UNDERSCORING THE LOOMING THREAT OF ANTIBIOTIC RESISTANT *ESCHERICHIA COLI*: A COMPARATIVE ANALYSIS ACROSS URINARY TRACT INFECTIONS, DIARRHEAL ILLNESSES AND HEALTHY CONTROLS

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ABSTRACT

Background: This study investigates the prevalence and characteristics of *E. coli* resistance patterns, focusing on isolates from patients with urinary tract infections (UTIs), diarrheal illnesses, and healthy controls. **Methods:** The study collected and identified *E. coli* isolates from 36 individuals: 17 with UTIs (urine samples), 6 with diarrhea (stool samples), and 13 healthy controls (stool samples). Antimicrobial susceptibility testing (AST) was performed to assess resistance against ten antibiotics from eight classes. Fluoroquinolone resistance was further evaluated against four drugs (nalidixic acid, ciprofloxacin, ofloxacin, and moxifloxacin). Additionally, molecular analysis of quinolone resistance-determining regions (QRDRs) in *gyrA, gyrB, parC*, and *parE* genes was performed on two selected isolates (U44 and U46) by genome sequencing. **Results:** Chloramphenicol and meropenem displayed the highest efficacy (>70% sensitivity), while AZM, AML, NA and SXT showed the highest resistance. UTI isolates exhibited higher resistance than diarrheal and healthy control counterparts. Worryingly, 58% of isolates exhibited multidrug resistance (MDR), with most (13/21) originating from UTI patients. The presence of MDR *E. coli* in five healthy individuals suggests potential carriage and community transmission. Fluoroquinolone resistance was particularly alarming, with moxifloxacin showing the highest resistance (80.95%). Molecular analysis confirmed mutations in all three fluoroquinolone resistance determining genes except *gyrB*. S84L and D87N dual mutations in the QRDR of *gyrA* was found in both isolates. S80I and S458A single mutations were observed in *parC* and *parE,* respectively. **Conclusion:** The study findings highlight the widespread prevalence of antibiotic resistance in *E. coli* and the urgent need for alternative treatment strategies.

KEYWORDS: Antibiotic resistance, *E. coli*, UTI, Diarrhea, Fluoroquinolone, Molecular analysis.

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Introduction

Escherichia coli (*E. coli*) occupies a versatile niche within the human microbiome, colonizing the gastrointestinal tract and serving a commensal role in healthy individuals. However, this bacterium can also be a formidable pathogen, implicated in a diverse array of infections including urinary tract infections (UTIs) and diarrheal illnesses (Zhou et al. 2023, Mueller and Tainter 2024). The emergence and dissemination of antibiotic-resistant *E. coli* strains pose a significant and growing threat to global public health. The escalating ineffectiveness of antibiotics against these pathogens complicates treatment options and jeopardizes patient outcomes.

Understanding the dynamics of *E. coli* resistance patterns necessitates a multifaceted approach, encompassing not only the prevalence of resistance but also the specific types of resistance encountered and their variations across distinct populations. This study delves into this critical question by investigating *E. coli* isolates from individuals in Dhaka, Bangladesh, a region with a high burden of infectious diseases. We hypothesize that *E. coli* isolates from patients with UTIs and diarrheal illnesses will exhibit higher levels of resistance compared to isolates from healthy controls. Furthermore, we posit that the specific resistance profiles will differ between these groups. To address these hypotheses, we have meticulously collected and identified *E. coli* isolates from patients diagnosed with UTIs and diarrheal infections, as well as healthy individuals serving as a control group. We employed a comprehensive antimicrobial susceptibility testing (AST) to evaluate the susceptibility profiles of the isolated *E. coli* strains.

