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Research Article

Molecular and *Insilico* Characterization of Antimicrobial Resistant *Escherichia fergusonii* and *Morganella morganii* causing Urinary Tract Infections

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Abstract: Urinary tract infections (UTIs) are a major public health concern causing mortality and morbidity worldwide. There are approximately 13,000 deaths that are linked to UTIs every year. Bacterial antibiotic resistance is increasing globally, making it difficult to treat urinary tract infections. Biochemical tests have resulted in inaccurate identification, due to which wrong empirical treatment of antibiotics is prescribed. The present research work aims to identify rare bacterial isolates along with the assessment of antibiotic resistance patterns. A total of 339 samples were examined for urine cultures. Based on biochemical testing, three positive samples were selected. Culture sensitivity results revealed two multidrug-resistant and one extensively drug-resistant bacteria. MDR strains showed sensitivity to meropenem, gentamicin, cefoperazone-sulbactam, nitrofurantoin, tigecycline and imipenem, and co-amoxiclav while XDR bacteria showed sensitivity to nitrofurantoin and tigecycline. The 16S rRNA sequencing results revealed two *Escherichia fergusonii* strains and one *Morganella morganii* strain. Our findings revealed the identification of rare and opportunistic bacteria less reported from Pakistan. In addition, it is the first report of *E. fergusonii* from Pakistan. In conclusion, using molecular identification of rare and neglected bacteria via PCR is more sensitive and highly specific than other identification methods. In the future, this approach will help in accurate diagnosis and will help in minimizing antibiotic resistance.

Keywords: Urinary Tract Infections (UTIs), *Escherichia fergusonii*, *Morganella morganii*, Antimicrobial Resistant, Antibiotics

1. INTRODUCTION

The word “urinary tract infection” (UTI) is a common term used extensively, indicating an infection across the urinary tract from the urethral meatus to the perinephric fascia, including ureters, bladder, renal pelvis, urethra, and parenchyma [1]. Various microbial species are the main source that gives rise to UTIs. Still, most of the infections in both inpatient and outpatient populations are caused by gram-negative, uropathogenic, and facultative anaerobic bacteria. *Escherichia coli* is the major and supreme cause of UTIs, a study revealed that 80% of UTIs are caused by uropathogenic *E. coli* in healthy women aged 18 to 39 years [2].

UTIs are the fifth most frequent kind of medical care-associated infection, with approximately 62,700 total UTIs in critical-care hospitals in 2015. In addition, UTI reports are higher than 9.5% of infections reported by critical-care-health centers [3]. Nearly all UTIs (health center-linked) are caused by urinary tract instruments. Relatively, 12 to 16% of hospitalized patients will possess an indwelling urinary catheter throughout their span of hospitalization. The duration of the catheter remained for more than 2 days which leads to a 3 to 7% risk of catheter-acquired urinary tract infection to the patient (CAUTI) [4,5]. Around 0.34% of manifestations occur due to contagious agents causing cystitis, which proliferates and multiplies and further mounts to the kidney through the ureters, where these agents infect calyces, cortex, and pelvis, ultimately leading to the symptoms and

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signs of pyelonephritis [6]. Approximately 27% of the clinical manifestations can be allocated to urinary isolates in patients visiting the emergency department while suffering from sepsis and thus are entitled to urosepsis. In clinical settings, the percentage of sepsis due to UTIs surged to 42% [7,8].

Antimicrobial resistance (AMR) arises because various pathogenic organisms including bacteria, fungi, viruses, and parasites, progress so that drugs of multiple classes do not alter them. Because of resistance, it is complicated or occasionally difficult to cure infections, leading to a high morbidity and mortality rate. There is a global threat due to multi, pan, and extensively drug-resistant bacteria. The resistant bacteria are called superbugs because they carry resistance to current and previous classes of antibiotics [9]. MDR bacteria are insusceptible to multiple regimens, while pan-drug-resistant bacteria are insusceptible to all previously existing antimicrobial drugs.

Similarly, XDR bacteria resist most crucial regimens while sensitive to several regimens [10]. Resistance in bacteria is a major challenge due to less naturally presenting drugs. In contrast, many drugs are synthetic, and it is hard to synthesize novel regimens as opposed to bacteria previously resistant to such antibiotics. This disaster is a consequence of overuse and misuse of these regimens and insufficient doses throughout the recovery course of infection [11].

