine the prevalence of UPEC, rate of MDR strains, to characterize phenotypic screening and genotypic characterization of the ESBL producing genes among uropathogenic *E. coli.* **Materials & Methods:** Urine samples from suspected UTI patients were inoculated in Blood agar, MacConkey agar, Hicrome agar for identification and confirmation done by biochemical reactions. Antibiotic susceptibility done using Kirby Bauer disc diffusion method against 14 antibiotics and phenotypic ESBL screening, confirmation detected using double disc synergy test. DNA extraction was done by boiling lysis method. ESBL genes - bla TEM, bla SHV, bla CTX-M detected using PCR. **Results:** 423 (45.8%) were culture positive from 924 samples, with *E. coli* 212 (50.1%). Based on the results of antibiotic susceptibility test 65 (30.6%) were MDR *E. coli*. Phenotypically, 133 were non-ESBL and 79 (37%) ESBL producing isolates. Genotypically, 81 (38%) isolates had bla TEM (55.6%), bla SHV (11.1%), bla CTX-M (61.7%). Isolates possessing single type of gene and combination among three genes were also noted. **Conclusion:** *E. coli* being more prevalent and CTX-M found in many isolates. Variation in phenotypic expression and genetic detection were observed. Further studies are necessary for insight knowledge of phenotypic and genotypic importance, emergence of other uncommon ESBL genes.

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INTRODUCTION

Urinary tract infection is the common bacterial infection for both female and male, especially *Escherichia coli* is a common UTI causing gram negative bacteria (Rezwana *et al.,* 2015; Nithyalakshmi, 2015). *E. coli* are divided into intestinal, extra intestinal pathogens and commensals of intestinal flora and distal urogenital flora. The varitype of *E. coli* causing UTI is called Uropathogenic *Escherichia coli* (UPEC), which accounts for up to 80% of UTI (Turner, 2005). The bacteria ascend through the urethra and gain entrance into the urinary tract and causes infection. Recurrence of infection based on the host immunity there are possibilities to develop irreversible kidney damage and death. The source of infection of organism is treated aggressively with antibiotics. Extended spectrum beta-lactamase producing Enterobacteriaceae is an emerging public health problem as the bacterial pathogens become resistant to conventional drugs. Complication in UTI is increasing nowadays because of prevalence of ESBL producing *E. coli* which limits the treatment options to the management of UTI making it deleterious. Uropathogenic *E. coli* possess various virulence factors, with increased prevalence of ESBL accounting up to 17% of community acquired UTI and 58% of nosocomial UTI infection (WHO, 2011). ESBL enzyme production confers resistant to all β-lactam antibiotics except carnapenems and cephamycins (Maninder, 2013). Genotypic characterization of ESBL in UPEC has become important objective in antibiotic resistance of infectious agents. The common genes responsible for resistance against β-lactam groups are TEM, SHV, CTX-M. TEM-1 was the first and SHV-1, the second plasmid mediated β lactamase in Gram negative bacteria. Transmission of TEM-1 gene from one bacterium to the other occurs quickly as it is plasmid and transposon mediated (Sekar and Shanthi, 2009; Alfraresi *et al.,* 2010). CTX-M gene possessing 291 aminoacid and any change in it results in emerge of a new variant CTX-M gene (Bauernfeind, 1990; Naseer *et al.,* 2011). Genotypic and phenotypic expression may differ in wild and laboratory conditions based on gene regulation. Patients with increased threat of colonization and infection with ESBL producing

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microorganism makes the patients fatally sick with prolong hospital stay (Paterson *et al.,* 2005). In last two decades, resistance to β-lactam antibiotics are significantly increased, guidelines for prescribing antibiotics are in need based on antibiotic susceptibility of bacteria. Prevalence of infections by MDR strains has become necessary for the rate of emergence of spreading antibiotic resistance (Tuladhar *et al.,* 2004). Based on this, the present study has been carried out to determine the prevalence of bacterial pathogens in UTI, rate of MDR strains, to characterize phenotypic screening and genotypic presence of the common ESBL producing genes among uropathogenic *E. coli*.

MATERIALS AND METHODS

Study design

The present study was a hospital based cross sectional study which was conducted in Microbiology laboratory in a tertiary care hospital during the period from October 2015 to July 2017. Institutional human ethical clearance (IHEC) has been obtained.