Another particular focus of this study was the prevalence and characterization of fluoroquinolone resistance in *E. coli*. Fluoroquinolones represent a valuable class of antibiotics which was previously reported as a first line of defense against acute uncomplicated cystitis and pyelonephritis (Hooton 2003). However, over-prescription of fluoroquinolones for

uncomplicated UTI, acute sinusitis, and acute bronchitis has lead the United States (US) Food and Drug Administration (FDA) to set warning on fluoroquinolones recommendations as a first line defense (FDA 2016). A 2013-2018 US study found no significant decline in fluoroquinolone prescriptions despite the FDA warning. Notably, the majority of these prescriptions were used to treat uncomplicated UTIs (Bratsman et al. 2020). These widespread and often inappropriate use of fluoroquinolones has fueled the emergence of resistant bacterial strains, threatening the continued efficacy of these crucial medications. To gain a deeper understanding of fluoroquinolone resistance in our isolates, we aim to assess resistance against a broad panel of fluoroquinolone drugs, as well as delve into the underlying resistance mechanisms. This will involve sequencing the quinolone resistance-determining regions (QRDRs) of specific genes (*gyrA, gyrB, parC, and parE*) to pinpoint the precise mutations conferring fluoroquinolone resistance.

By meticulously analyzing the prevalence and characteristics of *E. coli* resistance patterns across diverse populations in Dhaka, Bangladesh, this study seeks to contribute valuable insights to the ongoing battle against antimicrobial resistance. The comprehensive data generated will inform evidence-based interventions for managing *E. coli* infections and guide strategies for preserving the effectiveness of antibiotics in these crucial clinical settings.

Materials and Methods

Sample collection

Stool (n=6) and urine (n=17) samples from diarrheal and UTI patients, respectively, were collected in collaboration with Dhaka Medical College Hospital. Additionally, thirteen stool samples from the male staff of a residential hall of Dhaka University were collected as representative healthy control who had no known case of diarrhea or UTI at the study period. Patients or healthy individual taking antibacterial medications were rejected from this study. Both male and female participants of any ages were included. Samples were collected in a sterile, dry, clean container and processed within two hrs. post-collection.

Isolation and identification of E. coli from clinical specimens

Raw samples (stool/urine) were diluted by adding 1g(/ml) of sample into 9mL of normal saline. Diluted samples were grown on Eosine methylene blue agar media to presumptively identify *E. coli* colonies with green metallic sheen. Colonies from MacConkey agar media were subcultured to nutrient agar media to obtain pure isolated colonies. Gram staining, catalase test, oxidase test, kligler's iron agar test, MR-VP test, Citrate agar test, and indole test were performed for the biochemical characterization of the presumptively selected *E. coli* isolates (Cappuccino and Sherman 1992).

Antimicrobial Susceptibility Testing (AST)

A panel of ten antibiotics encompassing Gentamicin (CN), Meropenem (MEM), Cefixime (CFM), Cefepime (FEP), Ceftriaxone (CRO), Azithromycin (AZM), Nalidixic Acid (NA), Chloramphenicol (C), Sulfamethoxazole-trimethoprim (SXT), and Amoxicillin (AML), representing eight distinct antibiotic classes, were employed in the study. The classification of isolates into Resistant (R), Sensitive (S), and Intermediate (I) categories was determined based on the zone diameter measurements in accordance with the Clinical and Laboratory Standards Institute (CLSI) M100 (34th edition) guideline published on 2024 (CLSI 2024). Isolates resistant to nalidixic acid were additionally subjected to three other fluoroquinolone drugs (Ofloxacin, Moxifloxacin and Ciprofloxacin) to analyze the prevalence of fluoroquinolone resistance among diarrheal patients, UTI patients and healthy individuals.

