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Distribution and Phylogenetic Analysis of Bacterial Isolates from Urinary Tract Infection Patients of Pakistan

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ABSTRACT

Urinary Tract Infections (UTIs) are the second most common infections that infect millions of people worldwide. UTI can occur anywhere along the urinary tract and may cause symptomatic infection to complete renal failure. Most UTIs are caused by *Escherichia coli* (*E. coli*) which exhibit a high degree of both phenotypic and genetic diversity and disseminated in various geographical regions of the world. The aim of the present study was to determine the foremost pathogen responsible for UTIs and to investigate its genetic lineage in Pakistan. The clean clutch midstream urine samples of 174 (78% females and 22% males) patients with clinical symptoms of UTI from Gujranwala and Faisalabad districts of Pakistan, were cultured for the isolation of bacterial agents. The bacterial species were identified by standard biochemical and molecular tests. Out of total 174 samples, 84 (48%) were found urine culture positive with CFU $>10^4$. Among them, *E. coli* was the most frequent bacterium (n=51, 60.7%) isolated from patients followed by *Pseudomonas* sp. (n=13, 15.4 %), *Klebsiella* sp. (n=9, 10.7%), *Proteus* sp. (n=7, 8.3%), and *Staphylococcus aureus* (n=4, 4.7%). To perform phylogenetic analysis of *E. coli* isolates, partial 16S rRNA was amplified and sequenced. Sequences were aligned and edited using CodonCode Aligner. Phylogenetic tree constructed on the basis of 613 bp fragment of 16S rRNA, using MEGA6.1 through Neighbor-Joining method revealed that majority of *E. coli* isolates (n=16) had close relatedness with established uropathogenic strain ST-131. Eight isolates resembled closely and grouped with each of ST-101 and ST-14 strains while seven isolates showed high similarity with ST-648 strain followed by ST-73 strain indicating genetic closeness with four local isolates. It is concluded that genetically diverse uropathogenic *E. coli* are prevalent in the clinical setting of Pakistan in the studied area. The data of these isolated *E. coli* strains would improve our understanding about the epidemiology of this organism and may serve as baseline information for future researchers in the days ahead.

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Authors' Contributions
NS designed project and analyzed data. BA and TH collected samples. IH and MI performed bench work. FT and TH conducted phylogentic and statistical analysis. AT and MHR supervised the study. All authors contributed to writing of the article.

Key Words
Urinary tract infection, *Escherichia coli*, 16S rRNA, phylogenetic analysis.

INTRODUCTION

The urinary tract infection (UTI) is one of the most common infections of the human body affecting 8.1 million individuals per annum (Schapper *et al.*, 2008). UTI being responsible for almost 40% of all nosocomial infections are considered as a significant problem of health care systems (Johansen *et al.*, 2006). Among population, women are at higher risk of UTI because of some anatomical reasons (Daniele *et al.*, 2011). The UTIs are classified according to the site of infection such as pyelonephritis, cystitis and urethritis for infections of kidneys, urinary bladder, and urethra, respectively (Bien *et al.*, 2012). Leading symptoms of UTI include burning pain during urination, frequent urination, pain above the pubic bone and hematuria (Ronald *et al.*, 2001). Numerous bacterial species such as *Mycoplasma*, *Klebsiella*, *Enterobacter*, *Pseudomonas*, *Proteus*, *Acinetobacter*, Staphylococci, and *Citrobacter* may contribute towards UTI (Hooton and Stamm, 1997). However, *E. coli* is the most common pathogen being responsible for UTIs manifesting 90% urine positive cultures from UTI patients (Ronald, 2002).

E. coli are large group of bacteria mainly living in the intestine of human and other animals. *E. coli* strains are usually harmless being significant part of microbial flora of healthy population. However, they may cause diarrhea and other diseases outside the gastrointestinal tract. Pathogenic *E. coli* strains are classified into two main pathotypes; entero-pathogenic *E. coli* (EPEC) and extra-intestinal pathogenic *E. coli* (ExPEC) (Katouli, 2010). *E. coli* strains that cause the UTI are called uropathogenic *E. coli* (UPEC) and are the largest part of the *E. coli* population that causes the disease outside the

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gastrointestinal tract (Lloyd *et al.*, 2007). *E. coli* exhibit a high degree of both phenotypic and genetic diversity and disseminate in various geographical regions of the world (Mathew *et al.*, 2006). Likewise, diverse sequence types (STs) of uropathogenic *E. coli* strains have been reported in MLST database from different sources (Siu *et al.*, 2008) and are available at GenBank. Interestingly, some groups of pathogenic *E. coli* strains are associated with particular STs or ST complexes (Siu *et al.*, 2008).

