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Simultaneous detection of phylogroups and ESBL genes in *E. coli* using Multiplex PCR

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Abstract

Background: *Escherichia coli* (*E. coli*) strains harbor various allelic versions of beta lactamase genes and their identification using conventional phenotypic tests is a tedious and time consuming task. In the present study, multiplex PCR is performed for the simultaneous detection of *E. coli* phylogroups and extended-spectrum β -lactamase- (ESBL) genes.

Methods: A total of 128 *E. coli* isolates from urine samples were screened for antibiotic resistance and expression of ESBL activity using phenotypic and genotypic methods. Uniplex and multiplex PCRs were used to detect *E. coli* phylogroup determinants and *bla*^{CTX-M-15}, *bla*^{OXA-1} and *TEM1* genes. Chi Square test of independence was employed for evaluating significant levels at *P value* < 0.05.

Results: Phylogroup B2 was detected as the predominant group (36%) followed by group D (30%), A (25%) and B1 (9%). The highest resistance was seen against nalidixic acid (100%) and the lowest against amoxicillin-clavulanic acid (55%). Significant *P values* were observed for resistance against cefotaxime and cefepime in the phylogroup B2 while resistance against cefoxitin, sulfamethoxazole and fosfomycin was significantly associated with group D. Combination disc diffusion test (CDDT) showed ESBL activity in 42% *E. coli* isolates. A significant association of *bla*^{CTX-M-15} gene was observed for phylogroup B2 (*P* = 0.007). Moreover, a combination genotype of *bla*^{CTX-M-15} and *TEM1* was also found statistically prevalent in phylogroup B2 (*P* = 0.006).

Conclusion: The study highlights the alarming rise in antibiotic resistance and delineates B2 a predominant phylogroup with a high prevalence of *bla*^{CTX-M-15} and *TEM1* genes in urinary *E. coli* isolates.

Introduction

Co-existence of *Escherichia coli* (*E. coli*) as a commensal constitutes an integral part of the natural microflora in many mammalian gastrointestinal tracts [1]. The metabolic diversity in *E. coli* allows the organism to maintain its homeostasis in diverse environments. Genetic accretions, pathological adaptation to the host immunity and ability to translocate at multiple niches facilitates the transit from a commensal to the pathological state [2]. Heterogeneity in *E. coli* pathotypes makes it a most common intestinal and extra-intestinal pathogen [3]. Extra-intestinal pathogenic *E. coli* (ExPEC) are commonly associated with bacteremia, neonatal meningitis and urinary tract infections (UTIs) [4]. ExPEC affect large population with UTI throughout the globe [5]. It is very important to detect prevalent *E. coli* pathotype associated with UTIs and its epidemiology in UTI infections for monitoring and management of infection at the regional and global scale. Based on the detection of three genetic markers, i.e. *chuA*, *yjaA*, and *TspE4.C2*, Clermont et al., provided a rapid and convenient tool to categorize *E. coli* into four distinct phylogroups (i.e. A, B1, B2 and D) [6]. Phylogroups B2 and D are the most frequent ExPEC isolates from UTI with virulent determinants whereas *E. coli* belong to the phylogenetic groups A and B1 are usually witnessed as avirulent species [1,6,7]. However, resistance to multiple antibiotics is widely reported for both the pathogenic [7] and commensal *E. coli* [8]. The resistance against β -lactam antibiotics is mainly manifested through the production of β -lactamase enzymes. These enzymes are classified into different groups based on their phenotypic/functional characteristics (i.e. Group 1-3) and their amino acid sequences (i.e. A-D classes) [9]. Group 2 (classes A and D) includes extended spectrum of β -lactamase (ESBL) enzymes with the tendency to hydrolyze Penicillin, cephalosporins and monobactams. Since last few decades several variants have been observed for harboring ESBL genes like *bla*^{CTX-M}, *bla*^{OXA}, *bla*^{SHV} and *bla*^{AmpC} and a huge diversity has been reported across the world including Pakistan. Co-existence of one or combinations of these genes was also reported worldwide indicating prevalence of different functional groups of β -lactamase enzymes is an emerging resistant trait in *E. coli* pathotypes [10]. In the present study we tried to explore the association of different phylogroups with antimicrobial resistance and the related ESBL genes such as; *TEM1*, *bla*^{OXA-1}, *bla*^{CTX-M-15}. We propose here that in comparison to traditional phenotypic methods, multiplex PCR could be a reliable method for the direct detection of *E. coli* pathotype and the associated ESBL genotype. Previous studies showed multiplex PCR an ideal choice for the detection of multiple β -lactamase genes or their variants in single