Chromosomal DNA Extraction and purification

Boiling DNA method was used to extract the chromosomal DNA of all the *E. coli* isolates (Dimitrakopoulou et al. 2020). Well isolated colony selected from nutrient agar plate was inoculated into LB broth and incubated at 37°C. A 1.0 mL culture was transferred into a 1.5 mL Eppendorf tube and subjected to centrifugation at 10,000 rpm for 10 minutes using an Eppendorf centrifuge (Eppendorf, Germany). The resulting cell pellets were re-suspended in 1 mL of normal saline and centrifuged again at 10,000 rpm for another 10 minutes. Supernatant was removed and the pellet was resuspended in 500 µL of nuclease free water. After boiling the cell suspension for 10mins in a 100°C water bath, The Eppendorf tubes were immediately placed on ice for 10 minutes. Following this, the tubes were centrifuged at 12,000 rpm for 5 minutes. The supernatant (100-150 µL) was transferred to a fresh Eppendorf tube and stored at -20ºC. The concentration of the extracted genomic DNA was measured in ng/µL using a Nanodrop 2000 (Thermo Scientific, USA). The purity of the DNA was assessed by measuring the ratio of absorbance at 260 nm to 280 nm (A260/280) where a ratio of approximately 1.8 indicates that the DNA is not contaminated with RNA or protein.

Amplification and gel electrophoresis of Quinolone resistance determining region (QRDR)

The extracted chromosomal DNA for each isolate was used for the amplification of quinolone resistance determining regions (QRDRs) of *gyrA*, *gyrB*, *parC* and *parE* genes by Polymerase Chain Reaction (PCR). Primer used in the current study was selected from previous study (Nam et al. 2013). For all PCR reactions, a negative control containing all reaction components except the DNA template was included. In relevant cases, a positive control with a known DNA template carrying the target gene was also included. PCR amplicons were analyzed using agarose gel electrophoresis (Voytas 2000). Agarose was dissolved in 1X TBE buffer to give a final concentration of 1.5% agarose by heat. When the temperature dropped to 50°C, 3µg/mL of Ethidium Bromide was added to the gel. The gel was then poured onto the gel casting tray. The gel was submerged in 1X TBE buffer in a gel running tank. Electrophoresis was carried out at 70 volts until the tracking dye migrated sufficiently. The molecular marker used was 100 bases plus DNA ladder marker, 5 µl was loaded into the well. The stained gel was observed with a UV transilluminator (Gel Doc, Bio-Rad, USA) and photographs were taken.

Sequencing of amplified QRDRs of gyrA, gyrB, parC and parE genes

As UTI isolates showed the highest fluoroquinolone resistance, two UTI isolates (U44 and U46) which showed the presence of QRDRs of all the four genes (*gyrA, gyrB, parC*

and *parE*) were selected for sequencing by BTseq^{TM} method and further molecular analysis such as possible mutational impact. Raw sequences were cleaned using chroma software (Goodstadt and Ponting 2001) and compared with reference sequence to *E. coli K12* (NC_000913.3) by multiple sequence alignment using MEGA11.0 software (Tamura et al. 2021).

Results

Selection of E. coli from clinical samples

Six presumptively identified *E. coli* isolates through selective and differential plating were selected from the stool samples of diarrheal patients and another seventeen isolates from UTI samples. Thirteen *E. coli* colonies were identified from healthy controls. All the 36 isolates were lactose fermenter; showed positive reaction for MR, Indole and Catalase tests; while negative for VP, Citrate and Oxidase tests. The observed biochemical profile of the isolates validates their identity as *E. coli*.

Antimicrobial resistance (AMR) profiles of the selected isolates

Chloramphenicol and meropenem were found to be the most effective antibiotics against most of the isolates (>70% sensitivity), while highest resistance was noticed for AZM (67%), closely followed by AML, NA and SXT. Although majority of the isolates were sensitive to CN (5 resistant isolates only), six isolates (n=36) developed intermediate resistance which warrants about their potential conversion into resistant ones. Intermediate resistance to AML (6), CRO (5), MEM (4) and C (4) were also noticeable (Figure 1A).

Isolates obtained from UTI patients should higher resistance to all the tested antibiotics than the diarrheal patients, except for CN, CFM and C. Highest resistance was exerted by UTI isolates against AZM (88%). Isolates from healthy individuals showed less resistance than both UTI and diarrheal patients (Figure 1B). Isolate U-46 resisted the effect of nine tested antibiotics which render it to be the most resistant strain. Although isolates from healthy control showed less resistance, one particular isolate (HC-11) showed resistance against 6 tested antibiotics (Figure 1C). Supplementary Table 1 enlists the AMR profile of all isolates.