Numerous antibiotics have been utilized as a wide-range regimen to fight against UTIs, *i.e.,* community and hospital-acquired. However, the persistent antibiotic resistance and the researcher's regard for the function of dependent representative of the host micro-flora have emphasized the crucial demand for regimens or strategies that can particularly recover UTIs without any modifications and changing the gut structure and vaginal micro-flora. In the digestive tract, utilization of antibiotics escalates inflammation, weakens the immune surrounding of the host, and further progresses pathogen proliferation by escalating substrate accessibility for instance, *E. coli* proliferation by escalated nitrate accessibility while survival in the digestive tract is additionally linked with a high risk of recurrent UTI [12,13,14]. Recovery with an antibacterial regimen may hinder the vaginal micro-flora (that protects the surface from pathogens) by disrupting colonization with *lactobacillus spp, w*hich produces peroxides that suppress ascension and colonization [15,16]. Consequently, antibacterial drugs can also be a risk factor for recurrent UTIs due to the contagious effect on vaginal and gut micro-flora [12,17,18]. The study aimed to isolate and identify rare, opportunistic, and neglected XDR and MDR bacteria via biochemical testing and molecular characterization.

2. MATERIALS AND METHODS

2.1. Design and Setting

Patients with UTIs were examined at Khyber Teaching Hospital (KTH), Peshawar. A total of 339 urine culture samples were assessed from July 2021 to October 2021. Morphological analysis, biochemical testing, gram staining, and culture sensitivity of the selected isolates were performed in the microbiology section of KTH. At the same time, a molecular study was subsequently conducted at the Department of Biotechnology Quaid-i-Azam University, Islamabad.

2.2. Inclusion Criteria

Specimens with urease and/or mannose-positive bacterial isolates (n=3) were included in the study.

2.3. Exclusion Criteria

Specimens without urease and/or mannose-positive bacterial strains were excluded from the study.

2.4. Sample Collection and Primary Screening

Urine samples were collected from the patients admitted to various wards and diagnosed with UTIs. Samples were processed, and cultured in the Microbiology laboratory.

2.5. Isolation of Bacteria

A drop of urine from each sample was taken and placed on freshly prepared MacConkey agar plates. The streaking was done using a sterile inoculating loop 3 to 4 times. The plates were labeled and covered to prevent any contamination. The plates

were then incubated at 37 ℃ for 24 hours until the growth of bacterial colonies. The isolated strains were labeled as QAU. 1011, QAU. 1012 and QAU. 1013 respectively.

2.6. Gram Staining

Isolated bacteria were stained to identify the type of bacteria. Initially, a drop of normal saline was added to the microscopic slide. A pure colony was picked, mixed with normal saline, and smeared. The gram staining procedure was then performed. Finally, the slide was observed under the microscope at 1000 X magnification power.

2.7. Biochemical Tests

Biochemical classification was executed following standard protocols. Urea agar, Simmon's citrate agar, triple sugar iron (TSI) agar, and one phenol red mannose broth were prepared. For the urease test, *Proteus vulgaris* (ATCC 13315) and *Escherichia coli* (ATCC 25922) were used as positive and negative controls. Agar slants and broth containing pure bacterial isolates were then incubated at 37 ℃ for 24 h to examine test results.

2.8. Antibiotic Sensitivity Test

Culture sensitivity testing was done to assess the activity of various antibiotics. For this purpose, Muller Hinton agar media was prepared. Pure bacterial isolates were initially streaked on MHA plates. A total of 12 antibiotic discs with various concentrations were used (Table 1) and placed on the agar plates containing pure bacterial isolates. The plates were then incubated at 37 ℃ for 24 h. The next day, plates were examined for antibiotic resistance following the guidelines of the Clinical and Laboratory Standards Institute (CLSI) and Antimicrobial Susceptibility (AST).

2.9. Molecular Characterization

For molecular identification of bacteria, genomic DNA from the bacterial isolates was extracted via the plain boiling method as defined previously [19] with slight modifications.

2.9.1. PCR Amplification

The extracted DNA was amplified with 16S rRNA gene universal primers, i.e., 27F (AGAGTTTGATCCTGGCTCAG) and 1492R (TACGGCTACCTTGTTACGACTT) as described previously [20]. The reaction mixture consisted of 3 µl of template DNA, 1.5 µl forward primer, 1.5 µl reverse primer 10 µl of 2X GoTaq® Green Master Mix, and 4 µl PCR water (total reaction volume = 20 µl). PCR (peqSTAR 96x gradient-VWR) amplification conditions were: 95 ˚C for 3 min; 35 cycles of 95 ˚C for 30 s, 54 ˚C for 30 s, and 72 ˚C for 60 s; and a final extension of 72 ˚C for 15 min before holding at 8 ˚C.

After the PCR reaction, the amplified DNA products were tested on 1.5% gel. A ladder of 1kb was used to compare the product sizes (GeneRuler

Table 1. Concentrations of various antibiotics utilized for culture sensitivity testing.

