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THE CHARACTERIZATION OF MULTIDRUG RESISTANT TYPE 1 S-FIMBRIATED *ESCHERICHIA COLI* FROM WOMEN WITH RECURRENT URINARY TRACT INFECTIONS (RUTIS) IN BANGLADESH

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The characterization of multidrug resistant type 1 S-fimbriated *Escherichia coli* from women with recurrent urinary tract infections (RUTIs) in Bangladesh

ABSTRACT

Background: *Escherichia coli* is a bacterial species that is most frequently associated with urinary tract infections (UTIs) worldwide. Recurrent UTIs (RUTIs) pose a major threat to health, especially in women. However, studies on the pathogenic potentials of *E. coli* isolates associated with RUTIs has yet to be done. The present study was designed to characterize the isolates of *E. coli* from women, suffering from repeated UTIs in Bangladesh.

Methods: A total of 15 isolates of *E. coli*, collected from women with RUTIs in the Sylhet city of Bangladesh, were analyzed by a series of biochemical and molecular tests.

Results: The PCR amplification of the mannose-resistant type 1 S-fimbriae gene (*sfa1*) confirmed all *sfa1*$^+$ isolates of *E. coli*. In an antibiogram assay, all the isolates of *E. coli* were found to be completely resistant to at least five of the ten commercial antibiotics and drug resistance were found to be correlated with the weight of the RUTIs patient. Analysis of the 16S rRNA data of the two most drug-resistant and clinically significant isolates were found to be clustered with the drug-resistant UTI-causing *E. coli* isolates, circulated in India.

Conclusions: This study could pave the way of RUTIs diagnosis and treatment strategy for women in Bangladesh.

Keywords: RUTIs; *E. coli*; *sfa1*; multidrug-resistant isolates; 16S rRNA gene sequencing; correlation analysis.

LA CARACTERISATION DES ESCHERICHIA COLI MULTIRESISTANTES DE TYPE 1 S-FIMBRIEES CHEZ DES FEMMES AYANT DES INFECTIONS URINAIRES RECIDIVANTES (IUTR) AU BANGLADESH

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ABSTRAIT

Contexte: *Escherichia coli* est une espèce bactérienne associée le plus fréquemment aux infections urinaires dans le monde entier. Les infections urinaires récurrentes (IVU) constituent une menace majeure pour la santé, en particulier chez les femmes. Cependant, les études sur le potentiel pathogène des isolats d'E. Coli associés aux RUTI n'ont pas encore été réalisées. La présente étude visait à caractériser les isolats d'E. Coli provenant de femmes souffrant d'infections urinaires répétées au Bangladesh.

Méthodes: Au total, 15 isolats d'E. Coli, prélevés chez des femmes atteintes de RUTI dans la ville de Sylhet au Bangladesh, ont été analysés par une série de tests biochimiques et moléculaires.

Résultats: L'amplification par PCR du gène S-fimbriae résistant au mannose (sfa1) a confirmé tous les isolats de sfa1 + d'E. Coli. Dans un essai d'antibiogramme, tous les isolats d'E. Coli se sont révélés complètement résistants à au moins cinq des dix antibiotiques commerciaux et la résistance aux médicaments s'est avérée être corrélée au poids du patient RUTI. L'analyse des données de l'ARNr 16S des deux isolats les plus résistants aux médicaments et cliniquement significatifs s'est avérée être regroupée avec les isolats d'E. Coli causant des infections urinaires résistantes aux médicaments, diffusés en Inde. Conclusions: Cette étude pourrait ouvrir la voie au diagnostic et à la stratégie de traitement des RUTI chez les femmes au Bangladesh.

Mots-clés: RUTIs; *E. coli*; *sfa*1; des isolats multirésistants; Séquençage du gène de l'ARNr 16S; analyse de corrélation.

INTRODUCTION

Urinary tract infections (UTIs) are the systemic bacterial infections that are known to affect the urethra, urinary bladder, and kidneys. Females are mostly infected due to their anatomical arrangement- a shorter urethra, resulting in an easier travel by the bacteria (1). Previous studies have revealed that around 50–60% of women are likely to develop UTIs in their lifetime (2). The numerous causative agents, responsible for this disease; however, *E. coli* alone accounts for 80–85% of the global UTIs (3, 4). Recurrent urinary tract infections (RUTIs) are the reinfections that are generally caused by the original bacterial isolates at a young age. Frequent sexual intercourse increases the chance of getting RUTIs (5). Approximately one third of women are found to be positive for RUTIs by the same bacteria (6, 7). In some cases, RUTIs can be lethal when the bacteria persist for a long time in a position (5).