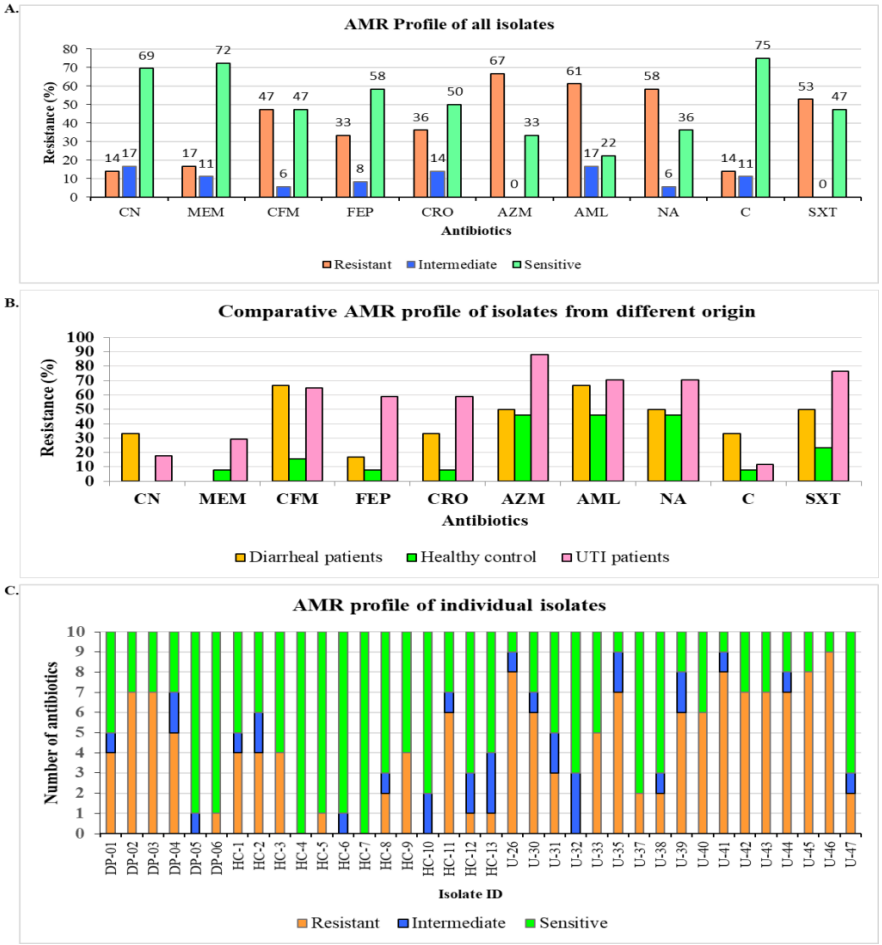

Figure 1. Graphical representation of the antimicrobial resistance (AMR) profile of *E. coli* isolates. A. Cumulative AMR profile of all isolates against ten different antibiotics. B. Comparative AMR profile of isolates representing diarrheal, UTI and healthy control origins. C. Individual AMR profile of 36 isolates. Here, CN: Gentamycin; MEM: Meropenem; CFM: Cefixime; FEP: Cefepime; CRO: Ceftriaxone; AZM: Azithromycin; AML: Amoxicillin; NA: Nalidixic Acid; C: Chloramphenicol; SXT Sulfamethoxazole-trimethoprim

Multidrug resistance (MDR) profiles of the selected isolates

Among the 8 tested antibiotic classes, Macrolide showed the least efficacy as depicted by 67% resistant strain. Efficacy of Penicillin, Quinolone, Cephalosporin and Sufonamide were also greatly compromised (>50% resistant isolates). Choramphenicol and Aminoglycosides were found to be the most effective antibiotic class (14% resistant), closely followed by Carbapenem (17% resistant) (Figure 2A). 21 isolates (58.33%) exhibited MDR profile by resisting the effect of three or more antibiotic classes, while 15 isolates (41.66%) were non-MDR. Majority of the MDR isolates (13) belongs to UTI origin, which only have 4 non-MDR isolates. Contrastingly, five MDR isolates could be isolated from healthy individuals. 50% of the diarrheal patient associated isolates (n=6) were MDR (Figure 2B).