S. No.	Antibiotics discs	Concentrations (µg)
1.	Meropenem (MEM)	10
2.	Ceftazidime (CFM)	5
3.	Azithromycin (ATM)	30
4.	Ciprofloxacin (CIP)	5
5.	Cefepime (FEP)	30
6.	Co-trimoxazole (CTX)	30
7.	Imipenem (IPM)	25
8.	Gentamicin (GN)	120
9.	Cefoperazone-sulbactam (SCF)	105
10.	Nitrofurantoin (NA)	30
11.	Tigecycline (TGC)	15
12.	Co-amoxiclav	25

DNA ladder 1kb, Fermentas). The amplified products were then eluted and were checked on 1.5% agarose gel to confirm the amplification before sequencing.

2.10. DNA Sequencing

The amplified products were sequenced via Sanger sequencing (Macrogen Inc., Korea).

2.11. Bioinformatics Analysis

Consequently, the sequences obtained after Sanger sequencing were analyzed using BLAST (Basic Local Alignment Search Tool) from the National Centre for Biotechnology Institute (NCBI) (www.ncbi.nlm.nih.gov/BLAST). Similarly, sequences were aligned using the clustal W program, phylogenetic tree construction was done via the Neighbor-Joining method of MEGA-X with a bootstrap value of 0.010 [21], and were submitted to the NCBI gene bank.

3. RESULTS

In this research study, three urine samples were collected from various patients with UTI from care units of KTH Peshawar, based on gender distribution. Of the three samples, 2 were females, and one was male (Table 2).

Table 2. Patient's Attributions.

S. No.	Isolate name	Age	Gender
1.	QAU. 1011 (*Escherichia fergusonii*)	8 months	Girl (child)
2.	QAU. 1012 (*Escherichia fergusonii*)	10 months	Girl (child)
3.	QAU. 1013 (*Moranella morganii*)	19 years	Male

3.1. Morphology and Gram Staining

After the incubation period, the MacConkey agar plates were checked in a sterile environment. All the bacterial isolates appeared colorless on media plates which revealed the isolates as non-lactose fermenting bacteria (Figure 1 a,b,c). Similarly, gram staining results of isolated bacterial strains under microscopic with resolution (1000X) depicted that all isolates were gram-negative bacteria as the colonies for all the isolates appeared pink in color (Figure 1d).

3.2. Biochemical Tests

After gram staining, the morphology observation on MacConkey agar plates, bacterial morphology was identified through various biochemical tests to differentiate desired bacteria from other gram-negative rods and bacilli bacteria. Two bacterial strains were urease negative as no color change was observed after incubation, indicating the bacteria were within the *Escherichia* family. At the same time, one isolate was urease positive as a positive reaction was observed after incubation (pink color indication) depicting the non-*Escherichia* family. The results were compared with the positive and negative controls. Similarly, the TSI test for two isolates revealed a change in the slant color depicted sugar fermentation with color change.

In contrast, no change occurred in the control (TSI without inoculated colony). At the same time, 1 sample revealed no sugar fermentation, as no color change was observed after incubation for both the inoculated colony and control. Citrate for all the isolated bacterial strains was negative, as no color change was observed after incubation. The mannose fermentation test revealed one isolated mannose fermented bacteria depicting *Morganella* species as the culture changes its color after incubation. In contrast, 2 strains showed mannose non-fermented bacteria indicating *Escherichia* species with no color change in the medium (Table 3).

3.3. Antibiotic Sensitivity

Out of 3 specimens, 2 species (*E. fergusonii* and *Morganella morganii*) were found to be multidrug-resistant (MDR), while 1 sample (*E. fergusonii*) was extensively drug-resistant (XDR) bacteria. Of the 2 MDR strains, the first showed sensitivity to meropenem, gentamicin, cefoperazone-sulbactam, nitrofurantoin, tigecycline, and imipenem while resistant to all other regimens. Similarly, the second revealed sensitivity to meropenem, nitrofurantoin, tigecycline, co-amoxiclav, and imipenem, while the XDR strain revealed sensitivity to nitrofurantoin and tigecycline as detailed in (Figure 2 and Table 4).