run [11,12]. In the present study, we optimized the protocol of multiplex PCR for simultaneous detection of *E. coli* phylogroup (A, B1 B2 & D) and the most prevalent *bla*^{CTX-M-15}, *bla*^{OXA-1} genes in a single reaction. We propose this multiplex PCR assay would help in probing the molecular epidemiology, diagnosis and management of *E. coli* infections in the community.

Methods

Isolation and Identification of *E. coli*

Urine specimens with bacterial count of more than 10⁵ Colony Forming Units (CFU) per ml were considered positive for bacterial UTIs. The isolation of *E. coli* from urine samples was performed in collaboration with Diagnostic research laboratory at Civil hospital Hyderabad, during the period of February, 2022 to July, 2022. A total of 475 urine samples were processed to isolate *E. coli* either on MacConkey's agar medium/CLED agar medium (Oxoid, UK). *E. coli* were recovered from 128 samples and their identity was confirmed with biochemical phenotype of lactose and indole positive but citrate negative. All of the 128 isolates were stock cultured in 50 % glycerol for the antimicrobial susceptibility check and molecular characterization in the Molecular Microbiology and Genetics Research laboratory at the Institute of Microbiology, University of Sindh, Jamshoro.

Antibiotic Susceptibility test and phenotypic detections of ESBL

Antibiotic susceptibility test was performed according to the CLSI guidelines on Muller Hinton using Kirby Baur-disc diffusion method [13]. Antibiotics ($n=18$) used from varying groups include, Beta-lactamase inhibitors [piperacillin tazobactam (TZP), amoxicillin-clavulanic acid (AMC)]; Cephalosporins [ceftazidime (CAZ), cefotaxime (CTX), ceftriaxone (CRO), cefpodoxime (CPD), cefepime (FEP), cefoxitin (FOX)]; Fluoroquinolones [ciprofloxacin (CIP), nalidixic acid (NA)]; Aminoglycosides [amikacin (AK)]; chloramphenicol (CM); trimethoprim sulfonamides (SXT); imipenem (IMP) and fosfomycin (FOS) (Oxoid, UK). ESBL phenotype were confirmed by standard double disc synergy test (DDST) and combination disc diffusion tests (CDDT) using ceftazidime (30 μ g), cefotaxime (30 μ g), ceftriaxone (30 μ g), cefpodoxime (10 μ g) antibiotic discs with 27 mm apart in synergy with AMC (30 μ g) at center. Additional discs of cefepime (30 μ g) was also placed. All the results were interpreted according to the CLSI guidelines and a published data for Enterobacteriaceae [14]. Expansion in the inhibition zone towards β -lactam inhibitor (AMC) for the otherwise decreased susceptibility to ceftazidime, cefotaxime, ceftriaxone, cefpodoxime and cefepime was considered a positive synergy test for ESBL production.

Detection of phylogroup markers and β-lactamase genes

Genomic DNA was extracted from *E. coli* isolates using boiling method as described before (15). Each isolate was subject to triplex PCR using protocol by Clermont et al, to detect different phylogenetic groups of the *E. coli* strains [6]. For the detection of ESBL genes, initially single PCR reactions were set with one set of primers and for multiplex PCR five different pairs of primers were used.

