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 catch 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 >105. 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.

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, Staphylococcus, 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
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 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 et 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 phylogenetic 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 37°C 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 37°C 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 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. 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 centrifuged 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 H2O. 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'-ATCACCAGGTAGACGATGTGC-3') and reverse primers (5'-CACCAGATGCCATGT 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'-AGAGTTTGTATCCTGCTCAG-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 MgCl₂ (Thermo
Scientific), 1U DNA Taq polymerase (Thermo
Scientific), 200µM 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 rDNA 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 uropathogen
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 10⁵ 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
Staphylococcus aureus (n=4, 4.7%), respectively.

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

<table>
<thead>
<tr>
<th>Isolated bacteria</th>
<th>Number of isolates (N)</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>51</td>
<td>60.7%</td>
</tr>
<tr>
<td>Pseudomonas sp.</td>
<td>13</td>
<td>15.4%</td>
</tr>
<tr>
<td>Klebsiella sp.</td>
<td>9</td>
<td>10.7%</td>
</tr>
<tr>
<td>Proteus sp.</td>
<td>7</td>
<td>8.3%</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>4</td>
<td>4.7%</td>
</tr>
<tr>
<td>Total</td>
<td>174</td>
<td>100%</td>
</tr>
</tbody>
</table>

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).](image)

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).

**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 (Meggard, 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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