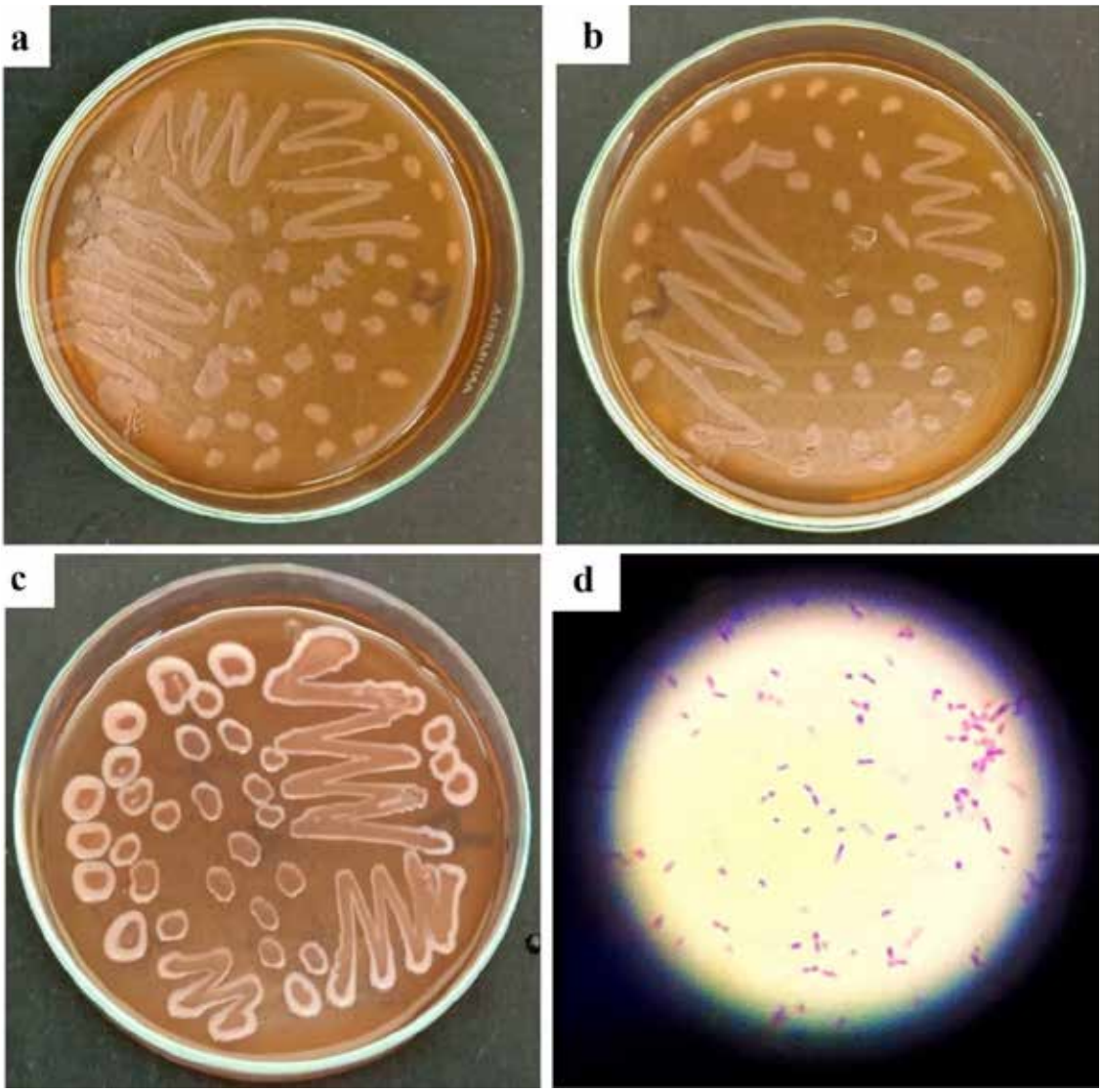

Fig. 1a. Image of pure bacterial isolate QAU.1011 of the urine sample. **b.** Pure bacterial isolate QAU.1012. **c.** Isolate QAU.1013. The colorless colonies of bacterial isolates indicate non-lactose fermenting bacteria. **d.** Small rod-shaped, pink-colored, and gram-negative bacteria under the light microscope (with a resolution of 1000X).

Table 3. Biochemical tests indicating positive and negative results.

S. No.	Biochemical tests	Results		
		Escherichia fergusonii **(QAU. 1011)**	***Escherichia fergusonii*** **(QAU. 1012)**	***Morganella morganii*** **(QAU. 1013)**
1.	Triple sugar iron (TSI)	+	+	-
2.	Urease	-	-	+
3.	Citrate	-	-	-
4.	Swarming	-	-	-
5.	Mannose	-	-	+

3.4. Molecular identification

The amplified product size was approximately 1500bp for all the samples, as shown in (Figure 3). After PCR product purification and sequencing, the isolate QAU. 1011 and isolate QAU. 1012 were identified as *E. fergusonii* with accession No. ON076882 and OP810623, and the isolate QAU.