Drug resistance in *E. coli* is one of the most common barriers for treating UTI patients worldwide. However, the problem is more severe in countries like Bangladesh due to an improper tendency of frequently prescribing antibiotics for the treatment of UTIs (8). The multidrug-resistant strains of *E. coli* have been reported to further add to the complications in the UTI patients and decrease the effectiveness of the treatment (9, 10). In addition, patients with RUTIs have been reported to have a higher prevalence of antimicrobial resistance due to the evolution and spread of more virulent strains by various genetic mechanisms (11). The frequency of RUTIs caused by the multidrug resistant strains of *E. coli* has increased recently and has sparked strong attention from the government, medical practitioners, and health agencies (12). Therefore, regional studies on the patterns of antibiotic sensitivity are much needed for selecting proper treatment strategies to overcome the massive problem of drug resistance.

Virulence factors play a major role in the pathogenicity of *E.coli* associated RUTI infections. The S- fimbriae adhesin (*sfa*) genes, encoded by sfa operon are common in all the types of UTIs found to be strongly associated with *E.coli* pathogenicity (13). The *sfa* gene is associated with UTIs, gestational pyelonephritis, recurring cystitis, pregnancy complications, and diarrhea (13, 14). The isolates of *E. coli*, especially from the uropathogenic (UPEC) and the diffusely adhering (DAEC) groups are known to produce this virulence factor that mediates the host-pathogen interactions (13, 15).

Recent development in molecular techniques, especially the 16S rRNA gene sequencing tool has been extensively used for the analysis of bacterial species in clinical samples (16, 17). In addition, the introduction of computer-aided bioinformatics tools in sequence analysis has simplified the understanding of the strains that are poorly characterized and rarely isolated. Therefore, the aim of the present study was the characterization and phylogenetic positioning of the isolates of *E. coli* from women with RUTIs, by using the

computer-aided bioinformatic analysis of their 16S rRNA gene sequences.

MATERIALS AND METHODS

Ethics statement

This work has been conducted in accordance with "The Code of Ethics of the World Medical Association". The Graduate Research Ethics Committee (GREC) of the School of Life Sciences, Shahjalal University of Science and Technology, Sylhet 3114, Bangladesh, approved and monitored the study. The patients consent data were handled according to human privacy rights.

The collection and culture of the bacterial isolates

A total of 15 isolates of *E. coli* (E1-E15) were collected from three different hospitals: the Popular Medical Diagnostic Centre (PMDC), Sylhet, Bangladesh, the Jalalabad Ragib-Rabeya Medical College and Hospital (JRRMCH), Sylhet, Bangladesh, and the MAG Osmani Medical College and Hospital (MOMCH), Sylhet, Bangladesh from January, 2017 to December, 2017. After screening the patient data, samples were collected from only those women, who were having a history of RUTIs. In addition, other information about the patients, including patient ID (PID), age, and the types of infection were also recorded from the patient consent forms (**Table 1**). Primarily, all the patient samples were inoculated onto a chromogenic medium, containing Eosin Methylene Blue (EMB) and incubated at 37 °C for 24 h at the Biochemistry and Microbiology laboratory of PMDC, Sylhet, Bangladesh. The samples were then transported, at a low temperature, to the laboratory for further studies.

The biochemical characterizations

All the isolates of *E. coli* were subjected to several biochemical and microbiological tests, following the Bergey's manual for the presumptive identification of *E. coli* (18). The isolates were assayed for Gram staining, catalase test, oxidase test, oxidative-fermentative (OF) test, H_2S production test, Methyl Red (MR) test, Voges-Proskauer (VP) test, citrate test, urease test, gelatin test, gas production test, etc. Following these biochemical tests, the positive isolates of *E. coli* were preserved and cultured for further identifications through the PCR technique.

