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Research Article
Molecular Investigation of Multidrug-Resistant *Escherichia coli* Clinical Isolates from Patients with Urinary Tract Infections

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Abstract

Background and Objective: Urinary tract infections believe to be one of the main acquainted infections by *Escherichia coli* in hospitals with an excessive incidence of illness. This study aimed to analyze the antibiotic resistance profile and molecular characteristics of *E. coli* isolates recovered from patients with urinary tract infection at different hospitals in Taif Governorate, Saudi Arabia. **Materials and Methods:** Out of 143 isolates collected for 11 months, from February-December 2019, 24 isolates were identified as *E. coli* by API system and 16S rRNA sequences techniques. An antibiotic sensitivity test was performed using the disk diffusion method. Besides, the repetitive sequence repeat-PCR (Rep-PCR) technique was used for genotyping the 24 isolates. **Results:** Almost all isolates were resistant to most tested antibiotics such as ampicillin, ceftazidime, cefepime, trimethoprim/sulfamethoxazole, amox/clavulanic. The PCR results show that virulence genes *kpsII* and *yaiO* were detected in all *E. coli* isolates. *Stx1*, *fimH*, *hly* and *uidA* were moderate detected in all isolates. **Conclusion:** The high frequencies of antibiotic-resistant *E. coli* isolates in patients with urinary tract infections in the current study suggest that continuous surveillance of the use of appropriate antibiotics is required and that control of infections is necessary.

Key words: Molecular characterization, virulence genes, 16S rRNA, rep-PCR, *E. coli*, polymorphic, urinary tract infection

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Urinary Tract Infections (UTIs) are described as bacteriuria with urinary[1]. UTIs, consider as one of the main familiar infections in hospitals with a great frequency of morbidity[1]. UTIs may affect half of all people during their lifetime[2,3]. The factors that cause UTI differ from one place to another and even differ in their patterns of susceptibility to resistance. Several types of microbial pathogens can cause UTIs[3,4]. These pathogens included *Escherichia coli*, *Klebsiella pneumoniae Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Proteus* sp. and *Enterococci* spp[3,5,6]. *E. coli* has been stated as the most prevalent pathogen that causes UTI and is a very common cause for consultation and prescription of antibiotics in current practice[2,7]. Many strains of *E. coli* have adapted to become opportunistic and commensal pathogens[5]. Huge and improper use of antibiotics to treat UTI generates selective pressure followed by rapid onset and spread of multidrug-resistant bacterial strains[2,8]. Undesirably, numerous bacterial isolates have developed and become resistant to antibiotics over the last several decades[4]. Several international health institutions like WHO or the United States Institute of Medicine officially stated anxiety against antibiotic-resistant bacteria that represent health and economical risks[9,10].

It was published that *E. coli* strains are divided into 4 categories: A, B_1, B_2 and D. The low pathogenic strains are located in Group A and B[11]. Extraintestinal pathogenic strains mainly belong to B_2 and D groups and intestinal pathogenic strains belong to groups A, B_1 and D. Based on the virulence gene profiles, *E. coli* has been classified into four clusters (I-IV)[11,12]. These *E coli* strains have been reported to produce two types of Shiga toxins (*Stx*), *Stx1* and *Stx2*, which can cause severe diarrhea and hemolytic uremic syndrome[13,14]. The main virulence factors include the Shiga Toxin-producing *Escherichia coli* (STEC) *hlyA* gene, which is often associated with severe clinical disease in humans. Also, the intimin, the product of *eaeA* gene, is involved in bacterial attachment and effacing adherence[7,12]. STEC as a virulence factor is a major cause of food-borne infections and is thus a major public health concern[2]. The virulence factors also include certain capsular antigens (*kpsII* and *K1*), which have been recognized as uropathogenic genes and *FimH*, a major determinant that facilitates colonization and survival in host cells and has a high tropism for urinary tract receptors[14]. Multiplex PCR has been used elsewhere to detect virulence genes in *E. coli*, particularly *uidA, stx1*, *stx2, eaeA, fimH* and *hlyA*[6,13].

The main objective of this study was to identify the virulence genes in *E. coli* isolates obtained from UTI patients. Besides, the repetitive sequence repeat (Rep-PCR) technique was used for genotyping the *E. coli* isolates.

MATERIAL AND METHODS

Sampling: The current study was carried out at Department of Biology, College of Science, Taif University, Saudi Arabia from February-December, 2019. Out of 143 isolates collected from UTI patients, 24 were identified as *E. coli*. Eighty percent of the patients were females. The urine samples were collected in a clean and sterile tube. The ethics committee of Obstetrics Hospital in the King Faisal Complex, Taif, Saudi Arabia, approved the study experiment.