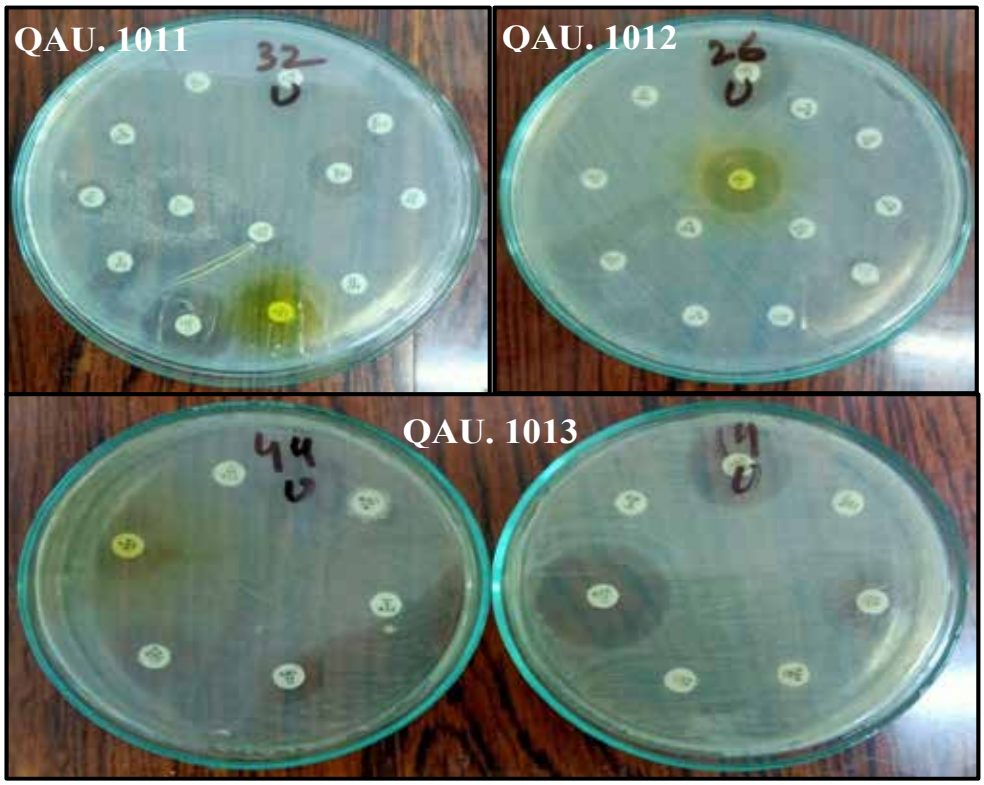

Fig. 2. Culture sensitivity test results of bacterial isolates from patients depicting sensitivity to some while resistance to several antibiotics. QAU. 1011 and 1012 are *E. fergusonii,* while QAU. 1013 is *Morganella morganii*.

Table 4. Drug profile of isolates showing resistance and sensitivity pattern.

S. No.	Antibiotics	Isolates		
		QAU. 1011 (*Escherichia fergusonii*)	**QAU. 1012** (*Escherichia fergusonii*)	**QAU. 1013** (*Morganella morganii*)
1.	Meropenem (MEM)	S	R	S
2.	Ceftazidime (CFM)	R	R	R
3.	Azithromycin (ATM)	R	R	R
4.	Ciprofloxacin (CIP)	R	R	R
5.	Cefepime (FEP)	R	R	R
6.	Co-trimoxazole (CTX)	R	R	R
7.	Gentamicin (GN)	R	R	S
8.	Cefoperazone-sulbactam (SCF)	R	R	S
9.	Nitrofurantoin (NA)	S	S	S
10.	Tigecycline (TGC)	S	S	S
11.	Co-amoxiclav	S	R	R
12.	Imipenem (IPM)	S	R	S

Abbreviations: S: Sensitive, R: Resistant.