Inclusion and exclusion criteria

Urine samples collected from the suspected UTI patients and sent to the Microbiology laboratory. The samples which showed polymicrobial growth and insignificant growth are excluded from the study.

Specimen collection, Processing and Identification of the bacteria

The sterile leak proof wide mouthed containers were labeled and given to the patients after giving proper instruction to collect midstream urine sample with sterile precautions. Urine sample was streaked in Blood agar, MacConkey agar and Hichrome agar by the semi-quantitative culture method using standard loop (0.001 mm) and were incubated at 37C and the colony forming unit was counted for significant bacteriuria as per the criteria and were identified by confirming with biochemical properties and.

Screening and Confirmation of ESBL *E. coli*

Antibiotic susceptibility pattern were analyzed using Kirby Bauer Disc Diffusion technique as per CLSI guideline (Clinical and Laboratory Standards Institute (CLSI 2015)). The strains which showed resistant to one antibiotic in at least three or more antibiotic category were considered as MDR isolate. Resistance to third generation cephalosporins was suspected as ESBL producers. The screening of ESBL was done by Ceftazidime and Ceftazidime plus clavulanic acid and were further confirmed by Cefotaxime and Cefotaxime-clavulanic acid by double disc synergy method (Clinical and Laboratory Standards Institute (CLSI 2015)).

PCR amplification for ESBL genes

DNA extraction was performed using the boiling method (Farzaneh Firoozeh *et al.,* 2014). PCR amplification of common ESBL genes were used to reveal the prevalence of bla TEM, bla SHV, bla CTX-M using specific oligonucleotide primers (Table 1). Amplification of genes was carried out in a Thermal Cycler (Eppendorf Master) after standardizing the PCR conditions, with initial denaturation at 94°C for 2 min, followed by 30 cycles of denaturation at 94 °C for 1 min annealing at 60C for 30s and extension at 1 min 30s and final extension at 72°C for 5min. PCR product were then loaded in 1% agar gel electrophoresis and amplified DNA fragments were detected by UV fluorescence transilluminator and the size of the amplicons was estimated by comparing

Table 1. Primer sequences for ESBL detection

Primer	Primer sequence (5'- 3')	Product size (bp)	Reference
SHV-F	TCAGCGAAAAACACCTTG	231	Akila et al., [15]
SHV-R	TCCCGCAGATAAATCACC	231	
TEM-F	CTTCCTGTTTTTGCTCACCCA	800	
TEM-R	TACGATACGGGAGGGCTTAC	800	
CTX-M –F	ACCGCCGATAATTCGCAGAT	593	Mahbobeh et al., [16]
CTX-M –R	GATATCGTTGGTGGTGCCATA	593	

Statistical analysis

The analysis of data was done by using SPSS 20.0 version and Microsoft Excels 2007. The p-value<0.001 was considered statistically significant.

RESULTS

A total of 924 urine samples have been collected, the prevalence of microbial infection in UTI showed 423 (45.8%) of culture positive isolates among suspected UTI patients, of which *E. coli* accounting up to 50.1% and 49.9% were other microbial isolates represented.

MDR *E. coli* and ESBL *E. coli*

Of the total 212 *E. coli*, 65 isolates were found to be MDR *E. coli* of which 41 isolates were confirmed as ESBL producers (Figure 1).

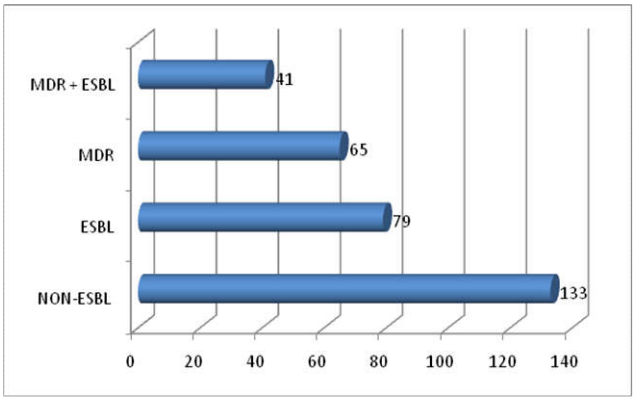

Fig. 1. Occurrence of ESBL and Non-ESBL isolates (Phenotypic) and MDR *E. coli* producing ESBL enzyme

Screening and confirmation of ESBL *E. coli*

Out of 212 isolates, 37.3% were screen test positive for ESBL and confirmed as ESBL producers using double disc approximation test (Table 2).