The extraction and quantification of genomic DNA

Using a commercial bacterial genomic DNA extraction kit (Bio Basic Inc., Markham, Ontario, Canada), genomic DNA from the isolates of *E. coli* was extracted. Following the manufacturer's instructions, Proteinase K and RNase A were added to remove impurities from the DNA samples. Using a lambda (λ) DNA molecular weight marker, the quantification of the extracted genomic DNA samples was done on an agarose gel and considering protein-DNA absorbance recorded using NanoDrop UV-Visible measurements for nucleic acid (ThermoFisher Scientific, 2000c). The DNA was then diluted accordingly to make the final concentration 30 ng/µl. The extracted genomic DNA samples were then preserved in an ultrafreezer at –20° C for further use.

The amplification of the *sfa* gene

PCR amplification was performed in 50 µL reaction mixtures, containing 25 µL of the prepared 2X master mixtures (Fermentas, GeneRuler™, USA), 2.5 µL of each of the forward and reverse primers (*sfa*F, 5'-CTCCGGAGAACTGGGTGCATCTTAC–3' and *sfa*R 5'- CGGAGGAGTAATTACAAACCTGGCA–3'), 5 µL of the template DNA (100 ng), and 15 µL of nuclease-free water. The PCR conditions were optimized as follows: an initial denaturation step at 94 °C for 4 min, a denaturation step at 94 °C for 1 min, an annealing step at 65 °C for 1 min, an extension period at 72 °C for 1.5 min, and a final extension step at 72 °C for 10 min, followed by storage at 4 °C. A total of 35 serial cycles of the amplification reaction were performed in a MultiGene Gradient Thermal Cycler (Labnet International Inc., USA). The PCR products were separated by electrophoresis on an agarose gel, stained with ethidium bromide solution, and visualized in a gel documentation system(8).

The antibiogram profiles of the isolates of *E. coli*

The antibiotic profiles of the *E. coli* isolates against ten commercial antibiotic discs were determined by the Kirby-Bauer Disc Diffusion Method (19). An aliquot of 30 µL of overnight bacterial cultures (10^6 CFU/mL) was spread on Tryptic Soy Agar plates (Micromaster Laboratories Pvt. Ltd., Maharashtra, India) and the antibiotic discs were placed aseptically onto the culture media, in order to observe the antibiogram profiles of all the isolates of *E. coli*. The antibiotic discs, used in this study were ampicillin (10 µg/disk), cotrimoxazole (10 µg/disk), tetracycline (30 µg/disk), erythromycin (15 µg/disk), ciprofloxacin (30 µg/disk), cephradine (25 µg/disk), gentamicin (10 µg/disk), streptomycin (10 µg/disk),

chloramphenicol (10 µg/disk), and sulfamethoxazole (25 µg/disk). After 24 h of incubation at 37 °C, the zones of inhibition were measured (8).

The amplification and sequencing of the16S rRNA gene

The amplification of the target sequences of the 16S rRNA gene specific for the isolates of *E. coli* was carried out following the standard method (20). The PCR reaction mixture was prepared in the same concentration as that used for the amplification of the *sfa* gene with 2.5 µL of each of the universal primers, 27F and 1492R (5′-AGAGTTTGATCCTGGCTCAG–3′ and 5′-GGATACCTTGTTACGACTT–3′). For this reaction, a total of 30 serial cycles were programmed and the PCR parameters were adjusted like the previous amplification reaction. The amplified full-length PCR products, having a size of 1465 bp were visualized in an agarose gel and purified with the PureLink PCR Purification Kit (Thermo Scientific, Waltham, MA, USA), according to the manufacturer's instructions. The purified 16S rRNA PCR products were then sequenced from the "1st BASE" sequencing center, Malaysia. The raw sequences were extracted, edited, assembled, and run for further analysis, using the various sequence analysis tools (16).