Single PCR reactions were set with the specific primer at their respective annealing temperature to yield the PCR products of *TEM1* (297 bp); *bla^{CTX-M-15}* (586 bp) and *bla^{OXA-1}* (814bp) separately. For multiplex PCR five pairs of primers including *bla^{CTX-M-15}*, *bla^{OXA-1}*, *ChuA*, *yjaA* and *TspE4.C2* were added for simultaneous detection of phylogroup and ESBL genes. Due to the close proximity in base pair size of *TEM1* to *chuA*, a separate PCR reaction was performed to detect TEM-1 β-lactamase. Primers for the genes are presented in Supplementary Table 1. PCR reactions contained 4 μl of GoTaq PCR master mix (Promega), 0.5 μl of each forward and reverse primers, 2 μl bacterial DNA extract and water required to adjust the total 50 μl volume. Thermocycler was programmed as follows: initial denaturation at 95 °C for five minutes followed by 25 consecutive cycles at 95 °C for 30 seconds 55 °C for 30 seconds and 72 °C for 1 minute with a final 2 minutes extension at 72 °C. The amplified products were run on 2% agarose gel electrophoresis in Tris–borate–EDTA (TBE) buffer and visualized after ethidium bromide (EtBr) staining in gel doc system (BioBase).

Statistical analysis

The statistical analysis was performed using SPSS (IBM) version 21 and online statistical calculator [Select Statistical Services UK (www.select-statistics.co.uk)]. Chi square test for independence was used to assess differences by assuming cut-off *P value* of < 0.05 as significant.

Results

Phylogenetic determination of *E. coli* isolates using triplex PCR

A total of 128 *E. coli* isolates were categorized into the four different phylogenetic groups (A-D) according to the original Clermont's phylogenetic scheme [6]. A representative gel image displaying the amplification of phylogenetic specific genes is shown in Figure 1. Triplex PCR targeting *chuA*, *yjaA* and *TspE4.C2* DNA fragment indicated phylogroup B2 as the predominant group (n = 46, 36%), followed by group D (n = 38, 30%), A (n = 32, 25%) and B1 (n = 12, 9%) (Table 1). From the 128 *E. coli* isolates, 36% (n = 46) were isolated from male and 64% (n = 82) were from female group. Isolates belonging to

phylogroup A and B2 were more prevalent in male group of patients (35 and 39% respectively) while the isolates belonging to phylogroup B1 and D were more prevalent in female group of patients (12 and 34% respectively). The *P value* (< 0.05) determined for each phylogroups were non-significant indicating that their prevalence is independent of gender (Table 1).

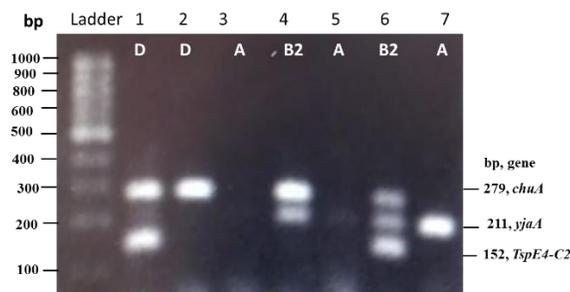


Figure 1: Agarose gel image showing triplex PCR amplified products specific for *E. coli* phylogrouping genes. Ladder: 1000-100 bp DNA marker. Lane 1-7 showing amplified PCR products for the detection of *E. coli* specific phylogrouping genes; *chuA* (279 bp), *yjaA* (211 bp) and *TspE4C2* (152 bp).

The Phylogenetic groups were assigned according to the following genotypes: group A (*chuA*-/*yjaA*-/*TspE4.C2*-or *yjaA* +), group B1 (*chuA*-/*TspE4.C2*+), group B2 (*chuA*+/*yjaA*+ or *chuA*+/*yjaA*+/*TspE4.C2*+), group D (*chuA*+/*yjaA*-or *chuA*+/*TspE4.C2*+), as per the original Clermont's scheme [6].