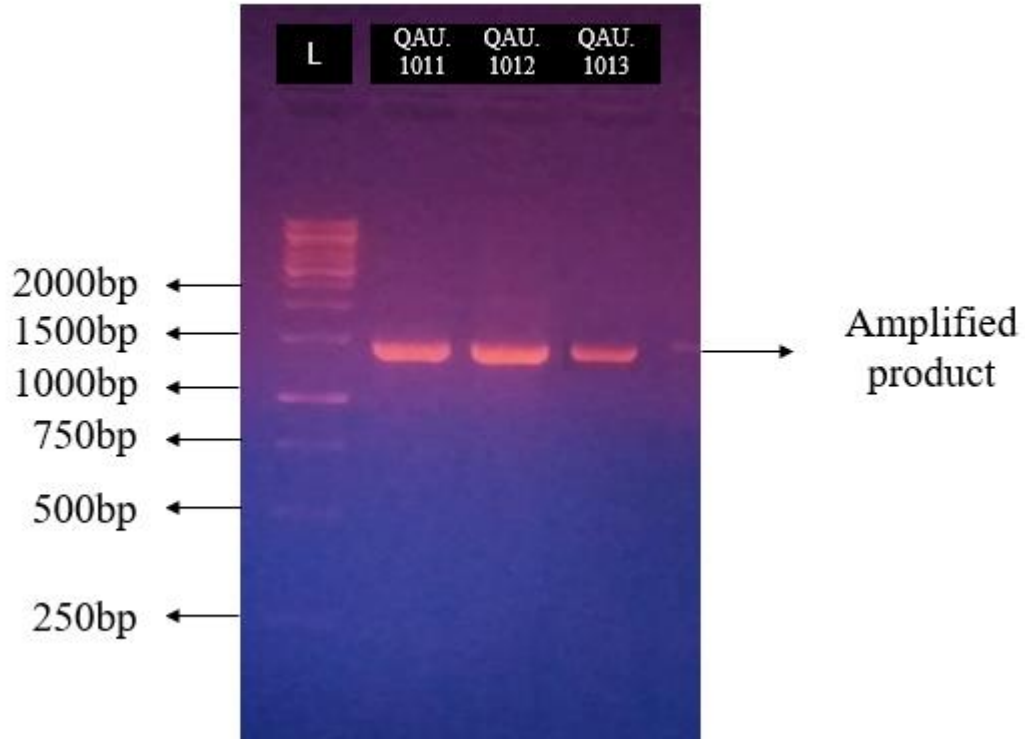

Fig. 3. Agarose gel electrophoresis (1.5% agarose) of Amplified PCR products via universal primer sets. Lane 1-3 depicts the sample strains *(Escherichia fergusonii* and *Morganella morganii). Lane L illustrates the DNA ladder of 1kb.*

1013 was identified as *M. morganii* with accession No. OP810942 after submitted to NCBI gene bank.

3.5. Phylogenetic Analysis

Partial sequences (16S rDNA) genes of the genus were recognized. The obtained sequences were aligned with the genera i.e., *Escherichia, Shigella, Salmonella, Kosakonia, Citrobacter, Morganella, Providencia,* and *Proteus*. Based on BLAST results, isolate QAU. 1011 and QAU. 1012 were closely related to *Escherichia species* with 99% similarity. The genus *Escherichia* formed a distinct clade with the highest sequence similarity found among *E. fergusonii*. In contrast, QAU. 1013 was closely associated with *Morganella species* with a similarity index of 97%. The genus *Morganella* arose from the similar clade of *M. morganii* depicting higher similarity with the strain of Japan. Based on phylogenetic analysis, the isolate QAU. 1011 (*E.scherichia fergusonii* with accession No. ON076882) and QAU. 1012 (*E. fergusonii* with accession No. OP810623) were genetically related to *E. fergusonii* strain ATCC 35469 of Canada (Accession No. NR 027549) with 98% maximum identity. The sublines were determined in the *Escherichia* clade, both included *E. fergusonii* (Figure 4). Similarly, isolate QAU. 1013 (*M. morganii* with accession No. OP810942) was closely related to *Morganella morganii* strain JCM1672 (Accession No. NR112191) belonging to Japan. The subline was determined in *Morganella* clade included *M. morganii*. The clade depicts the evolutionary bacteria associated with the strain of *M. morganii* with 86% maximum identity (Figure 5).

4. DISCUSSION

UTIs are one of the extreme and usual causes of bacterial infections, influencing approximately 150 million individuals worldwide yearly and establishing greater than six billion dollars in immediate medical management costs [22]. Resistance against antibiotics has emerged with destructive effects on useful microflora of the host via antibiotic use, revealing a deficiency in the treatment for UTIs [23]. The existing impact of bacteria causing urinary tract infections is a major challenge to evaluate due to the misdiagnosis of bacteria with other gram-negative bacterial isolates. At the same time, identification can be done via