Analysis of the 16S rRNA gene sequence data and the status of phylogenetic relationship

According to the antibiogram assay, the two most multidrug-resistant isolates of *E. coli*, E8 and E10—also associated with other complications in the patients such as cystitis, hypertension, and pregnancy—were selected for the universal 16S rRNA gene sequencing to analyze their phylogenetic positions. After sequencing, the raw sequences were extracted and assembled using the BioEdit (v7.0.4), Chromas (v2.01), and SeqMan Pro (v15.0) sequence editing tools. The web-based database, DECIPHER (v9.20), was used to remove the chimeras from the raw sequences. Using BLASTn in the NCBI database, the edited DNA sequences were then run for the homology matching of the highly similar sequences. Using the ClustalW program in MEGA (v7.0), the assembled sequences were aligned and using the nearest neighbor method with 1,000 bootstrap replicates, a phylogenetic tree was constructed. The GenBank accession numbers of the similar sequences were indicated in parenthesis to most of the *E. coli* species. The evolutionary divergence among the sequences was calculated as the Kimura J-Model in MEGA (v7.0).

Statistical analysis

Data collected from RUTI patients were plotted in corrplot (v0.84) for finding of graphical correlations among variables in RStudio of R package. A total of 15 observations from four variables were tested for Pearson(s) correlation ($p<0.5$), structured in matrix (mat) form, then mixed (corrplot.mixed) and finally visualized using color method where blue signified positive correlations and red demonstrated negative correlations.

RESULTS

The biochemical characterization of the isolates of *E. coli*

Although all the isolates were initially supplied as *E. coli* in the EMB agar medium, the specific details of the morphological and biochemical characterizations confirmed that all of them belonged to the same species. The results showed isolates were positive for catalase, indole, methyl-red, and motility tests while the alpha-hemolysin producing assay exhibited partial lysis of the sheep blood agar and appeared greenish in color following overnight incubation. The isolates were negative for Gram's test, oxidase, voges-proskauer tests; couldn't utilize citrate, urease, gelatin and H_2S for production of gas.

The amplification of the *sfa* gene in the isolates of *E. coli* by PCR

In the PCR amplification, all the 15 *E. coli* isolates were positive for the *sfa* gene and gave an expected product size of 410 bp upon visualization on the agarose gel. The results of the PCR amplification of the *sfa* gene in the isolates of *E. coli*, collected from RUTIs women are shown in **Figure 1**.

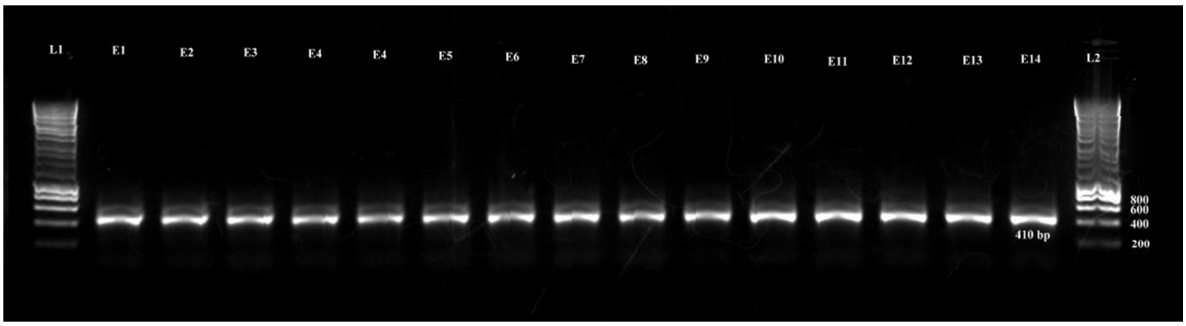

FIGURE 1: PCR AMPLIFICATION OF *AFA*1 GENES IN *E. COLI* ISOLATES. ALL OF THE ISOLATES WERE POSITIVE FOR *SFA1* AND SHOWED 410 BP PCR PRODUCTS ON AGAROSE GEL.

The antibiotic sensitivities of the isolates of *E. coli*

Among the ten antibiotics tested by the Kirby-Bauer Disc Diffusion Assay, all the isolates of *E. coli* were completely resistant to ampicillin (AMP), erythromycin (ERY), streptomycin (STP), sulfamethoxazole (SXT), and tetracycline (TET). Out of 15 isolates, five (E2, E5, E8, E9, E10) were resistant to gentamicin (GEN) and cephradine (CEP) and two (E8, E10) displayed resistance to cotrimoxazole (COT). The isolate E8 showed sensitivity to only ciprofloxacin (CIP) and E10 was inhibited by chloramphenicol (CPL) and ciprofloxacin (CIP).