Phylogenetic Group	Total [n (%)]	Male [n (%)]	Female [n (%)]	<i>P value</i>
A	32 (25)	16 (35)	16 (20)	0.05557
B1	12 (9)	2 (4)	10 (12)	0.143885
B2	46 (36)	18 (39)	28 (34)	0.572832
D	38 (30)	10 (22)	28 (34)	0.140432
Total	128	46 (36)	82 (64)	-

Table 1: Absolute count and relative values for various phylogroups in both genders along with calculated *P values*.

Prevalence of antibiotic resistance pattern and ESBL phenotype in different phylogroups

Except co-amoxiclav, more than 70% resistance was witnessed against each antibiotic used in this study (Table 2). All the isolates were resistant to nalidixic Acid (100%) followed by ciprofloxacin and ceftazidime (95%). The lowest percentage of resistance was seen against co-amoxiclav 55% followed by imipenem (62.5%). Isolates of the phylogroup B2 expressed statistically significant dependence for cefotaxime (*p* = 0.017) and cefipime (*p* = 0.001) resistance. Similarly, the isolates belonging to Phylogroup D showed a statistically significant dependence for cefoxitin (*p* = 0.006), sulfomethoxazol (*p* = 0.035) and fosfomycin (*p* = 0.006) resistance. A significant independence was seen for cefoxitin (*p* = 0.001), fosfomycin (*p* = 0.001) and tazobactam (*p* = 0.035) in phylogroup A, sulfamethaxazole (*p* = 0.014), amikacin (*p* = 0.019), chloramphenicol (*p* = 0.017) in

phylogroup B2, and cefotaxime ($p = 0.009$) and cefepime ($p = 0.045$) resistance in phylogroup D isolates (Table 2).

Combination disc diffusion test (CDDT) showed extended spectrum of β -lactamase (ESBL) activity in 54 *E. coli* isolates (42%) whereas 74 (58%) were detected ESBL negative (Figure S1). A chi-square test calculation and *P* value analysis was performed to evaluate any significant difference between ESBL phenotype and the phylogroups (A-D). Table 3 shows a significant association of ESBL positive with phylogroup D ($p = 0.019$) and non-ESBL phenotype with phylogroup B2 ($p = 0.005$).

Antibiotic resistance pattern in different phylogroups					
Antibiotics	A (n=32)	B1 (n=12)	B2 (n=46)	D (n=38)	Total
	N (%)	N (%)	N (%)	N (%)	N (%)
Amoxiclavate	18 (56)	6 (50)	30 (65)	16 (42)	70 (55)
<i>P</i> value	0.837	0.731	0.073	0.065	-
Cefpodoxime	24 (75)	12 (100)	42 (91)	28 (74)	106 (83)
<i>P</i> value	0.176	0.366	0.056	0.075	-
Cefotaxime	26 (81)	12 (100)	44 (96)	28 (74)	110(85)
<i>P</i> value	0.378	0.505	0.017*	0.009	-
Ceftriaxone	26 (81)	10 (83)	36 (78)	26 (68)	98(76.5)
<i>P</i> value	0.469	0.560	0.734	0.157	-
Cefepime	22 (69)	6 (5)	40 (87)	22 (58)	90(70)
<i>P</i> value	0.823	0.105	<0.001*	0.045	-
Ceftazidime	30 (94)	12 (100)	44 (96)	36 (95)	122(95)
<i>P</i> value	0.629	0.646	0.891	0.841	-
Cefoxitin	18 (56)	12 (100)	40 (87)	38 (100)	108(84)
<i>P</i> value	< 0.001	0.427	0.546	0.006*	-
Imipenem	22 (69)	8 (66)	26 (57)	24 (63)	80(62.5)
<i>P</i> value	0.399	0.754	0.295	0.920	-
Sulfamethoxazole	26 (81)	12 (100)	34 (74)	36 (95)	108(84)
<i>P</i> value	0.573	0.427	0.014	0.035*	-
Amikacin	28 (88)	12 (100)	38 (83)	38 (100)	116(91)
<i>P</i> value	0.483	0.826	0.019	0.067	-
Chloramphenicol	30 (94)	12 (100)	40 (87)	38 (100)	120(94)
<i>P</i> value	1.000	0.852	0.017	0.205	-
Nalidixic Acid	32 (100)	12 (100)	46 (100)	38 (100)	128(100)
<i>P</i> value	0.410	0.046	0.676	0.526	-
Ciprofloxacin	30 (94)	12 (100)	42 (91)	38 (100)	122(95)
<i>P</i> value	0.629	0.646	0.108	0.358	-
Fosfomycin	20 (63)	12 (100)	38 (83)	38 (100)	108(84)
<i>P</i> value	< 0.001	0.427	0.680	0.006*	-
TazoPperacillin	26 (81)	12 (100)	40 (87)	38 (100)	116(91)
<i>P</i> value	0.035	0.826	0.286	0.067	-