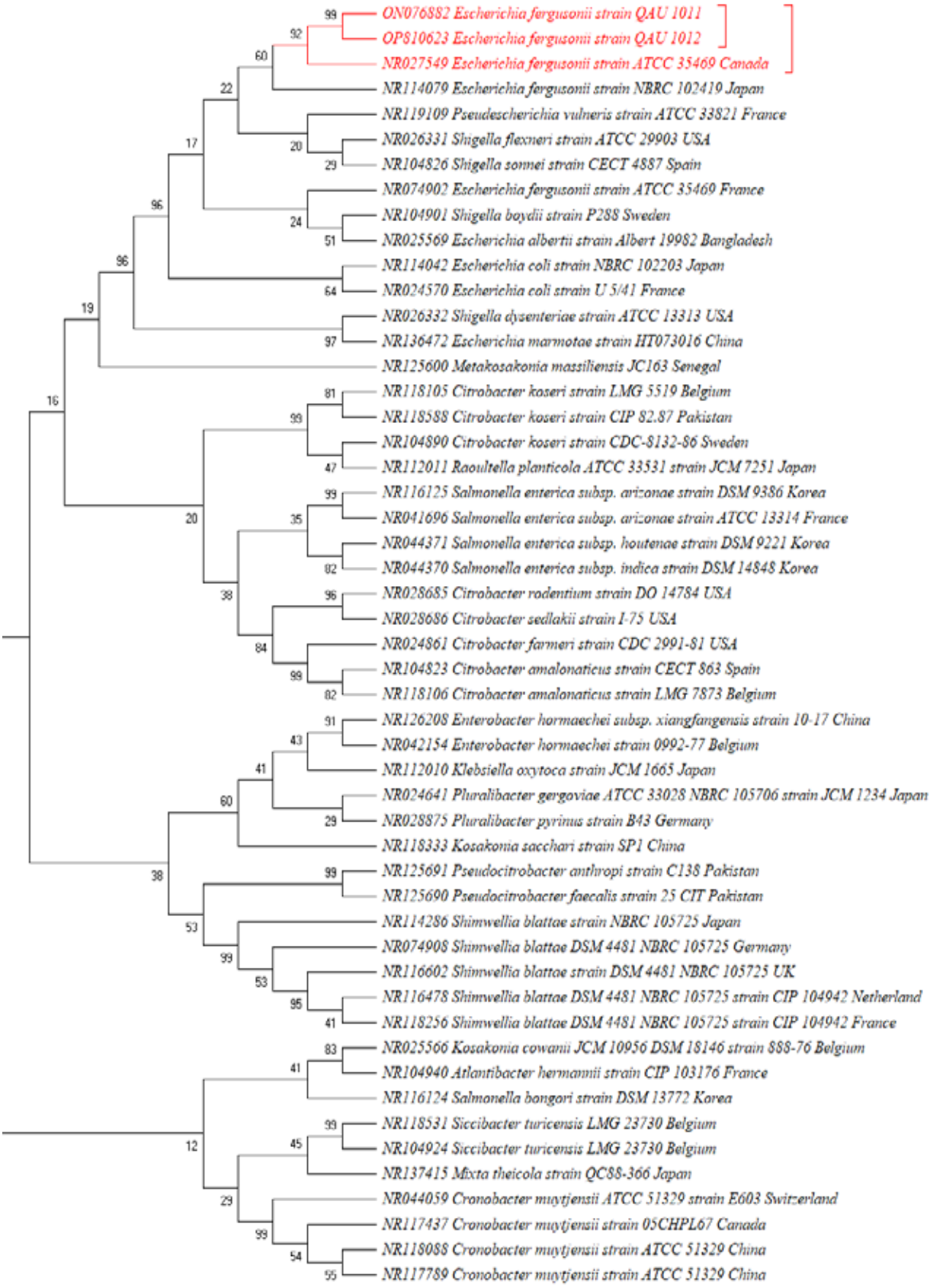

Fig. 4. Phylogenetic relationship of isolate QAU. 1011 and QAU 1012 with the closely related strains of Escherichia fergusonii with a bootstrap value of 0.010.

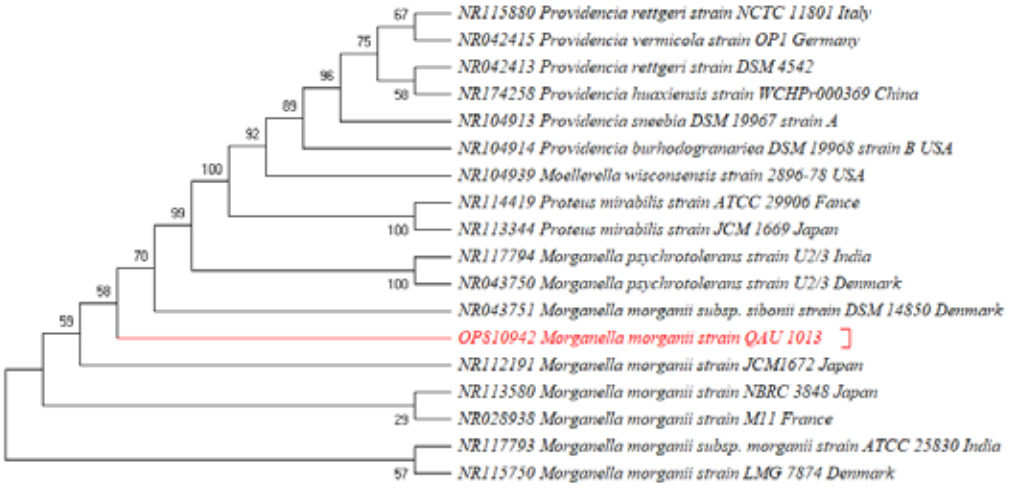

Fig. 5. Phylogenetic relationship of isolate QAU. 1013 with the closely related strains of *Morganella morganii* with a bootstrap value of 0.010.

biochemical and molecular detection using PCR assay [24].