Isolation of bacterial strains: Urine samples were cultured on MacConckey agar media and then were incubated for at least 24 hrs in positive cases ($\geq$105 CFU mL^{-1}) or 48 hrs in negative cases. *E. coli* isolates were identified using standard biochemical tests and the results were confirmed using the API 20E system (Biomerieux, Inc., Missouri, USA).

Antibacterial susceptibility test: Antibiotic susceptibility of *E. coli* was achieved by the roles of the National Committee for Clinical Laboratory Standards using the disk diffusion method as previously reported[5]. All bacterial strains were cultured on nutrient agar at 37°C for overnight. The *E. coli* ATCC 25922 was used as Extended Spectrum Beta Lactamase (ESBL)-negative control. Antibiotic discs that used in the present experiment were: Piperacillin-tazobactam (100 µg), amoxicillin/clavulanic acid, Cefoxitin, Cefepime, cefalotin, Nitrofurantoin, Trimethoprim-Sulfamethoxazole, piperacillin/tazobactam, Ceftazidime (30 µg), Gentamicin (10 µg), Ceftriaxone (30 µg), Ciprofloxacin (5 µg) and Imipenem (10 µg).

DNA extraction: *E. coli* isolates were grown in Luria Bertani broth at 37°C overnight. Bacteria were then harvested from the broth, resuspended in sterile distilled water and genomic DNA was extracted for each isolate using a DNA extraction kit (Promega, German) according to the manufacturer's instructions. The DNA template was stored at -20°C until used for PCR.

Sequencing of 16S rRNA gene of *E. coli* isolates: Two PCR primers were designed (Macrogen, Inc., Seoul, South Korea) to amplify approximately 1465 bp of a consensus 16S rRNA gene as described previously[15], including forwarding primer 27f (5-AGAGTTTGATCMTGGCTCA-3) and reverse primer 1492r (5-TACGGYTACCTTGTTACGACTT-3). PCR amplicons of the 16S rRNA gene were purified from gel bands using a QIAquick PCR Purification Kit (QIAGEN) and were sequenced commercially. Raw sequences were edited and assembled using MEGA 5.2.

The sequences were deposited in GenBank under accession numbers KY780336-KY780359. The nucleotide sequences of the 16S rRNA genes obtained in the present study and from GenBank were aligned, sequence identities were calculated and a phylogenetic tree was generated as previously described[14].

Virulence genes PCR amplification: *E. coli* isolates were examined through duplex PCR using specific primers to determine the presence of *stx1* and *sxt2* genes according to Alsanie *et al*.[16] Samples were also tested for the presence of *uidA*, *yaiO, eaeA, hlyA, fimH* and *kpsII* according to Moyo *et al*.[17].

Repetitive sequence repeat-PCR (Rep-PCR): Rep-PCR conditions were standardized according to Hassan and Belal[16]. Thirty repetitive sequence primers were tested. Among them, six primers that presented the strongest band resolution were chosen for this study (Rep1-6). PCR conditions and DNA amplicons resolution was accomplished according to Hassan and Belal[14].

Association between Rep-PCR markers, antibiotic resistance and virulence genes: All genotypic data obtained from Rep-PCR primers for all isolates were converted to the presence (1) or absence (0). For analysis purposes, antibiotic sensitivity was converted to numerical values, where 1 represented sensitive (S), 2 represented resistant (R) and 3 represented Immune (I). Besides, virulence gene responses were also converted to the presence (1) or absence (0). We tested associations between individual pairs of Rep markers and both antibiotic and virulence genes by assessing the nonparametric Kendall's tau-b (τ) correlation[18], to determine the relationship between genotypic and phenotypic patterns. Following data conversion, Kendall's tau-b (τ) correlation was conducted as described in XLSTAT[19]. The correlation was analyzed to determine the relationship between Rep markers and both antibiotic and virulence genes. Besides, the correlation between antibiotic resistance and virulence genes was also assessed to determine whether there was any association between both variables. The significance of the correlations was determined at $p = 0.001$ using Kendall's rank correlation critical values[19].

Data analysis: To identify the genetic relationship among the 24 *E. coli* isolates, the Rep-PCR banding pattern was converted to the presence (1) or absence (0). Following data conversion, NTSYS-pc 2.1e software[20] was used to perform cluster analysis based on Nei's genetic distance[20], by the Unweighted Pair Group Method with Arithmetic mean (UPGMA).