TABLE 1: LIST OF SAMPLES AND INFORMATION

E. coli isolates	Age	No. of antibiotic resistance (out of 15)	Weight	Sources
E1	37	6	56	1*
E2	40	7	48	2†
E3	34	5	52	3
E4	40	6	58	1*
E5	35	7	50	1*
E6	33	6	54	2†
E7	38	6	49	2†
E8	35	9	62	1*
E9	43	7	58	3
E10	33	8	60	2†
E11	36	6	52	1*
E12	42	6	54	1*
E13	45	6	53	2†
E14	48	5	50	2†
E15	40	6	56	3

Footnotes: *Popular Medical and Diagnostic Centre, Sylhet, Bangladesh; †Jalalabad Ragib-Rabeya Medical College and Hospital, Sylhet, Bangladesh; MAG Osmani Medical College and Hospital, Sylhet, Bangladesh.

The amplification of the universal 16S rRNA gene sequences

The genomic DNA of the isolates of *E. coli* was used to amplify the universal 16S rRNA gene sequences by PCR. The multidrug resistant and complications-associated isolates, E8 and E10, were amplified by the universal amplification primers, 27F and 1492R. Both the isolates gave positive amplifications and displayed an expected product size of 1465 bp on the agarose gel.

Analysis of the 16S rRNA gene sequences of the multidrug-resistant isolates of *E. coli*

In the NCBI database, BLASTn of the assembled sequences of the *E. coli* isolates, E8 and E10, showed 99.0% homology to the sequences of the *E. coli* isolates, extracted from clinical samples. The evolutionary divergence score revealed no significant distances (0.000) among the isolates in the present study and the two most recent UTI-causing strains of *E. coli* (IBB1 and U744) are circulating in India since August 2017. However, evolutionary distances were found to exist among the study isolates and the other strains, previously circulating in countries such as Pakistan and the USA (0.001–0.002). The phylogenetic tree also supported the close relationship of the four strains—E8, E10, IBB1, and U744, circulating in 2017, according to the cluster they formed (**Figure 2**). The outgroup bacteria, *Klebsiella pneumoniae,* were positioned in a completely separate cluster in the phylogenetic tree and had significant evolutionary distances with the study isolates (0.048). The sequences of the isolates of the present study are now available at the NCBI database under the GenBank accession numbers, MG857840.1 (*E. coli* strain E8) and MG857757.1 (*E. coli* strain E10).

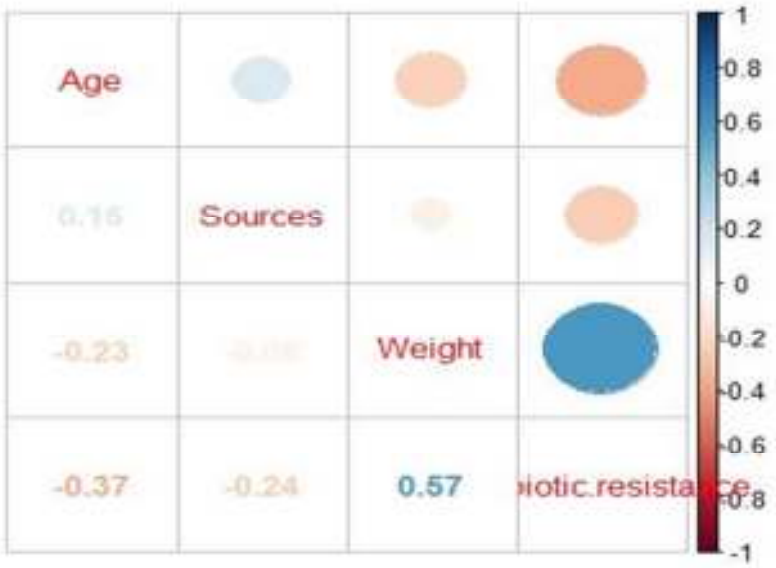

FIGURE 2:. CORRELATION MATRIX PLOT SHOWED ASSOCIATION AMONG OBSERVATIONS. DRUG RESISTANCE WERE FOUND TO HAVE AN ASSOCIATION WITH WEIGHT BUT INDEPENDENT OF AGE AND SOURCES (HOSPITALS).