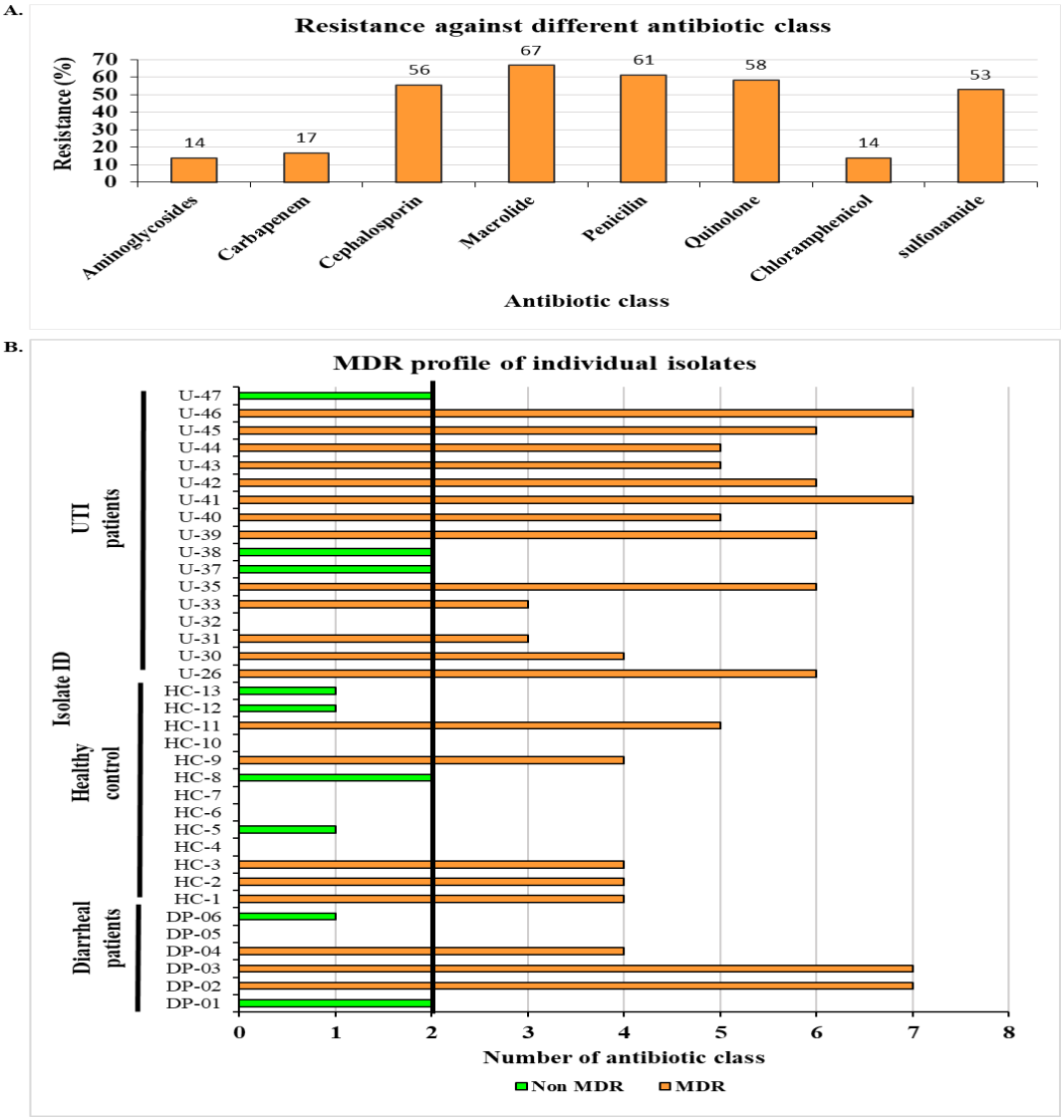

Figure 2. Graphical representation of the resistance pattern of *E. coli* isolates against eight antibiotic class. A. Cumulative resistance profile of isolates against eight antibiotic class. B. Multidrug resistance (MDR) profile of individual isolates representing diarrheal, UTI and healthy control origins. MDR has been defined as resistance against three or more antibiotic classes.