Table 2: Antibiotic resistance pattern in different phylogroups. *P* value analysis was performed to evaluate any significant difference in the resistance pattern for each antibiotic among all the assessed four groups (A-D). *=statistically strong association.

Phylogenetic Group	ESBL Positive N (%)	ESBL Negative N (%)	<i>P</i> value
A (n=32)	14 (26)	18 (24)	0.836273
B1 (n=12)	6 (11)	6 (8)	0.564856
B2 (n=46)	12 (22)	34 (46)	0.005735*
D (n=38)	22 (41)	16 (22)	0.019379*
Total (n=128)	54 (42)	74 (58)	

Table 3: Absolute count and relative values for various phylogroups in ESBL positive and negative isolates along with calculated *P* values. (Note = * represent Significant dependence).

Multiplex PCR for detection of ESBL genes and phylogroups

Different combinations of ESBL genes were visualized after the multiplex PCR amplification of *TEM1*, *bla^{CTX-M-15}* and *bla^{OXA-1}* genes (Figure 2). ESBL genes were identified in 90.6% (114/128) *E. coli* strains. *TEM1* was observed as the most prevalent genotype (89%) followed by *bla^{CTX-M-15}* (78.1%) and *bla^{OXA-1}* (34.3%) (Table 4).

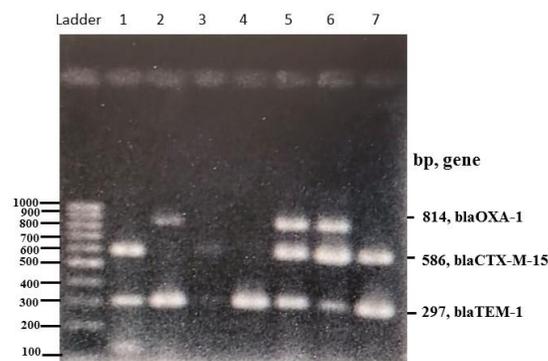


Figure 2: Multiplex PCR for the detection of ESBL genes. Ladder: 1000-100 bp DNA marker. Lane 1: *bla^{CTX-M-15}* and *TEM1*; Lane 2: *bla^{OXA-1}* and *TEM1*; Lane 3: no gene detected; Lane 4: *TEM1*; Lane 5-6: *bla^{OXA-1}*, *bla^{CTX-M-15}* and *TEM1* Lane 7: *bla^{CTX-M-15}* and *TEM1*.