Detection of ESBL (TEM, SHV, CTX-M) genes using PCR

All the 212 isolates were subjected for ESBL detection. The overall percentage of genes and the isolates possessing a single gene were represented in Figure 2 & 3.

Table 2. Criteria for screening ESBL enzyme production in bacteria

Double disk synergy method	ESBL confirmation Criteria	No. of suspected ESBL	No. of confirmed ESBL isolates	Total no. of E. coli ESBL producers	Negative for ESBL production
Ceftazidime (CAZ) 30µg and CAZ 30µg + Clavulanic acid (CV) 10µg	Increase in zone size of >5mm with > 1mm of the combinational disk	90	79	79	11
Cefotaxime (CTX) 30µg and CTX 30µg + Clavulanic acid (CV) 10µg			79		

Table 3. Total occurrence of genotypically confirmed ESBL among UPEC ESBL (N = 80) isolates

ESBL genes	n (%)	X^2 value	p value
blaCTX-M	50 (61.7%)	93.879	
blaTEM	45 (55.6%)	90.034	<0.001
blaSHV	9 (11.1%)	14.749	

Table 4. Phenotypic and genotypic detection of Non-ESBL and ESBL producing E. coli

E. Coli	Phenotypic confirmation of ESBL	Molecular detection of ESBL genes
Non- ESBL isolates	133 (62.7%)	131 (61.7%)
ESBL isolates	79 (37.3%)	81 (38.3%)