Resistance against Fluoroquinolone drugs

21 isolates that showed resistance against quinolone drug nalidixic acid were selected for analyzing their resistance pattern against three other fluoroquinolone drugs (Ofloxacin, Moxifloxacin and Ciprofloxacin). Among these 21 isolates, 12 represents UTI isolates, another 3 and 6 represent diarrheal patients and healthy controls, respectively. Highest resistance was noticed against moxifloxacin (80.95%, n=21), followed by Ofloxacin and Ciprofloxacin (57.14% in both cases). Ofloxacin and ciprofloxacin showed efficacy against 8 and 7 isolates respectively. Intermediate resistance was also noticed in 2 isolates (Figure 3A, Supplementary Table 1). Cumulatively, 12 isolates (57%, n=21) showed resistance against all the fluoroquinolone drugs. Among these 12 isolates, 75% represents UTI isolates, while 16.67% and 8.33% isolates represent the origin from healthy controls and diarrheal patients respectively (Figure 3B).

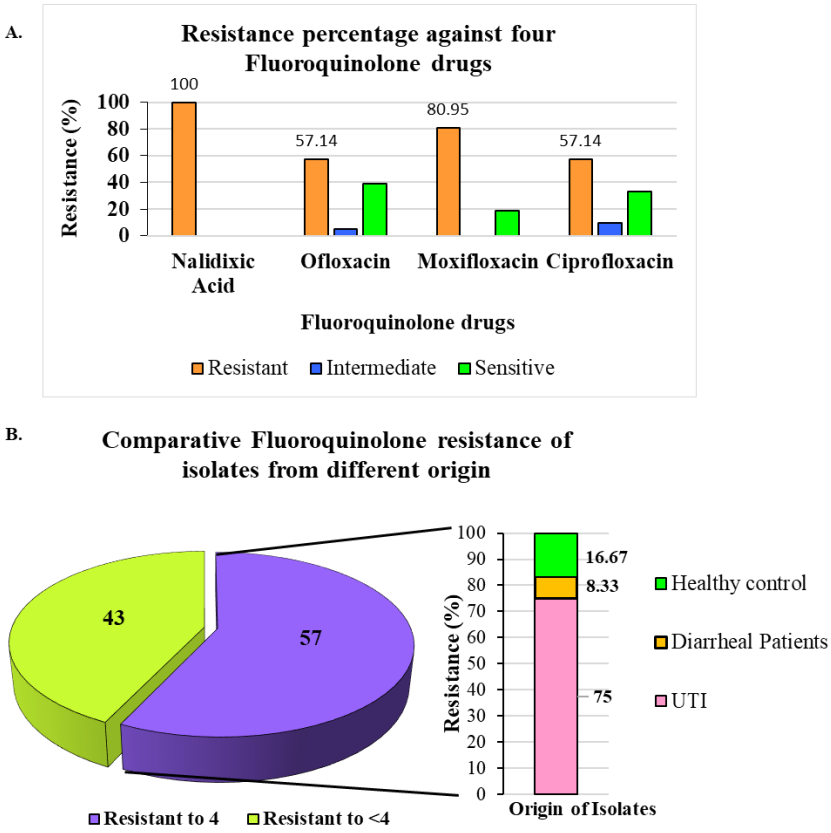

Figure 3. Resistance of *E. coli* isolates against four fluoroquinolone drugs. A. Cumulative resistance percentage of isolates against four fluoroquinolone drugs- Nalidixic acid, Ofloxacin, Moxifloxacin and Ciprofloxacin. B. Comparative fluoroquinolone resistance of isolates representing diarrheal, UTI and healthy control origins.