Many molecular and genetic techniques employing different genomic regions have been used extensively to study the phylogenetic relationships among different bacteria. The comparative analysis of various ribosomal RNA genes due to their highly conserved nature and ease of amplification are being used in routine to study the phylogenetic diversity of different bacterial species (Phumudzo *et al.*, 2013). However, the comparison of 16S rRNA gene sequence has become a gold standard genetic technique to differentiate various organisms, to identify unknown bacteria, and to compare the genetic relatedness between isolates, thereby grouping the closely related organisms into clonal complexes (Michael *et al.*, 2007).

In Pakistan, several studies have been conducted to examine the antibiotic profiling of UTI isolated strains (Tanveer *et al.*, 2012; Yasir *et al.*, 2014; Khalil *et al.*, 2014; Sabir e*t al*., 2014). However, no significant data to our best knowledge is available regarding the surveillance of UTIs and about the genetic lineage of prevalent UPEC strains. Therefore the present study was aimed at investigating the bacterial etiology of urinary tract infected patients in Gujranwala and Faisalabad districts of Pakistan and to analyze their phylogenetic relatedness with other established uropathogenic strains circulating across the world. Since variation in bacterial strains plays an important role in determining the outcome of infection, strain characterization and phylogenic analysis therefore would enhance our understanding about the distribution of locally isolated strains and will be important in monitoring the UPEC.

MATERIALS AND METHODS

Sample collection

A total of 174 clean clutch mid stream urine samples (5 ml volume) were collected aseptically from the patients with clinical symptoms of UTI from public and private hospitals of Gujranwala and Faisalabad districts of Punjab, Pakistan, between January to December, 2015. The patients already receiving antibiotic treatment for UTI were excluded from the study. The patients included were 135 (78%) females and 38 (22%) males with ages ranging from 20-55 years.

Isolation and identification of bacterial isolates

After mixing well, a loopful from each urine sample was inoculated on nutrient agar (Difco, USA) and incubated at 37ºC for 24 h. To differentiate further, the bacteria were inoculated on selective medium *i.e.* MacConkey agar (Difco, USA) plates. The plates were incubated at 37ºC for 24 h. The cultures were considered UTI positive if the colony count of a single or two potential pathogens was equal or more than 10^4 CFU/ml. The bacterial isolates were identified by conducting conventional biochemical tests (Hemraj *et al.*, 2013; Pezzlo M., 1988; Bonadio *et al.*, 2001).

DNA extraction from isolates

Bacterial cultures were inoculated in 4 ml of Lauria broth (Difco, USA). After overnight incubation at 37°C, cultures were centrifuged at 4000g at 4°C for 10 min. The pellets were washed with 2ml TEN buffer (10mM Tris-Cl, 1mM EDTA, 10mM NaCl) and centrifuged at 4000g for 10 min at 4°C. The pellet was re-suspended in 1ml SET buffer (Sucrose 20%, 50mM Tris-Cl, 50mM EDTA) with 100 ul of lysozyme (5mg/ml) and incubated at 37°C for 30 min. After incubation, 500 µl of TEN buffer and 50 µl of 25% SDS were added. The mixture was incubated at 60°C for 15 min. After cooling, 100 µl of 5M NaCl was added. An equal volume of phenol/chloroform was added in the mixture and centrifuged at 4000g for 10 min at 4°C. Upper layer was transferred to a new tube and 1.5 ml chloroform added and centrifuge at 4000g for 10 min at 4°C. Upper layer was transferred to a new tube and DNA was precipitated by adding 2 volume of ice cold absolute ethanol. The DNA was allowed to precipitate for 1 h and rinsed with 70% ethanol. The pellet was air dried for 15 min and dissolved in 40µl H_2O. The DNA quantity and quality were measured by spectrophotometric analysis and visualizing on 1% agarose gel.