The PCR-based assay is highly specific and sensitive for the detection of isolates compared to biochemical testing. Moreover, phenotypic identification using various automatized equipment or biochemical tests may lead to false identification [25]. The molecular method for the identification of bacteria remains the best and most appropriate approach. *E. coli* with respected representatives, i.e., *E. Alberti* and *E. fergusonii* were accurately distinguished from *E. coli* based on specific primers to certain genes in both strains. PCR results with specific primers revealed accurate identification of both strains. Similarly, beta-lactam genes, i.e., antibiotic resistance strains, can be identified accurately via molecular approach as the previous study demonstrated that various isolates of bacteria producing extended-spectrum beta-lactamase (ESBL) were detected using 16S rRNA gene sequencing [26, 27]. In contrast to these studies, our experiment revealed false identification of the strains via biochemical tests, while results of 16S rRNA gene sequencing and phylogenetic analysis of all the isolates revealed the strains as *E. fergusonii* and *M. morganii* indicating PCR-based assay (16S rDNA) as highly specific and sensitive.

Resistance to antibiotics and drug regimens is progressive and constantly emerging in healthcare settings [28]. UTI-causing bacterial isolates, specifically *E. coli* contribute to total infections of 80% with resistance to first-line antibiotics and other regimens such as nitrofurantoin, ciprofloxacin, trimethoprim and trimethoprim-sulfamethoxazole (TMP-SMX) in both community and hospital-acquired clinical-care settings. Similarly, urease-positive bacterial isolates previously showed resistance against nitrofurantoin, nalidixic acid, ceftriaxone, and cefotaxime while revealing sensitivity to gentamycin, norfloxacin, ciprofloxacin, vancomycin, amikacin, ciprofloxacin, and imipenem [29,30]. Another study demonstrated the susceptibility pattern of XDR and MDR gram-negative isolates in kidney transplant patients with UTI. Results revealed a total of 88 events of gram-negative XDR and MDR bacteria. All MDR and XDR isolated bacteria resisted first-generation and second-generation beta-lactam (monocyclic) and cephalosporin. The sensitivity pattern of all isolated bacteria was observed for amikacin, tigecycline, and meropenem. Among them, 12 XDR bacteria showed meropenem resistance, while tigecycline resistance was up to 25%. Among all the XDR isolated strains *E. coli* and *Acinetobacter baumannii* were tigecycline sensitive [31]. Our findings revealed all the isolates as urease and mannose-positive bacteria, in which 2 were MDR strains while one isolate was XDR. MDR strains showed sensitivity to meropenem, gentamicin, cefoperazone-sulbactam, nitrofurantoin, tigecycline, imipenem, and co-amoxiclav while resistant to ceftazidine, azithromycin, ciprofloxacin, cefepime and co-trimoxazole. Similarly, the XDR strain showed sensitivity to nitrofurantoin and tigecycline while

resistant to meropenem, gentamicin, cefoperazone-sulbactam, imipenem, co-amoxiclav, ceftazidine, azithromycin, ciprofloxacin, cefepime and co-trimoxazole respectively.

PCR-based assays and phylogenetic analysis previously revealed that UTI-causing bacteria are characterized by the genus *Escherichia, Streptococci, Enterobacter, proteus, and Morganella* [32]. The phylogenetic analysis previously revealed 98% similarity with *E. fergusonii* when isolated from dairy cattle causing diarrhea [33]. It was depicted in *Escherichia* clade as it was similar to *E.coli*. In contrast to the study, our finding revealed that the clades depicted *Escherichia* while the sublines indicated *E. fergusonii*. Similarly, another MDR strain revealed 98% similarity with *Morganella* and the subline depicted *M. morganii*. To the best of our knowledge, it is the first study that reported *E. fergusonii* as a UTI-causing bacteria in Pakistan with a high ratio of antibiotic resistance.

5. CONCLUSIONS

Escherichia fergusonii and *Morganella morganii* are rare bacteria with a high resistance rate. It is the first study that reported *E. fergusonii* as a UTI-causing bacteria for the first time from Pakistan. Our study concluded that these neglected and rare bacteria can pose major challenges at the lateral stage. Similarly, molecular diagnostic approaches are mandatory to overcome the problem of misdiagnosis. Further, rare antibiotic-resistant organisms should not be overlooked since these bacterial infections can lead to several morbidities.

6. ACKNOWLEDGMENTS

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7. CONFLICT OF INTEREST

The authors declared no conflict of interest.

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