Molecular detection of mutations in the fluoroquinolone resistant genes

21 isolates in total (Supplementary Table 1) that were found to be resistant against nalidixic acid were selected for PCR to detect the presence of QRDRs of *gyrA*, *gyrB*, *parC* and *parE* genes that cause resistance to quinolone antibiotics by mutations. Two out of three DP isolates; eight out of twelve UTI isolates; and all HC isolates were positive for *gyrA*, *gyrB*, *parC* and *parE* genes. Among the two sequenced isolates (U44 and U46), all three genes possessed non-synonymous mutations except *gyrB*. S84L and D87N dual mutations in the QRDR of gyrA was found in both isolates. S80I single mutation was observed in *parC*. *parE* possessed single mutation (S458A) in both isolates (Supplementary Figure 1).

Discussion

The rise of antimicrobial resistance (AMR) in *Escherichia coli* represents a critical global health concern. The emergence of MDR strains adds complexity to the treatment landscape, requiring a multifaceted approach to mitigate the escalating threat of bacterial resistance (Chinemerem Nwobodo et al. 2022). This comprehensive study delves into the prevalence and resistance patterns of *E. coli*, with a particular focus on distinguishing resistance dynamics between isolates from UTI and diarrheal patients, as well as healthy controls. Understanding these dynamics is crucial for informing evidence-based interventions and safeguarding the efficacy of antimicrobial therapies in these clinical scenarios.

E. coli, a versatile bacterium, is a frequent culprit in both UTIs and diarrheal infections (Cabrera-Sosa and Ochoa 2020, Majumder et al. 2022). In our study, we have collected and identified thirty-six *Escherichia coli* isolates representing origins from diarrheal patients (n=6), UTI patients (n=17) and healthy individuals (n=13). The antimicrobial susceptibility testing (AST) conducted against these isolates included a panel of ten antibiotics from eight distinct classes. The results revealed varying susceptibility patterns among the isolates. Chloramphenicol and meropenem exhibited high efficacy against most isolates, while azithromycin (AZM) displayed the highest resistance. The observed intermediate resistance to several antibiotics raises concerns about the potential for these strains to develop full resistance over time, additionally requirement of higher dose regimen and inaccessibility of these drugs to various body parts (Rodloff et al. 2008, CLSI 2019).

Treating multi-drug resistant (MDR) bacteria is becoming more difficult in various hospitals, particularly tertiary care facilities in the Indian subcontinent (Sultana et al. 2019). The study identified MDR profiles of the isolates, revealing that a significant proportion of isolates (58.33%) exhibited resistance to three or more antibiotic classes. Choramphenicol and Aminoglycosides were found to be the most effective antibiotic class, closely followed by Carbapenem (Figure 2A). MDR trait was more prominent with the UTI isolates (76.47%) compared to diarrheal patient isolates (50%), and healthy controls showed relatively lower percentage of MDR

isolates (38.46%) (Figure 2B). This finding aligns with previous reports suggesting increased resistance in urinary tract pathogens (Islam et al. 2022). The carriage of MDR strains among the healthy controls (n=5) (Figure 2B), as noticed by previous studies as well, raises questions about community transmission and underscores the need for preventive measures beyond clinical settings (Jamrozik and Selgelid 2020, Neut 2021). In the Indian subcontinent, empirical treatment for diarrheal and urinary tract infections often precedes urine or stool cultures, contributing to heightened pathogen resistance through antimicrobial misuse (Dash et al. 2013). Optimal antimicrobial selection relies on anticipated resistance patterns within a geographic region. Thus, periodic monitoring of resistance patterns within the community is imperative.