Correlations among variables

Among four variables, antibiotic resistance was found to be positively correlated (+0.57) with the weight of the RUTIs patients. The variables for antibiotic resistance pattern were independent of patient's age and sources of sample collection while the correlation between age and sources was insignificant to perspectives of the present study (**Figure 3**).

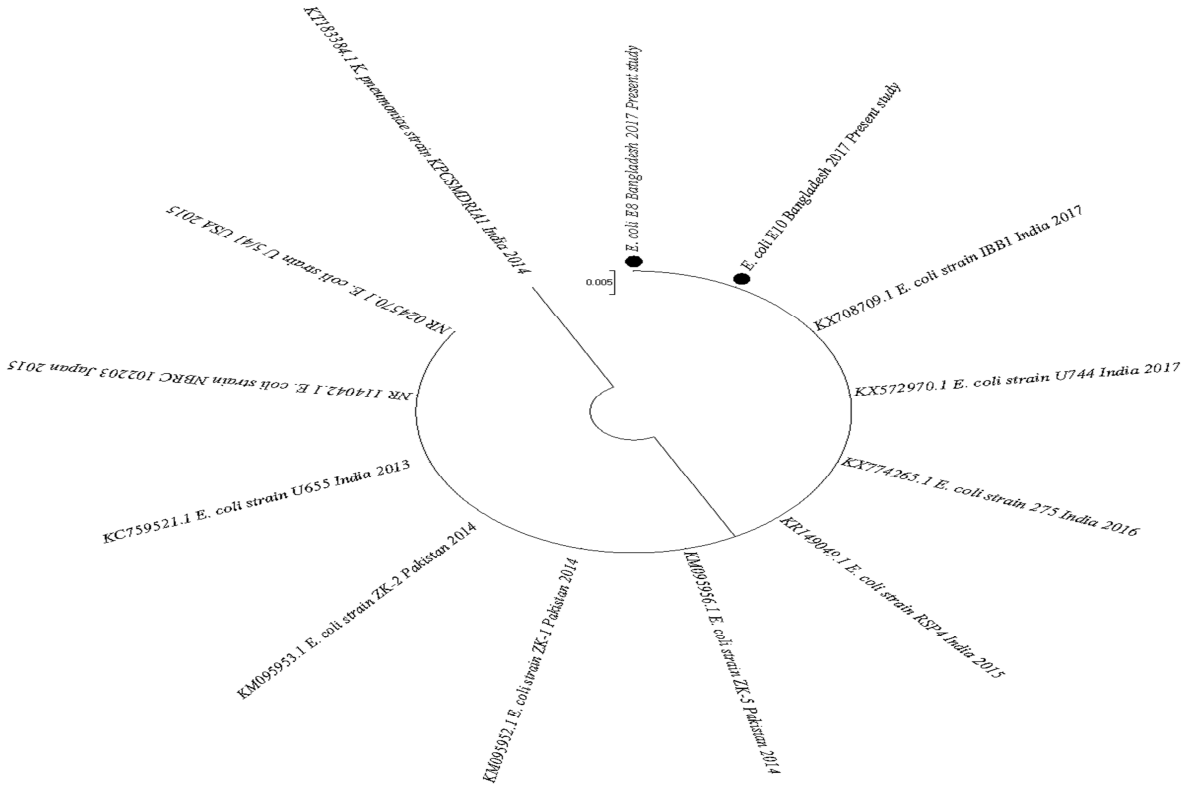

FIGURE 2: THE PHYLOGENETIC RELATIONSHIP OF *E. COLI* STRAINS E8 AND E10, ON THE BASIS OF 16S RRNA SEQUENCES, WITH THE *E. COLI* ISOLATES CAUSING UTI IN OTHER PARTS OF THE WORLD.

The evolutionary history was inferred using the neighbor-joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. all positions containing gaps and missing data were eliminated. *Klebsiella pneumoniae* signifies an outgroup bacteria in the tree. evolutionary analyses were conducted in mega7.