Molecular identification of E. coli *isolates*

For confirmation of *E. coli* at molecular level, isolates were subjected to PCR for the amplification of the species specific *uid A* gene which is reported to be expressed by 90% *E. coli* strains (Martin *et al.*, 1993). The 486 bps of *uid A* gene was amplified by using forward (5'-ATCACCGTGGTGACGCATGTCGC-3') and reverse primers (5'-CACCACGATGCCATGTT CATCTG-3') using PCR condition as described in Heninger *et al.* (1999).

Amplification of 16S rRNA gene

The genomic DNA isolated from *E. coli* strains was used to amplify a region of the universal 16S rRNA gene

by PCR. The primer sequences were
8F (5´-AGAGTTTGATCCTGGCTCAG-3') and
1492R (5´-GGTTACCTTGTTACGACTT-3´).
Each of 25 µl PCR reaction mixture consisted of 2.5 µl of 10X buffer (Thermo Scientific), 2.5 µl $MgCl_2$ (Thermo Scientific), 1U DNA Taq polymerase (Thermo Scientific), 200UM dNTPs (Thermo Scientific), and RNAse/DNAse free water was added in the reaction tube to make the volume up to 25µl. The reaction was run using the following cycling steps: 95°C for 10 min, 40 cycles each of 95°C for 60 seconds, 58°C for 45 seconds and 72°C for 30 seconds and final extension at 72°C for 10 min.

Purification of PCR products

The full-length amplicons (1500bps) generated from the 16S rRNA genes were visualized on agarose gel. The desired bands were cut under UV light and purified by gene clean kit (GeneAll Cat#102-102) according to manufacturer\s protocol. The purified products were quantified with NanoDrop (NanoDrop200, Thermo Scientific, USA).

Sequencing and phylogenetic grouping of strains

The purified PCR products of 16S rRNA were submitted for DNA sequencing to Macrogen, Korea. Resulting sequences were compared with uropathogenic reference sequences through NCBI BLAST. Briefly, sequences were aligned and edited using CodonCode Aligner. Phylogenetic tree constructed with highly identical reported uropathogenic strains on GenBank NCBI on the basis of 613 bp aligned fragment of 16S rRNA, using MEGA6.1 through Neighbor-Joining method with 1000 bootstrap value (Schumann *et al.*, 2013). The bacterial isolates were classified in different taxonomic groups on the basis of percentage similarity with the reported strains sequences.

RESULTS

Distribution of bacterial species in UTI patients

A total of 174 samples (n=174) were collected from the UTI diagnosed patients from the public and private hospitals of Gujranwala and Faisalabad districts of Punjab Pakistan, during 2015. Among 174 samples, 84 (48%) were found urine culture positive with CFU equal or more than 10^4 and 90 (52%) were recorded negative. Out of 84 positive samples, different bacterial species were isolated on the basis of colony characteristics and biochemical analysis (Table I). Briefly, *E. coli* was found the most frequent uropathogenic bacteria with 51 (60.7%) isolates followed by *Pseudomonas* sp. (n=13, 15.4 %), *Klebsiella* sp. (n=9, 10.7%), *Proteus* sp. (n=7, 8.3%), and *Staphylococci aureus* (n=4, 4.7%), respectively.

Table I.- Distribution of bacterial isolates in UTI patients of Pakistan.

Isolated bacteria	Number of isolates (N)	Percentage (%)
E. coli	51	60.7%
Pseudomonas sp.	13	15.4%
Klebsiella sp.	9	10.7%
Proteus sp.	7	8.3%
Staphylococcus aureus	4	4.7%
Total	174	100%

Molecular identification of E. coli *strains*

Since *E. coli* strains were abundant and proved to be major organisms responsible for UTI in the studied population. All 51 (100%) isolates showed the amplification of 486 bps of *uid A* gene confirming that isolates were *E. coli* **(**Fig. 1**).**

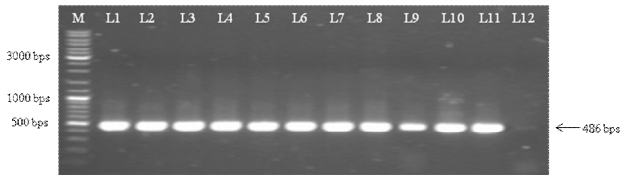

Fig. 1. Molecular identification of *E. coli* strains. The figure shows the presence of *uid A* gene in 10 representative *E. coli* strains. Marker (M), Line (L) 1, positive control; Lines 2–11, *E. coli* strains; Line 12, negative control. (Data is not shown for other strains).