RESULTS AND DISCUSSION

Antibiotic resistance: The 24 isolates identified microbiologically by the Application Programming Interface (API) system as *E. coli* were surveyed for the presence of multidrug resistance using the combination disk diffusion examination[21]. All 24 isolates were resistant to one or more antimicrobial agents except for isolates TU-19 and TU-23, which were sensitive to all tested antibiotics (Table 1). Overall, 22 (91.6%) of the isolates were resistant to ampicillin and 18 (75%) were resistant to ceftazidime and cefepime. In contrast, all *E. coli* isolates appeared to be completely sensitive to imipenem, meropenem, amikacin and tigecycline. The proportions of isolates resistant to trimethoprim/sulfamethoxazole, ciprofloxacin, cefoxitin and piperacillin/tazobactam were 50 (12/24), 45.8 (11/24), 29.2 (7/24) and 16.7% (4/24), respectively. The antimicrobial resistance of *E. coli* is an extraordinary concern around the world due to the increasing resistance of this bacterium to several

Table 1: Distribution of antibiotic resistance genes among *E. coli* and other isolates

Bacterial isolates	Antibiotic resistance profile
TU-1	Amp-Am/Cla-Cefo-Cefta-Cefe-Cip-Tri/Sulf
TU-2	Amp-Am/Cla-Cefo-Cefta-Cefe-Cip-Tri/Sulf
TU-3	Amp-pip/taz-Cefta-Cefe-Cip-Tri/Sulf
TU-4	Amp-Cefta-Cefe-Cip-Tri/Sulf
TU-5	Amp-pip/taz-Cefta-Cefe-Gen-Cip-Tri/Sulf
TU-6	Amp-Cefta-Cefe
TU-7	Amp-Cefta-Cefe-Cip-Nit -Tri/Sulf
TU-8	Amp-Cefta-Cefe-Gen-Tri/Sulf
TU-9	Amp-Cefo-Cefta-Cefe-Cip
TU-10	Amp-Cefta-Cefe-Gen-Tri/Sulf
TU-11	Amp-pip/taz-Cefta-Cefe-Cip-Nit
TU-12	Amp-Cefta-Cefe-Tri/Sulf
TU-13	Amp-Cefta-Cefe-Tri/Sulf
TU-14	Amp-Cefta-Cefe-Cip-Tri/Sulf
TU-15	Amp-Cefo-Cefta-Cefe
TU-16	Amp-Cefo-Cefta-Cefe-Gen
TU-17	Amp-Cefo-Cefta-Cefe-Cip
TU-18	Amp-Am/Cla-pip/taz-Cefo-Cefta-Cefe-Gen-Cip-Tri/Sulf
TU-19	-
TU-20	Amp
TU-21	Amp-Tri/Sulf
TU-22	Amp-Tri/Sulf
TU-23	-
TU-24	Amp

Amp (ampicillin), Am/Cla (amox/clavulin), Pip/Taz (piperacillin/tazobactam), Cefa (cefalotin), Cefo (cefoxitin), Cefta (ceftazidime), Ceftr (ceftriaxone), Cefe (cefepime), Imi (imipenem), Gen (gentamicin), Cip (ciprofloxacin), Nit (nitrofurantoin) and Tri/Sulf (trimethoprim/sulfamethoxazole)

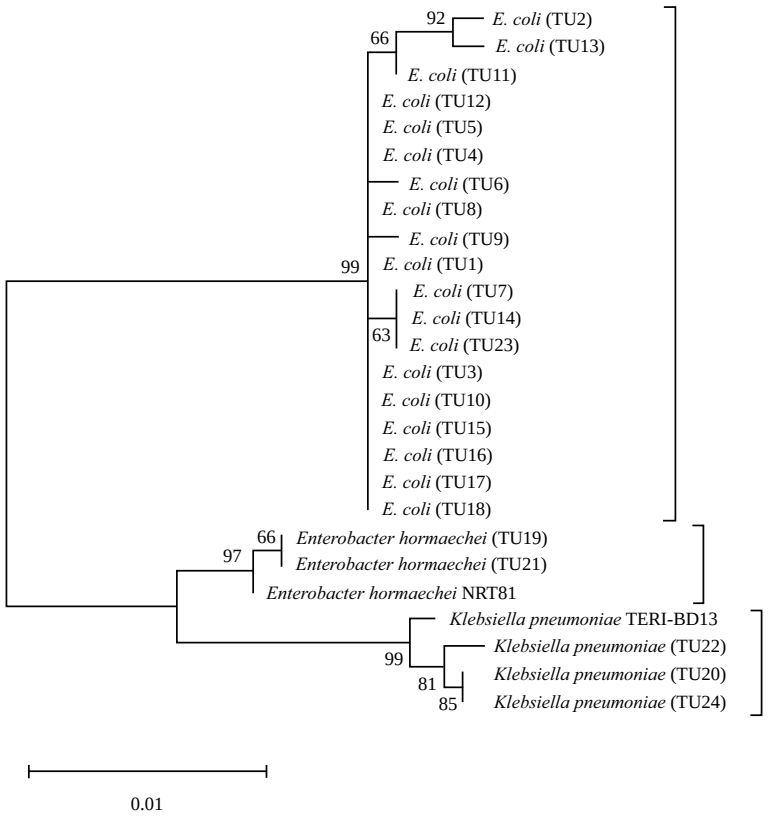

Fig. 1: Neighbor-Joining phylogeny based on 16S rRNA gene sequences of bacterial isolates