ESBL genotype	A (n=32)	B1 (n=12)	B2 (n=46)	D (n=38)	Total
	n (%)	n (%)	n (%)	n (%)	N (%)
<i>bla^{CTX-M-15}</i>	18 (56)	10 (83)	42 (91)*	30 (79)	100 (78.1%)
<i>bla^{OXA-1}</i>	12 (38)	0 (0)	16 (35)	16 (42)	44 (34.3%)
<i>TEM1</i>	24 (75)	10 (83)	44 (96)	36 (95)	114 (89%)
<i>bla^{CTX-M-15} bla^{OXA-1}</i>	10 (31)	00 (0)	16 (35)	12 (32)	38 (29.6%)
<i>bla^{CTX-M-15}, TEM1</i>	18 (56)	10 (83)	42 (91)*	30 (79)	100 (78.1%)
<i>bla^{OXA-1}, TEM1</i>	10 (31)	00 (0)	16 (35)	14 (37)	40 (31%)
<i>bla^{CTX-M-15}, bla^{OXA-1}, TEM1</i>	10 (31)	00 (0)	16 (35)	12 (32)	38 (29.6%)

Table 4: Absolute count and relative values for ESBL genotypes in various phylogroups along with calculated *P* values. (*= significant association of B2 with *bla^{CTX-M-15}* is 0.007*).

In Figure 3, multiplex PCRs show simultaneous detection of *bla^{CTX-M-15}* and *bla^{OXA-1}* ESBL genes and *E. coli* phylogroups using three identification markers (*chuA*, *yjaA* and *TspE4.C2*). A significant association was seen for the presence of *bla^{CTX-M-15}* gene in the isolates belonging to phylogroup B2 ($p = 0.007$), while none of the other phylogroups showed significant association for the presence of *bla^{CTX-M-15}*, *bla^{OXA-1}* or *TEM1* genes (Table 4). A combination of four different genotype patterns is analyzed in the individual phylogroups. Mixed genotypes of *bla^{CTX-M-15}*, *bla^{OXA-1}* and *TEM1* were detected among 100/128 (78.1%) *E. coli* isolates. A duo set of *bla^{CTX-M-15}* and *TEM1* was seen as the most predominant gene combination in the all assessed phylogroups (A-D). β -lactamase genes in triplet combination were largely observed among the

members of group B2 (n = 16, 35%), however, none of the group B1 members was found to display the triplet or dual presence of genes except *bla*^{CTX-M-15} and *TEM1* pattern (n = 12). A combination of *bla*^{CTX-M-15} and *TEM1* was observed statistically prevalent ESBL genotype [n=42 (91%), *P* value = 0.006] in B2 phylogroup (Table 4).

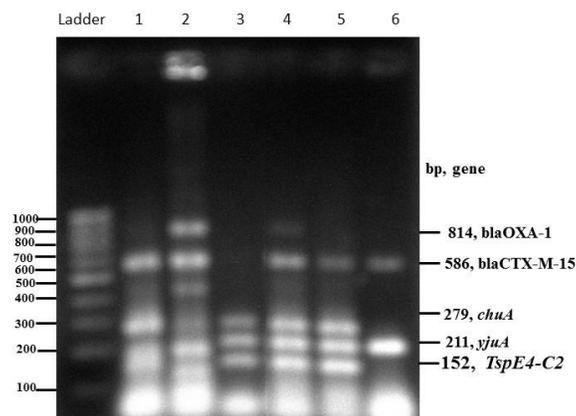


Figure 3: Agarose gel image showing multiplex PCR amplified products for the simultaneous detection of *E. coli* specific phylogroups and ESBL genes. The phylogenetic groups were assigned as per the original Clermont's scheme [6]. Ladder: 1000-100 bp DNA marker. Lane 1-7 showing amplified PCR products as: Lane 1: *bla*^{CTX-M-15}, group B2; Lane 2: *bla*^{OXA-1}, *bla*^{CTX-M-15} group A; Lane 3: group B2; Lane 4: *bla*^{OXA-1}, *bla*^{CTX-M-15}, group B2; Lane 5: *bla*^{CTX-M-15}, group B2; Lane 6: *bla*^{CTX-M-15} group A.

Discussion

Distribution of *E. coli* phylogroups and the epidemiology varies in different regions across the globe. For example, in Iran more than 50% of *E. coli* isolates belong to phylogroup D [16] whereas in Ethiopia and China prevalence of phylogroup B2 is observed as the highest followed by group D [17,18] which is similar to our findings in the current study. Interestingly, specific phylogroups have been shown missing in specific studied population, like B2 in Nigeria and South Korea and D in Benin, Africa [19].