The full length 16s rRNA gene was amplified by using PCR from all 51 *E. coli* isolates (Fig. 2).

Genetic lineage of uropathogenic E. coli *strains*

Phylogenetic tree constructed on the basis of aligned 613 bp sequence of 16S rRNA revealed two major groups. In first group the majority of *E. coli* isolates (n=16 *i.e.* U21, 26, 36 ,46, 41, 1, 29, 28, 23, 30, 31, 44, 15, 39, 33, and 3) had close relatedness (99% similarity) by clustering together in the same clade with established uropathogenic strains ST-131. The sequences of eight local isolates (U6, 7, 8, 11, 12, 24, 13, and 19) were genetically related with ST-101 strain sequence already reported on GenBank. A neighboring clad comprised of seven local isolates (U27, 9, 10, 32, 49, 34, 16) and established UTI strain ST-648 as well. The second major group comprised of two clades.

One small clade was formed by a small group of four isolates (U50, 51, 48, 35) with ST-73 strain while a comparatively bigger clade comprised of eight isolates (U22, 37, 38, 40, 4, 2, 5, 17) resembling closely with an already reported ST-14 strain. The phylogeny of our isolates with internationally reported strains from GenBank NCBI showed that genetically diverse population of uropathogenic *E. coli* are prevalent in the clinical setting of Pakistan in the areas of districts Gujranwala and Faisalabad of the Punjab province in Pakistan during the sampling year 2015. Analysis of 16s rRNA gene provided an overall view of the *E. coli* population (Fig. 3).

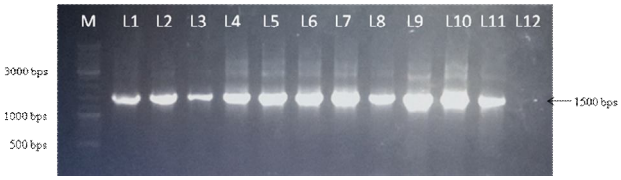

Fig. 2. Partially amplified 16S rRNA gene. The figure shows the amplification of 1500 bp fragment of 16S rRNA from10 representative *E. coli* strains. Marker (M) is on the extreme left, Lanes (L); L1-L11; *E. coli* strains. Lane 12 shows the negative control. (Data is not shown for other strains).

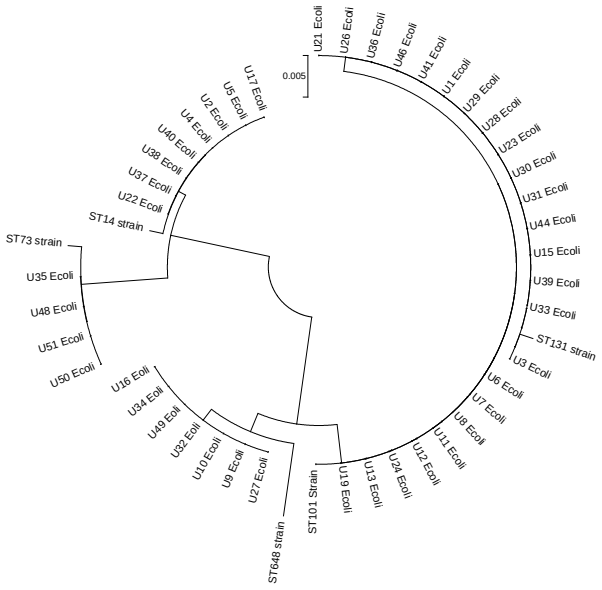

Fig. 3. Neighbor-Joining phylogenetic tree (circular view) of 43 local *E. coli* isolates based on 16S ribosomal RNA gene partial sequence constructed trough MEGA6.1 using 1000 bootstrap value. ''U'' represent the isolates from UTI patients.