Bacterial isolates TU1-TU24 are presented in blue colour, Number above branches represent bootstrap values. Brackets represent identified clusters

commonly prescribed antibiotics[18]. In the present research, *E. coli* isolates fluctuated in their sensitivity to different antibiotics (Table 1). These results are comparable to those of other local and global studies[19]. The high levels of resistance observed for certain antibiotics might be caused by the impulsive and intense use of these antibiotics[2,22]. In contrast, carbapenems (imipenem and meropenem) are stable toward ESBL enzymes and efficient in the therapy of infections due to the infection by ESBL-producing bacteria and the management methods of these antibiotics (either intravenous or intramuscular) limit their use by most patients[18].

16S rRNA gene of *E. coli* isolates: The 16S rRNA gene from the 24 clinical bacterial isolates was successfully amplified and sequenced. Individual BLAST searches of the 16S rRNA sequences confirmed 19/24 isolates as *E. coli*, whereas 3/24 isolates were found to be *K. pneumoniae* (TU-20, TU-22 and TU-24) and 2/24 isolates were found to be *Enterobacter* (TU-19 and TU-21). All sequences in the current study were deposited in GenBank under accession numbers KY780336-KY780359.

E. coli sequences were compared with the available 16S rRNA genes of selected published strains of the genus *Escherichia* that available in the GenBank database, including representative strains of the A, B_1, B_2, C, D and E subtypes of *E. coli*. The phylogenetic tree of different 16S rRNA sequences was not able to differentiate between different *E. coli* subtypes (Fig. 1). The phylogenetic relationships of the *E. coli* strains revealed four main clusters (Ia, Ib, II, III and IV). TU-1, TU-3, TU-4, TU-7, TU-14, TU-15, TU-16, TU-17 and TU-23 were related to Ia cluster, whereas TU-5, TU-6, TU-8, TU-9, TU-10, TU-12 and TU-18 were related to Ib cluster and TU-2, TU-11 and TU-13 were related to cluster III (Fig. 1). These results agree with the previous studies[7,16]. Alsanie *et al.*[17] reported that the traditional identification of bacteria using phenotypic characteristics is generally not as accurate as identification by genotypic methods.

Detection of antimicrobial resistance genes in *E. coli* using PCR: Using antibiotics accurately and distinguishing the resistance genes of bacteria that separated from UTI patients may assume a significant part in controlling the disease and its dangerous consequences. Throughout the previous decades, *E. coli* has been perceived as one of the main sources of nosocomial diseases worldwide[11]. On a fundamental level, a

Table 2: Virulence gene patterns among pathogenic *E. coli* and other isolates

Bacterial isolates	Present of virulence genes
TU-1	*KpsII/fimH/YaiO*
TU-2	*KpsII/fimH/UidA /YaiO*
TU-3	*Hly/KpsII/fimH/YaiO*
TU-4	*Hly/KpsII/fimH/UidA/YaiO*
TU-5	*Stx1/KpsII/fimH/UidA/YaiO*
TU-6	*Hly/KpsII/fimH/UidA/YaiO*
TU-7	*KpsII/fimH/YaiO*
TU-8	*Stx1/KpsII/fimH/YaiO*
TU-9	*KpsII/fimH/UidA/YaiO*
TU-10	*hly /KpsII/fimH/UidA/YaiO*
TU-11	*Hly/KpsII/fimH/UidA/YaiO*
TU-12	*Hly/KpsII/fimH/UidA/YaiO*
TU-13	*Stx1/KpsII/fimH/YaiO*
TU-14	*KpsII/fimH/YaiO*
TU-15	*KpsII/fimH/YaiO*
TU-16	*Hly/KpsII/fimH/UidA/YaiO*
TU-17	*Hly/KpsII/fimH/UidA/YaiO*
TU-18	*Hly/KpsII/fimH/UidA/YaiO*
TU-19	*KpsII/fimH/UidA/YaiO*
TU-20	*KpsII/fimH/UidA/YaiO*
TU-21	*KpsII/fimH/UidA/YaiO*
TU-22	*KpsII/fimH/UidA/YaiO*
TU-23	*KpsII/fimH/UidA/YaiO*
TU-24	*KpsII/fimH/UidA/YaiO*