Fluoroquinolones are frequently prescribed as empirical therapy and among the antibiotics that are considered for routine prescription in treating UTIs and other infections (Bader et al., 2020; Minarini & Darini, 2012). However, the excessive use of either oral or parenteral quinolones in recent years may enhance high rates of AMR (Holmes et al., 2016). In the current study, Twenty-one isolates that showed resistance against first generation quinolone drug nalidixic acid, were further selected for studying the prevalence of fluoroquinolone resistance against three fluoroquinolone drugs (Ciprofloxacin, Ofloxacin and Moxifloxacin). Highest resistance was noticed against moxifloxacin (80.95%, n=21), followed by Ofloxacin and Ciprofloxacin. Twelve isolates (57%, n=21) showed resistance against all the four fluoroquinolone drugs among which 75% represents UTI isolates, while 16.67% and 8.33% isolates represent the origin from healthy controls and diarrheal patients respectively (Figure 3).
From this study, it is evident that prescribing Fluoroquinolones to treat UTI needs further consideration, as the causative agents of UTI are showing high resistance to quinolone. The emergence of high fluoroquinolone resistance, particularly against moxifloxacin (80.95%), is of particular concern, as these drugs are commonly used in clinical settings. In a 2001 study, Colleen et al. reported better efficacy of moxifloxacin over other commercially available quinolones, such as ofloxacin, levofloxacin, sparfloxacin, and trovafloxacin (Culley et al. 2001). But current investigations suggest that the efficacy of moxifloxacin, as well as other fluoroquinolones have been greatly compromised (Stapleton et al. 2020, Islam et al. 2022) that highlights the urgency of addressing fluoroquinolone resistance.

The investigation into fluoroquinolone resistance in our study additionally involved molecular techniques, with sequencing of the QRDRs of *gyrA*, *gyrB*, *parC*, and *parE* genes of two UTI isolates (U44 and U46). The presence of non-synonymous mutations was noticed in all three genes except *gyrB*. Prevalence of S84L and D87N dual mutations in the QRDR of GyrA was noticed which is consistent with other previous reports (Ostrer et al. 2019, Mahmud et al. 2021). S80I single mutation was observed in ParC while ParE possessed S458A single mutation in both isolates. The identified mutations at the QRDR in this study suggest a genetic basis for the observed fluoroquinolone resistance. Investigating these mutations comprehensively will give the healthcare professionals a good understanding about the genetic underpinnings of fluoroquinolone resistance which is crucial for developing targeted interventions.

While this study provides valuable insights into the prevalence and resistance patterns of *E. coli*, it is crucial to acknowledge its limitations, such as the relatively small and unequal sample sizes from different sources. These factors may affect the comparative analysis of resistance patterns. Further studies with larger, more balanced, and diverse populations are warranted to enhance the robustness and generalizability of the findings.

Conclusion

Conclusively, the current study contributes valuable information on the prevalence and antimicrobial resistance of *E. coli* in clinical samples. The observed resistance patterns, especially in the context of fluoroquinolones, highlight the need for continuous surveillance, adaptive treatment strategies, and a concerted effort to address the complex interplay of bacterial resistance. The findings of this study can inform public health policies and guide future research endeavors aimed at mitigating the growing threat of antimicrobial resistance.

Author contribution statement

Mishu ID: Conceptualization; Methodology; Data analysis, validation, and result interpretation; Writing - Original Draft, Supervision, Project administration, Funding acquisition
Akter S: Methodology; Investigation; Data analysis, Writing - Original Draft; Review & Editing
Abonee FJ: Methodology; Investigation; Data analysis; Review & Editing
Rahman SR: Methodology; validation and result interpretation; Review & Editing; Supervision; Resources
Malek MA: Methodology; validation and result interpretation; Review & Editing; Supervision; Project administration, Funding acquisition

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Ethical Clearance Statement

This study was conducted in strict accordance with ethical guidelines and was approved by the Ethical Review Committee of the Faculty of Biological Sciences, University of Dhaka (Approval No. 218/Biol.Scs/2023). The research procedures were designed to minimize any potential risks to participants, and all data were handled with the utmost care to maintain privacy and integrity.

Declaration

The authors declare no conflict of interest. The study represents original research work carried out by the team and the contents of the paper are neither published nor submitted for publication to any other journal.

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