DISCUSSION

The accumulation of the multidrug-resistant clinical isolates results in significant health challenges worldwide, especially in women. Particularly in low-income countries, the prevalence and occurrence of the multidrug-resistant isolates of *E. coli* are much higher and more common (4). Previous studies have revealed that 69% of the UTIs are caused by *E. coli* in Bangladesh, where 83–85% of the infected patients are females (21, 22). Although many of these women have faced RUTIs in their lifetime, most of them were literally unaware of their reinfections (5). Currently, no clinical data about the percentages of women, suffering from RUTIs in Bangladesh are available because of the improper diagnostic and treatment strategies. Hence, we found only seven isolates of *E. coli* after 12 months of screening across three different hospitals. Due to the frequent use of antibiotics and rupture in the antibiotic dose regimens, the clinical isolates of *E. coli* have recently emerged as multidrug-resistant variants all over the country, thereby making the treatment of UTIs and RUTIs challenging (21, 23). Previous studies have discovered the failure of the commercial antibiotics against the clinical isolates of *E. coli* in the major cities of Bangladesh, including Dhaka, Rajshahi, and Sylhet, where 50–70% of drug resistance was noticed from 2010–2015 (24, 25). The present study found no effective agent that can potentially inhibit all the isolates of *E. coli*. Our results revealed that the pattern of drug resistance was increasing very rapidly in Bangladesh and varied even within a city, where drug resistance to the UTI-causing isolates of *E. coli* increased by

approximately 5–10% in every five years (23). Therefore, a careful use of the commercial antibiotics needs to be executed through appropriate monitoring systems only after a proper diagnosis of bacteria, in order to minimize the spread of the drug-resistant isolates.

In Bangladesh, the diagnosis of the isolates of *E. coli* from the UTI patients depends solely on the EMB Agar Base plate culture method, which often gives false positive results and requires significant technical skills, in order to confirm the bacteria (26). Although the 16S rRNA gene sequencing method is routinely used alongside plate-based screening for the analysis of bacterial sequences and phylogenetic relationship, the details of the virulence properties, molecular characterizations, and phylogenetic analysis, using PCR and the 16S rRNA gene sequencing tools are yet to be undertaken in Bangladesh. The detection of virulence factors is always a key aspect in analyzing the pathogenicity of any bacterial isolate because these factors act multifunctional (27). In the present study, we found very high frequency of the *sfa* gene in the hospitalized RUTIs patients, where all the isolates were positive in PCR amplification. Previous studies have concluded that more than 50% of the *sfa1*$^{+}$ isolates of *E. coli* in the UTIs have a high prevalence of virulence factors and pose a serious threat to the health of the patients (28). Adhesion and fimbrial virulence factors in *E. coli* such as *sfa*, *afa*, *fim*, etc., cause further risks in the development of RUTIs, especially in women (29). In 13 years (2002-2015), studies found approximately 13% increase of sfa prevalence in *E. coli* causing UTI (13). In contrast to the previous studies, our study revealed that the *sfa* gene frequencies are much higher (100%) in the Eastern part of Bangladesh and is probably responsible for other complications in women. In phylogenetic studies, the *E. coli* isolates in the present study clustered very closely to the *E. coli* strains found in India in 2017, suggesting the same evolutionary origin for these bacteria. The changing phylogenetic position of the isolates of *E. coli* each year indicates the accumulation of the more multidrug-resistant isolates in South Asia. Similar to the *E. coli* isolates, E8 and E10, the multidrug-resistant strains, IBB1 and U774 were also characterized from the chronic UTI patients and had a number of virulence factors (30, 31). In addition to drug resistance, obesity also increases the risk of RUTIs infection in women. One study revealed significant correlation of obesity with RUTIs ($p<0.001$) where fatness increases the risk of recurrent infections in women by 27% and drug resistance by 20% (32). In present study, the association between weight and drug resistance also significant compared to other variables. Thus, the overall trend and pathogenicity of the drug-resistant isolates of *E. coli* are truly alarming in Southeast Asia for the upcoming days.

CONCLUSION: In this comprehensive study, we effectively used the various traditional and molecular biology tools for the precise identification and analysis of the isolates of *E. coli* from women with RUTIs in the eastern part of Bangladesh that can be used for the further molecular analysis of these isolates, associated with UTIs and RUTIs in Bangladesh.

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CONFLICT OF INTEREST: The authors declare no conflict of interest.

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