few genes situated on the extraordinary plasmids in certain *E. coli* strains are viewed as being accountable for such problems[16,23]. Various studies have investigated the incidence of virulence genes in *E. coli* strains isolated from UTI patients. A study from Spain showed that almost 70% of the urinary strains carried at least one of the target virulence genes[2,8,23,24]. The classification of virulence genes can enhance the present awareness of the pathogenesis of diseases and reduce the resulting difficulties. All of the bacterial strains in the current study contained at least one virulence gene, among which 4 (16.7%), 8 (33.3%), 9 (37.5%) and 16 (66.7%) were found to harbour *stx1*, *fimH, hly* and *uidA* virulence genes, respectively (Table 2). Moreover, 100% of the tested isolates carried *kpsII* and *yaiO* virulence genes. Conversely, no isolates carried the virulence genes *stx2* or *eaeA*. The associations among different virulence factors in *E. coli* isolates were documented by Hassan *et al*.[2]. These genes were detected in a high proportion of bacterial strains isolated from mono-microbial cultures. Strains containing the genes *hly* and *fimH* exhibit an interesting relationship with uropathogenic *E. coli*[25]. Intestinal or extraintestinal isolates of clinical and symbiont *E. coli* separated from different patient's areas have been discovered to be particularly varied in their genetic makeup. After some time, this genetic variation has been developed through selection and adaptation, such pathogenic strains tend to become host-specific or hospital-specific[2,26].

Repetitive sequence repeat-PCR (Rep-PCR): Molecular markers are efficient techniques for molecular classification via DNA fingerprinting. Out of thirty Rep-PCR primers, six were chosen to investigate the genetic similarities within the 24 isolates (Fig. 2). The average number of amplified bands was 16 bands per primer. Over 60% of amplified bands were polymorphic. Primer Rep-1 produced 18 polymorphic loci (Fig. 2a). Additionally, primer Rep-3 give 19 polymorphic loci (Fig. 2b), while, primer Rep-6 produced ten polymorphic loci (Fig. 2c). The Rep-PCR strategy has already been utilized for the assessment of *E. coli* strains in several reports[16,27,28]. Rashid *et al*.[27] applied this technique to examine drug resistance of ESBL strains and to distinguish extended-spectrum beta-lactamase makers at the genotype level. Korvin *et al*.[28] analyzed *E. coli* strains separated from basic sources of faecal contamination and examined genetic relations of strains in each host unit using the Rep-PCR molecular technique.

Seventy-three fragments from all Rep-PCR analysis were appropriate for the investigation of genetic similarities and for designing the phylogenetic tree for *E. coli* isolates analyzed in this study (Fig. 3). According to the dendrogram constructed using UPGMA based on Jaccard's similarity coefficients dependent on genetic similarity and intra-species differentiation ranged from 0.17-0.80. Based on cluster analysis, the 24 *E. coli* isolates were grouped into four different groups at a cutoff of approximately 0.690 (Fig. 3). The first group included isolates TU-1, 3,4, 8, 12, 13, 14, 15, 17 and 18, while the second group harboured isolates TU-5, 6, 7, 11, 16 and 23. The third group contained isolates TU-2, 9, 10, 20, 22 and 24, while the fourth group had only TU-19 and 21 (Fig. 3).

The clusters of isolated bacterial strains obtained using the repetitive sequence fingerprinting method performed through Rep-PCR (Fig. 3) were completely different in comparison to those obtained based on 16S rRNA sequences (Fig. 1). Although Rep-PCR is a powerful method for genotyping method[6,16], there is growing evidence regarding the accuracy and reproducibility problems in this method[28,29]. The current results indicate that the Rep-PCR fingerprint clustering method could not be compared with 16S rRNA sequencing clustering methods that are due to the greater level of data obtained from the conservative 16S rRNA gene. Additionally, the 16S rDNA gene is a conserved and fixed gene that didn't change throw time. On other hand, the rep-PCR profile could be changed by the environment that affects the bacterial genome.

Nonparametric correlation analyses using Kendall's tau-b (τ) were carried out to assess the association between each