Fig. 2. Gel electrophoresis of CTX-M, TEM, SHV genes

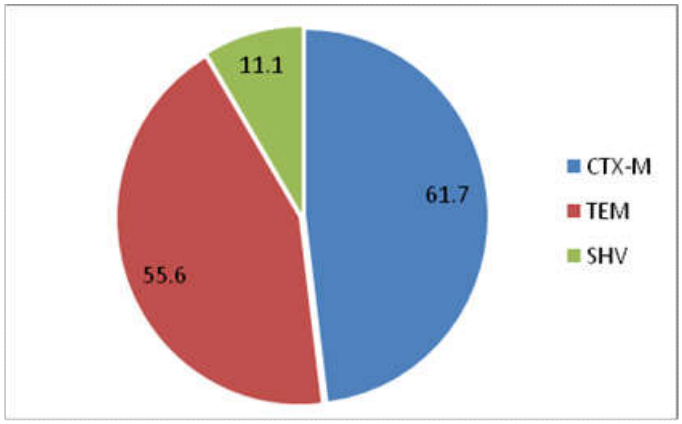

Fig. 3. Percentage of ESBL genes detected by PCR method

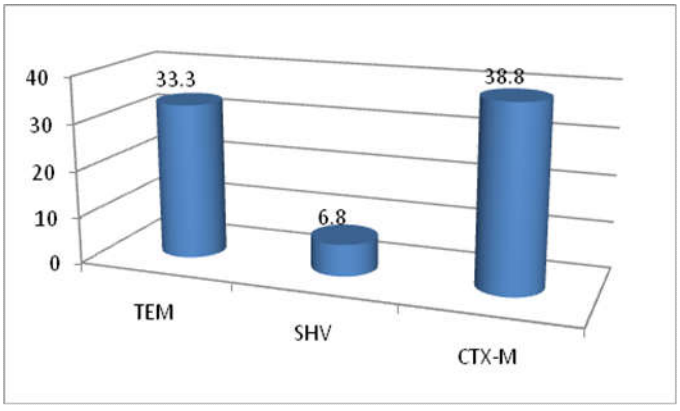

Fig 4. Occurrence (%) of single genes in *E. coli* isolates

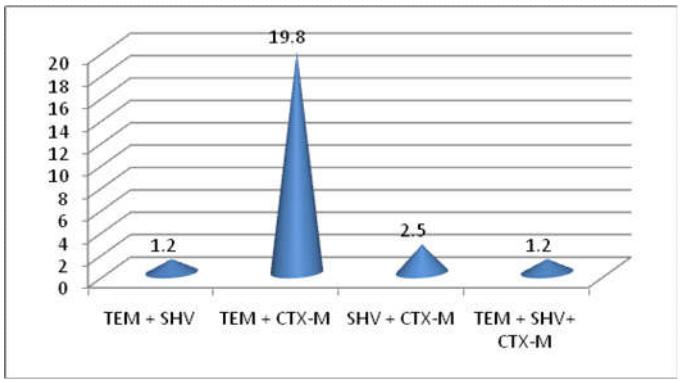

Fig. 5. Occurrence (%) of combination of ESBL genes in a single isolate

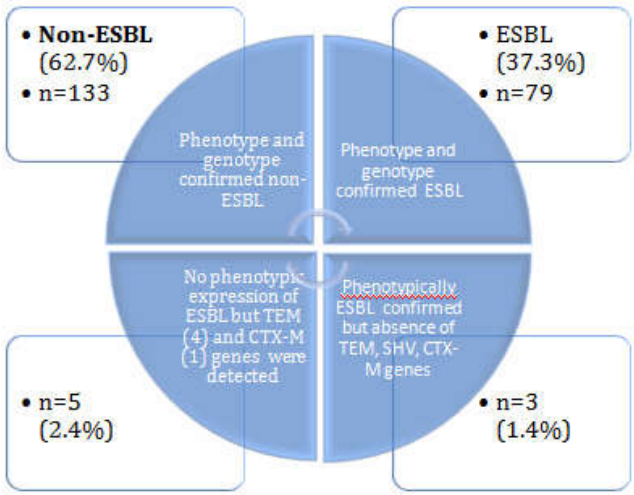

Fig. 6. Comparison of phenotypic and genotypic detection of ESBL

CHI square test combination of ESBL genes with significant p value (< 0.01) in TEM + CTX-M association is more than other associations in single isolates (Figure 4).

Genotypic detection and phenotypic expression: Three isolates were phenotypically confirmed for ESBL in the absence of TEM, SHV or CTX-M. Also in five isolates ESBL (4 TEM, 1 CTX) genes were present but it was not expressed phenotypically in antibiotic susceptibility test and double disk synergy test (Table 4) and the comparison were given in Figure 5.

DISCUSSION

One of the widespread community acquired and hospital acquired infection is UTI. Alteration in systemic and local immunity such as anatomical abnormalities, immune-suppression, diabetes, indwelling catheters, urethral sphincter mechanism incompetence is associated for the development of infection in urinary tract (Kate, 2011; Asadi *et al.,* 2014).

Prevalence of UTI: Totally 924 patients were suspected for UTI, the percentage of microbial growth was 45.8% which is higher compared to the study conducted by (Slama *et al.,* 2008) reported 27.1% of microbial growth.

UPEC and its role in UTI: *E. coli* causes intestinal and extra intestinal infections such as gastroenteritis, urinary tract infection, peritonitis, meningitis, septicemia by different pathotype variants (Von Baum and Marre, 2005). One pathotype Uropathogenic *E. coli* possessing virulence factors plays a role fitness and survival determinants for binding to gal-gal receptor on the uroepithelial cells, utilization of nutrients from host and toxin production making the UPEC as pathogenic (Ko *et al.,* 2007). In this study, out of 45.8% of microbial growth in urine, 50.1% were *E. coli.*

UTI and Beta lactam groups: Beta lactam antibiotics are used mainly for treating UTI. The enzyme, beta lactamase hydrolyzes beta lactam ring and denatures the action of antibiotics against bacteria mediated by acquired resistance. Emergence of ESBLs (Extended spectrum beta lactamase) enzyme makes the bacteria resistant to third cephalosporin groups, increasing resistance to broad spectrum antibiotics which are now predominantly found in *E. coli* and *Klebsiella pneumoniae*. This emergence of ESBL may also be due to over usage of third generation cephalosporins (Slama *et al.,* 2008; Pitout *et al.,* 2007). In the present study 38% of *E. coli* was ESBL producers which are confirmed by double disk screening and confirmatory test.

MDR (Multi drug resistant) *E. coli and ESBL isolates:* MDR *E. coli* are nothing but the isolate showing non-susceptibility to at least one agent in three or more antimicrobial categories. In this study, 65 isolates (31%) were found to be MDR out of 212 *E. coli* and suspected for β lactamase producers. 41(63.1%) isolates were confirmed as ESBL producers among the MDR isolates. The infections caused by MDR pathogens, the rate of emergence and spread of antibiotic resistance cannot be reduced without gathering information about the existing MDR strains (Tuladhar *et al.,* 2004).

