ORIGINAL ARTICLE

Unraveling the resistance puzzle: Antibiotic susceptibility and genetic insights into extra-intestinal E. coli Strains in UTIs.

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ABSTRACT… Objective: To investigate the antibiotic resistance patterns and genetic factors associated with ExPEC strains isolated from UTI patients. **Study Design:** Cross sectional Study. **Setting:** Microbiology Lab in Lahore, Pakistan. **Period:** December 2021 to June 2022. **Methods:** A total of 300 urine samples were collected from patients with UTIs, sourced from various hospitals and labs. E. coli identification was confirmed using biochemical tests and the API 20 E standardized method. **Results:** Antimicrobial susceptibility testing was performed using the disc diffusion technique following CLSI guidelines. The findings revealed varying levels of resistance among the isolates. High resistance rates were observed for ampicillin (92.6%), amoxicillin (93.6%), ceftriaxone (88.9%), and ciprofloxacin (82.4%). Lower resistance rates were found for gentamycin (66%), meropenem (8.2%), tigecycline (4%), imipenem (12.4%), and colistin (0.0%). However, no significant association was detected between the AER gene and fluoroquinolone resistance. **Conclusion:** This study sheds light on the prevalence of antibiotic resistance in ExPEC strains causing UTIs. The high resistance rates to commonly prescribed antibiotics highlight the need for judicious antibiotic use and alternative treatment strategies. Moreover, the absence of a clear link between the AER gene and fluoroquinolone resistance suggests the involvement of other mechanisms in conferring resistance. Understanding the resistance patterns and genetic factors associated with ExPEC strains is crucial for devising effective treatment strategies and combating the growing problem of multi-drug resistance in UTIs caused by E. coli.

Key words: Escherichia Coli, Fluoroquinolone Resistance, Multi-drug Resistance.

INTRODUCTION

Urinary tract infection (UTI) is a common bacterial illness, particularly prevalent among women due to the shorter female urethra, which allows easier access for bacteria like E. coli to reach the bladder.[1] Although various bacterial and fungal infections can cause UTIs, E. coli remains the most frequently identified pathogen. UTIs can have significant health effects, resulting in four days of restricted activity and two days of missed work. Individuals with underlying medical conditions, such as diabetes, may experience more complicated infections, including paraurethral or renal abscesses, which can lead to increased morbidity and hospitalization rates.[2] Moreover, UTIs have the potential to enter the bloodstream, causing bacteremia and a higher risk of fatality.[3]

Humans harbor commensal E. coli in the gastrointestinal tract (GIT), which can cause various diseases.[4] Pathogenic strains cause urinary tract infections (UTI), meningitis, diarrheal diseases, and infections in immunocompromised individuals.[5] Extra-intestinal pathogenic E. coli (ExPEC) causes illnesses outside the intestine, while commensal E. coli can also cause infections in weakened hosts. E. coli strains causing gastrointestinal infections are classified as obligate pathogens.[6] E. coli is a versatile bacterium belonging to the Enterobacteriaceae family. It is categorized into commensal, intestinal pathogenic, and extraintestinal pathogenic strains.[7] ExPEC is a common Gram-negative pathogen causing UTIs and bloodstream infections.[8] Antibiotic resistance is a growing concern in E. coli, with resistance mechanisms

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including beta-lactamase production and fluoroquinolone resistance. The prevalence of multi-drug resistant (MDR) E. coli, including extended-spectrum beta-lactamase (ESBL)-producing strains, is increasing. The acquisition of resistance genes on plasmids contributes to MD.[9,10] Iron acquisition, facilitated by aerobactin, plays a crucial role in E. coli infection.[11]

The rationale of this research is to study the common uropathogens and antibiotic susceptibility pattern among Urinary tract patients from the Gujranwala Hospitals and to detect the presence of aer gene in fluoroquinolones resistant uropathogenic Escherichia coli.

METHODS

This study was conducted at the IMBB University of Lahore, Pakistan's microbiology lab after approval from ethical committee (CRIMM/23/Research/7-8-23). Between December 2021 and June 2022, a total of 300 urine samples from patients with urinary tract infections in different age groups (ranging from 11 to 60 years) were collected. Samples were obtained both from the collaborative laboratories of Gujranwala hospitals, which includes Civil hospital, Government Social Security hospital and Chughtai's Lab.
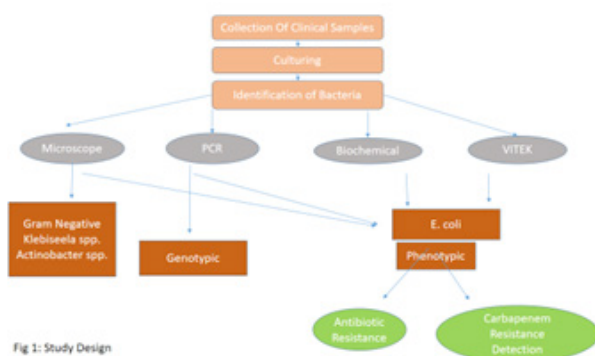

Figure-1. Study Design

The gathered samples were processed as soon as possible and transported to the lab to prevent the formation of normal flora. 3.2 Acquisition of samples Patients visiting Chughtai's Medical Laboratory (Lahore, Pakistan) from November 2021 to July 2022 provided a total of 500 clean catch midstream urine samples. Immediately after samples were collected in sterile plastic bottles,

the bacteria underwent culture processing and an antibiotic susceptibility test. In inclusion criteria, Urine samples from individuals of various ages with urinary tract infections yielded positive results demonstrating isolates. Clinical samples that don't demonstrate any growth, samples besides E coli and Poor sample handling were excluded. Patient histories were obtained from individuals with acute, simple UTIs or recurring UTIs, who had previously used antimicrobial medications. Pyrex glassware was cleaned and dried, and conical flasks were autoclaved for sanitation before conducting the study. Preparation of Media: Culture media were prepared according to manufacturer's instructions, sterilized in an autoclave, and supplemented with urea and blood as required. Petri dishes and test tubes containing the media were incubated at 37°C for 24 hours for sterility before use.