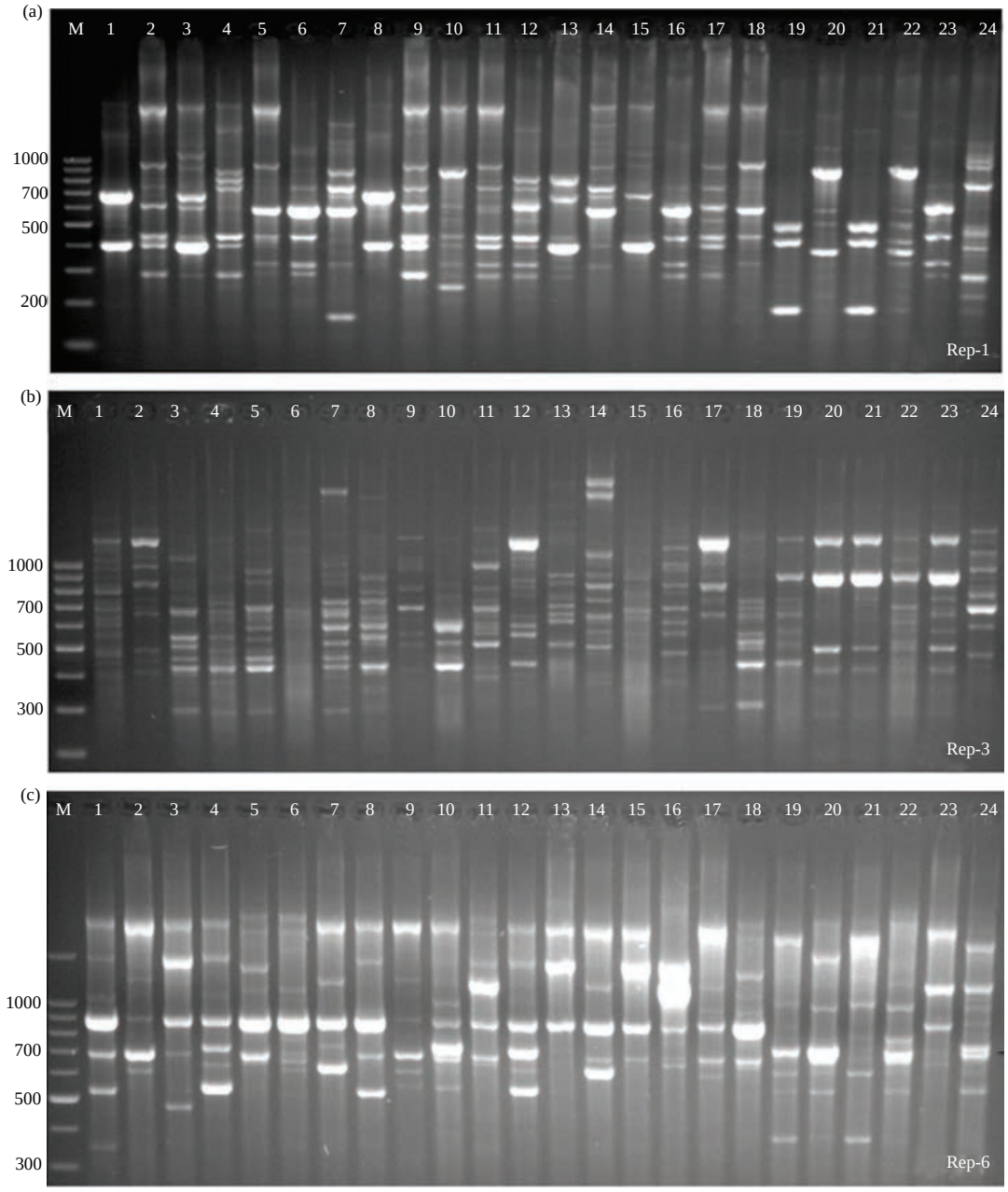

Fig. 2(a-c): Rep-PCR profiles of 24 antibiotic-resistant bacterial isolates
A: Using of Rep-1 primer; B: Rep-3 primer and C: Rep-6 primer, M: 100-bp DNA ladder

pair of rep-PCR markers, antibiotic resistance and virulence genes (Fig. 4-6)[16,19]. The result of Fig. 4 shows the correlation between Rep markers and antibiotic resistance. All correlation coefficients were low but highly significant ($p<0.001$). The lowest correlations were found between Rep4 and Nit antibiotics as well as between Rep5 and Gen antibiotic, with a correlation coefficient value of 0.11. The highest correlation was between Rep4 and each of the Cefta and Cefe antibiotics, with a correlation coefficient value of 0.33. Correlation coefficients between Rep markers and virulence genes are graphically presented in Fig. 5. Again, the correlation between all pairs was low but significant ($p<0.05$). The lowest correlations were found between Rep2 and each of the *hly, kpsII* and *fimH* virulence genes, with a correlation coefficient value of 0.02. The highest correlation was found between Rep4 and each of the *uidA* and *yaiO* genes, with a correlation coefficient value of 0.27. Correlation coefficients between antibiotic resistance and virulence genes are presented in