DISCUSSION

UTIs are among the most widespread bacterial infections and associated with millions of death across the world. Coupled with certain other complications, UTIs may also lead to severe outcomes such as renal failure. Various microbial agents have been well described to reside and infect the urinary tract. However, the list of bacterial species infecting the urinary tract is getting longer day by day. As a matter of fact, the distribution of microorganisms in UTIs varies among different parts of the world. Moreover, strain variations have also been reported in the same species. Therefore, the current study was designed to investigate the microbial flora responsible for urinary tract infections in UTI patients of Gujranwala and Faisalabad districts of Pakistan and to study the phylogeny of the most commonly isolated bacteria.

Our study described the prevalence of various bacterial species in the urine samples of UTI patients. Briefly, *E. coli* showed the highest prevalence (60.7%) followed by *Pseudomonas* sp. (15.4%), *Klebsiella* sp. (10.7%), *Proteus* sp. (8.3%) and *S. aureus* (4.7%), respectively. The present study corroborates the previous studies which describe the mixed growth of bacterial community in patients with lower urinary tract infection (Rajvinder *et al.*, 2013). Surprisingly, *E. coli* has also been reported in previous studies conducted in Pakistan and around the globe as the foremost bacterial pathogen associated with UTI and its prevalence is 60-80% (Ziad *et al.*, 2015; Laupland *et al.*, 2007; Kahlmeter *et al.*, 2003; Noor *et al.*, 2014). However, variation exists in studies reporting the prevalence of other bacterial species (Yasir *et al.*, 2014). Our results are completely in accordance with previous reports in terms of *E. coli* prevalence while slightly variable results were observed in the present study regarding the distribution of other bacterial species such as *Pseudomonas* sp., *Klebsiella* sp., *Proteus* sp. and *S. aureus*. This variation in the distribution of various organisms could be due to various factors such as socioeconomic status of the patients, environmental circumstances, practices at health care units, and hygienic conditions of the studied population.

One of the important findings of this study is the isolation of the Gram positive staphylococci from UTI patients. These bacteria are usually commensals of vaginal mucosa but recent reports have pointed out their role in UTI (Megged, 2014; Nobbs *et al.*, 2009). So, the results of our study endorsed the etiological role of *Staphylococcus aureus* in UTIs.

Since *E. coli* showed the highest prevalence in the present study, we performed phylogenetic analysis of *E. coli* strains on the basis of the 16S rRNA sequences.

Accuracy and precision of ribotyping is well established as compared to other conventional typing techniques (Clermont *et al.*, 2000; Carson *et al*., 2001). The phylogenetic tree showed close relatedness of our 16 isolates with ST-131 by grouping together in the same clade. Our eight isolates resembled each with ST-101 and ST-14 strains, followed by seven and four isolates grouping with ST-648 and ST-73 strains, respectively. As a whole our isolate sequences showed high similarity with five strains; ST-131, ST-101, ST-14, ST-486, and ST-73, which have been reported in previous studies as well-established and most frequent uropathogenic strains (Siu *et al*., 2008). Noteworthy, the only available previous study to our best of knowledge on uropathogenic *E. coli* strains from Pakistan, also reported the major resemblance of *E. coli* isolates with ST-101 and ST-468 (Mushtaq *et al.,* 2011). Interestingly, another study conducted in England on UPEC isolates revealed that the predominant ST was ST-131 followed by ST-73 (Siu *et al*., 2008). The results of our study are comparable with above mentioned as well as other studies recently performed in Asian countries such as India and Korea (Yun *et al*., 2015; Arif *et al*., 2014). It is important to mention here that the results of the present study are preliminary and based on the similarities between 16s rRNA sequences of the studied strains with the established sequence types. This may serve as baseline information to conduct future research employing other techniques such as MLST, in the days ahead.

CONCLUSION

UTI is a health problem with pressing importance and diverse bacterial etiology. Among various bacterial culprits, *E. coli* with genetically diverse population acquires the highest prevalence in hospital and clinical settings of Pakistan. Other bacteria associated with UTI also showed the upturned trend. Better strain characterization and phylogenetic analysis of locally isolated *E. coli* strains would enhance our understanding about the epidemiology of this pathogen and will allow us to develop improved strategies to monitor the uropathogenic *E. coli*.

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Statement of conflict of interest

All authors declared that no conflict of interest.

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