Occurrence of three ESBL genes: The most common studied ESBL genes are bla TEM, bla SHV, bla CTX- responsible for ESBL production in *E. coli*. In this study 38% were found with ESBL genes genotypically but phenotypic expression showed 37% ESBL positive with CTX-M 61.7%, TEM 55.6% and SHV 11.1%. CTX-M is the common ESBL gene detected in *E. coli* which is similar to the study 82% in (Ponnusamy, 2015), 68.97% in a study conducted in Iran (Mahbobeh Mohammad Tabar *et al.,* 2016).

Single and the association of the ESBL genes in a single isolate: "Association of three ESBL (bla TEM, bla CTX-M, bla SHV) genes in single bacteria" was noted with 1.2% but not significant (p value >0.01) which may be based on the prevalence of no. of ESBL genes as well as their percentage of combination, this type of combination were also noted in the study (Ponnusamy *et al.,* 2015). This association of genes in a single isolate coding for ESBL enzyme may prove that if one gene is in off mechanism and the other may be in active state depending on the environmental, host factors etc., but always the strain remains resistant to the β lactam antibiotics. If all three genes express at the same time the resistant mechanism will be high and persistent but it depends on regulation of gene operons.

Comparison of phenotypic expression and genotypic detection of ESBL: The presence of combinational genes signifies the rate of increase in β-lactamase production also high and persistent resistance. Presence of ESBL genes without phenotype expression may be due to mutation, varying substrate affinity or inoculum effect, influence of environmental factors, ESBLs may fail to reach a level to be detectable by disk diffusion tests. The reason behind, phenotypic expression in the absence of studied genes may be because of different β-lactamase genes (PER, VEB, GES, BES, TLA, OXA genes which are low prevalent currently. But always, phenotypic expression of a gene occurs based on gene regulation noting that genotypic method of gene detection has 100% sensitivity and specificity. Regulation of genes depends on an adaptive response to an environmental change, so that genes are expressed whenever required under stimulation. Most of the genes are under indirect control, expression of gene responds to signals that are not related directly to the function of gene. Performance of indirect control is poor in artificial conditions and shows that often gene regulation in the laboratory is maladaptive. There is no necessity that genes which have closely related functions should express at optimum or in a similar pattern. The related genes may show dissimilarity in their pattern of expression, regulation in the wild as well as in the laboratory due to suboptimal control of genes. An evolutionary change takes place in both indirect and suboptimal control more often than adaptive control. So in this study, the reason behind the absence of ESBL phenotype expression even with standard conventional techniques in spite of presence of ESBL coding gene may be under indirect control which works to the least in invitro condition (Morgan *et al.,* 2013).

Conclusion

The present shows that the bacteria *E. coli* being more prevalent among the uropathogens, becoming resistance towards common antibiotics as reported in many other national and global studies. Variation in phenotypic expression and genetic detection may involve the difference in setup of in vitro and in vivo conditions. But both these limitations highly confuse the treatment as all the treatments were based on conventional phenotypic reports as the genes express by adaptive response. Switch off and on mechanism of gene may differ based upon the genetic makeup of the individual and the conditions opting for a gene to express. This resistance makes the clinicians and patients in a challenge environment due to limited antibiotics which is also cost effective and side effective. Early detection of infection, proper and prescribed use of antibiotics with correct specification of duration makes the bacteria less resistance and reduces the recurrence of infection, damage and failure of functioning of the infected organ and mortality rate. Surveys and monitoring should be organized routinely for recognizing the antimicrobial

resistance and to setup the antibiotic policy. Further studies are to be carried for insight knowledge of resistance mechanism, phenotypic and genotypic importance, and emergence of other uncommon ESBL genes.

Future Scope of the study

- Different phenotypic disc diffusion method should be trailed and should follow the same which has higher sensitivity and specificity, as each method may vary in it.
- The genotypic method has higher sensitivity and specificity but the cost of equipment and other requirements are more compared to phenotype methods.
- In future the phenotypic method which gives the maximum result of ESBL findings more or less equivalent to genotype should be improved.
- The ESBL genes which are low prevalent are emerging apart from common (blaCTX-M, blaSHV, blaTEM) genes and with varied two and three combinational genes in a single isolate can be determined only by genotypic method but not phenotypically.

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