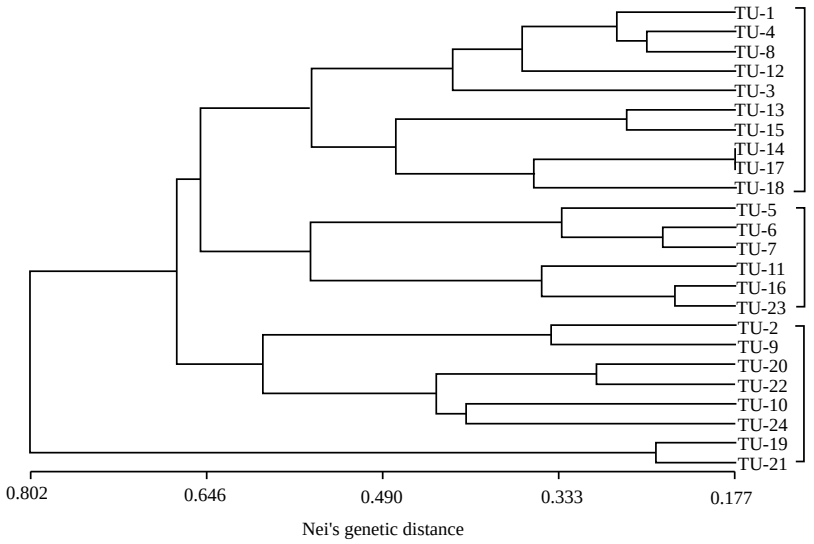

Fig. 3: UPGMA dendrogram representing the genetic relationships among the 24 isolates based on Nei's genetic distance
Brackets represent identified clusters

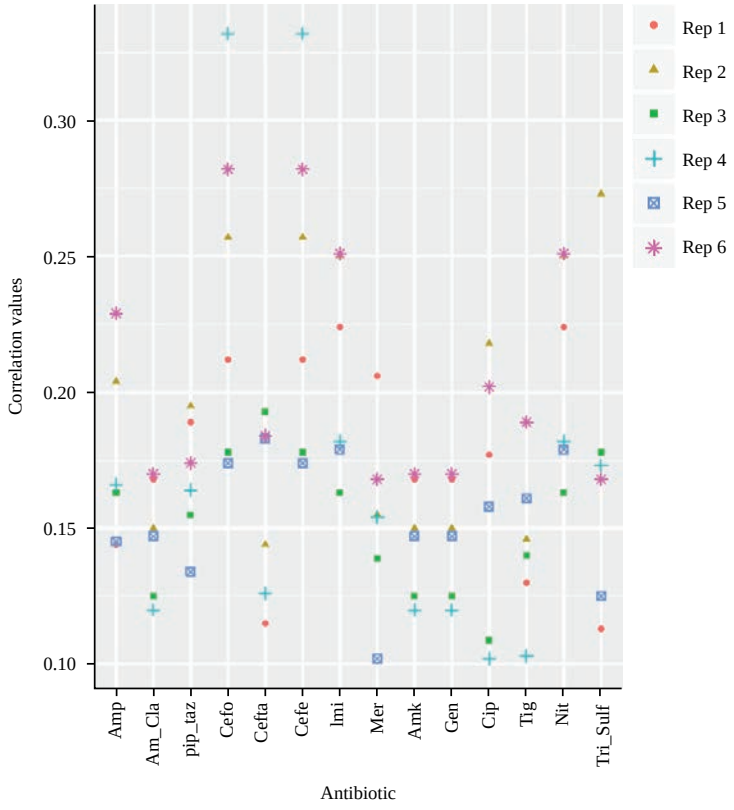

Fig. 4: Correlation between antibiotic profile and rep-PCR profile in 24 UTI isolates

Fig. 6. Surprisingly, the pattern of correlations varied greatly between pairs of antibiotic resistance and virulence genes. That is, no correlation was found between Mar, Amk and Tig antibiotics and all the 8 studied virulence genes. For the rest of the pair combinations, the correlation ranged from low negative correlation to moderate positive correlation. However, all these correlation coefficients were highly significant ($p<0.001$). The lowest negative correlation was