One of the limitations in the present study is that we used original triplex PCR method which classified *E. coli* into four phylogroups (A, B1, B2 and D). Clermont's initial phylogroup scheme is advanced to quadruple PCR, categorizing *E. coli* species into extended phylogroups (i.e. A, B1, B2, C, D, E, F) [20]. However, the traditional triplex PCR is still widely used as a simple identification scheme [21] perhaps being a more resource-efficient in terms of time and cost. This validation study showed that 80–85% of the phylogroup assignments using the Clermont's original method are correct. For strains with group B2 genotype, the accuracy of assigning phylogroup is found 95% valid [22].

In the present study, B2 was detected as the highest phylogroup 36% (n=46), followed by group D 30% (n=38), in agreement with the findings that UTI is mainly associated with group B2 and D [6,23]. However, it is notably different from the other study conducted in Pakistan where phylogroup B2 is the predominant group followed by groups A and B1 [23].

In the present study, extreme antibiotic resistance was noticed against different groups of antibiotics. Almost all the *E. coli* isolates were resistant to nalidixic acid and ciprofloxacin, once considered an ideal choice for the treatment of uncomplicated UTI and pyelonephritis [24]. More or less 70-95 % *E. coli* strains were resistant to second, third and fourth generation cephalosporin; protein and folate synthesis inhibitors which is alarmingly higher than previously reported in Pakistan [25, 26]. Although the genetic localization of these resistance determinants was not the focus of this study, however restricted use of these antibiotics can limit the dissemination and curtail the cost of *E. coli* associated UTI management. Carbapenem resistance is swiftly emerging in the clinical isolates of *E. coli* [27]. In the present study, 62.5% *E. coli* isolates were found resistant to imipenem which is similar to the findings in Iran where carbapenemase production was detected in 63.2% *E. coli* strains [28]. Intriguingly, in the current findings, lowest resistance was observed for amoxiclavate (54%) and it is also comparatively lower than the other studies conducted in Pakistan [29, 30].

To investigate any significant associations of β -lactamase genes, the individual and a combination set of ESBL genotypes were analyzed in each phylogroup. Statistically significant association was seen for the presence of *bla*^{CTX-M-15} - *TEM1* genes in group B2 while none of the other phylogroups showed significant association to any one of the assessed ESBL genes. The *bla*^{CTX-M-15} fall into functional group A according to the Bush and Jacoby classification [09] and are characterized by increased hydrolysis of oxyimino- β -lactams (i.e. cefotaxime, ceftazidime, ceftriaxone, cefepime, aztreonam) which is also in agreement with our phenotypic observations.

The current study propose multiplex PCR can be used as a rapid diagnostic method to delineate the molecular epidemiology of ESBL genes and *E. coli* phylogroups. Moreover, a frightening increase in antibiotic resistance also suggests to explore the alternate therapeutic agents with maximum efficacy for the management of bacterial infections in the community.

Acknowledgement

We show our gratitude to Diagnostic and Research Laboratory (Civil Hospital, Hyderabad) for providing a platform for this work. We are also grateful to University of Sindh to facilitate us to execute this research study.

Ethical Statement/ Study Approval

The study was approved from the Institutional Review Board at its 141 meeting held on 11-12-2021, vide resolution No. 12 (25), University of Sindh, Jamshoro. A verbal consent was taken from each patient before inclusion to this study. Each Patient's name was given a unique identification number to include the information about their gender and age.

Author Contributions

1. Dr. Bushra B. Patoli designed and supervised the reserach work and wrote a manuscript.
2. Dr. Atif A. Patoli did the statistical analysis and helped in performing PCR experiments.
3. Dr. Iram Nabi Begum helped in interpretation and writing of the results and reviewed the manuscript.
4. Zarafshan Majid processed *E. coli* isoaltes and performed ESBL work.

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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