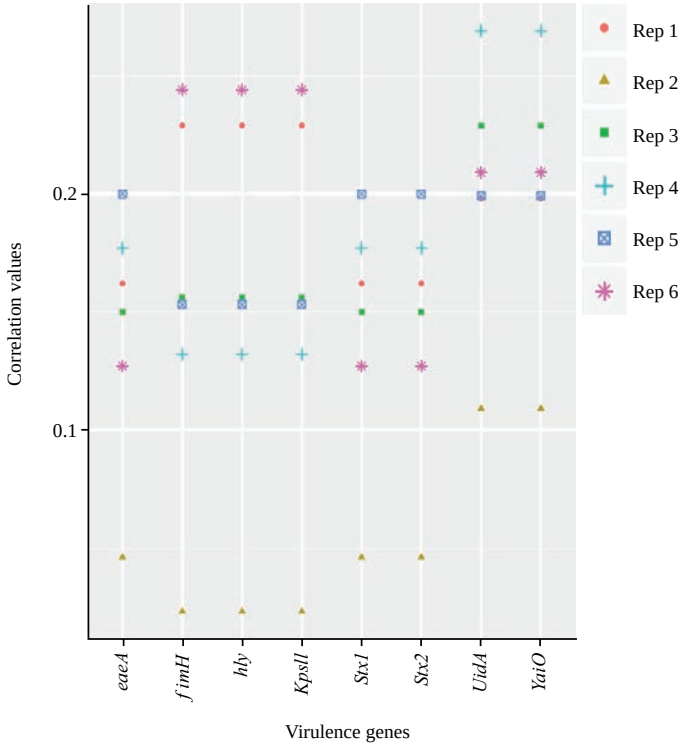

Fig. 5: Correlation between antibiotic resistance gene profile and rep-PCR profile in 24 UTI isolates

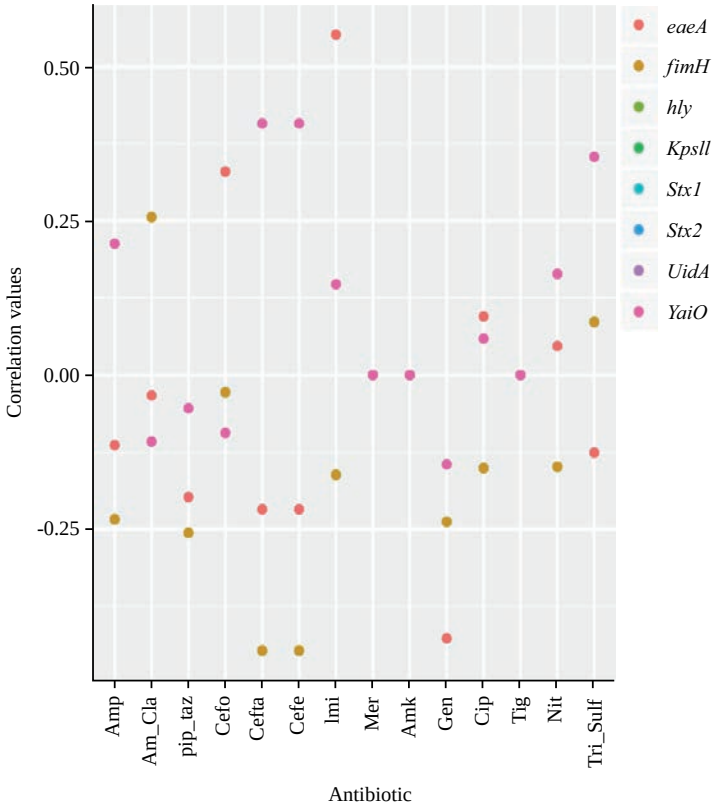

Fig. 6: Correlation between antibiotic resistance gene profile and antibiotic profile in 24 UTI isolates

found between (-0.45) each of the Cefta and Cefe antibiotics and *hly, KpsII* and *himH* virulence genes. The moderate correlations found between Cefta and Cefe antibiotics and each of *the uidA* and *yaiO* virulence genes were highly significant. These results indicate that the same resistance gene in different isolates is not always associated with the same plasmid in these isolates[25]. Finally, the high frequencies of antibiotic-resistant *E. coli* isolates in patients with urinary tract infections in the current study recommend that continuous investigation of the appropriate antibiotics is required for treatment of UTI patients and that control of the infections is essential. The limitations of this study can be concluded in the small number of samples that the study was conducted on as well as the focus of the study on one hospital only.

CONCLUSION

According to this finding, *E. coli* UTI clinical isolates showed high heterogenicity of resistance to antibiotics. PCR genomic fingerprinting based on (GTG)5 and BOX repetitive sequences and PCR detection for *Stx1, fimH, hly and uidA* genes revealed high genetic diversity of the isolates. Thus, these isolates can be circulating simultaneously. Genetic variation of *E. coli* is an important barrier to control public health risk associated with pathogen thereby this diversity should be taken into consideration when designing strategies for controlling *E. coli* outbreaks. Correlation, detected for the first time, between rep-PCR genotyping and antibiotic resistance patterns of *E. coli* could be valuable in the prediction of resistance patterns of *E. coli*

SIGNIFICANCE STATEMENT

This study discovers the possibility and effects of the PCR technique to fast detect antibiotic resistance genes and biotyping of *E. coli* from UTI patients. This study will help the researcher to understand the correlation among antibiotic-resistant genes, antibiotic-resistant profile and rep-PCR technique that many researchers were not able to explore.

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