Preparation of Reagents

Methyl red reagent was prepared by mixing methyl red, distilled water, and ethanol. Voges-Proskauer reagent was created by combining ethanol, -naphthol, distilled water, and KOH. Tris-EDTA (TE) buffer and Tris-Borate-EDTA (TBE) buffer were prepared following specific protocols. Ethidium bromide stain was prepared by dissolving Ethidium bromide in distilled water.

Isolation and Identification of Bacteria

Urine samples were incubated in CLED medium, and colonies were counted using a calibrated wire loop technique. Biochemical tests, Gram staining, and subculturing on MacConkey agar were performed to identify and confirm the presence of E. coli strains. API 20 E standardized identification method was used for further verification.[12]

Gram Stain

Air-dried, heat-fixed smears were stained with crystal violet, iodine, decolorized, and counterstained with safranin. Gram-positive bacteria appeared blue or purple, while Gram-negative bacteria appeared pink or red.

Oxidase Test

Bacterial colonies were tested for oxidase activity using a reagent, and a color change from rose to

purple indicated a positive result.

Other Tests
Various tests were performed to assess oxidation-fermentation, sugar fermentation, urease activity, indole production, methyl red test, Voges-Proskauer test, and motility. These tests provided information about bacterial metabolic activities and characteristics.

API 20 E Test
The API 20 E system, consisting of miniaturized biochemical tests, was used for identifying and distinguishing members of the Enterobacteriaceae family.

Gelatinase Test
Gelatinase activity was examined to determine if bacteria can liquefy gelatin.

Bacterial Isolate Maintenance
Short-term storage involved maintaining pure bacterial isolates in screw-capped universal tubes with brain heart infusion agar slants at 4°C for three months. Long-term storage was done by inoculating brain heart infusion broth with glycerol and storing at -20°C for 2-8 months.

Antibiotic Susceptibility Testing
Mueller-Hinton agar was used for antimicrobial susceptibility testing of E. coli using the disc diffusion technique following CLSI guidelines. Mueller-Hinton plates were prepared and antimicrobial discs were placed on the agar surface. Results were interpreted based on zone sizes and CLSI criteria.

Isolation of Plasmid DNA
Plasmid DNA was isolated using modified procedures, including cell lysis, centrifugation, phenol-chloroform extraction, and precipitation. Plasmid DNA was resuspended in buffer or water.

16S rRNA Sequencing and Analysis
Genomic DNA extraction, PCR amplification, purification of amplified products, and bidirectional sequencing were performed for 16S rRNA gene analysis. Sequences were compared with those in GenBank, and phylogenetic trees were generated.

Primer Information
Specific primers were used for amplification and sequencing of the 16S rRNA gene.

Sequencing Primer Name Primer Sequence	PCR Primer Name Primer Sequence
785F 5' (GGATTAGATAC-CCTGGTA) 3'	27F 5' (AGAGTTTGATC-MTGGCTCAG) 3'
907R 5' (CCGTCAATTC-MTTTRAGTTT) 3'	1492R 5' (TACGGY-TACCTTGTTACGACTT) 3'

Table-I. For the amplification of full length 16S Ribosomal DNA set of universal primers

Trace Chromatogram Analysis
Trace chromatograms were analyzed to interpret sequence data, including event descriptors, packet types, and nucleotide peaks.

BLAST Analysis
BLAST algorithm was used to search for similar sequences in biological databases. Input sequences were provided in FASTA format, and regional alignments were generated as the final output.

E. coli Phylogenetic Analysis
Phylogenetic analysis was performed to determine the phylogeny of E. coli, confirming its genus, subclass, and defined species.

RESULTS
The samples were collected from hospitals (Civil hospital and Government Social Security hospitals) and Chughtai's laboratory, in Gujranwala. Patients ranged in age from 31 to 90, with the majority being elderly. Overall, five hundred (500) clinical samples were collected from patients in laboratory departments of both hospitals. 100 samples were collected from Chughtai's laboratories.
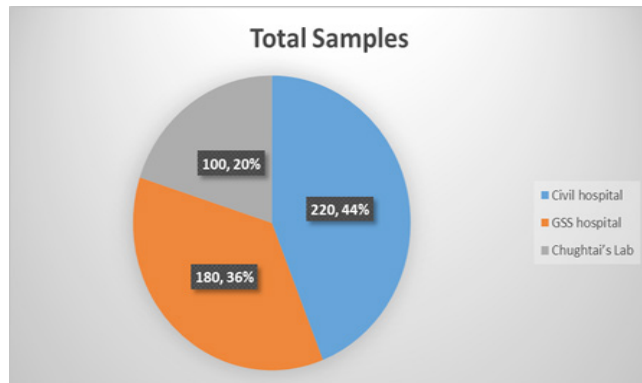
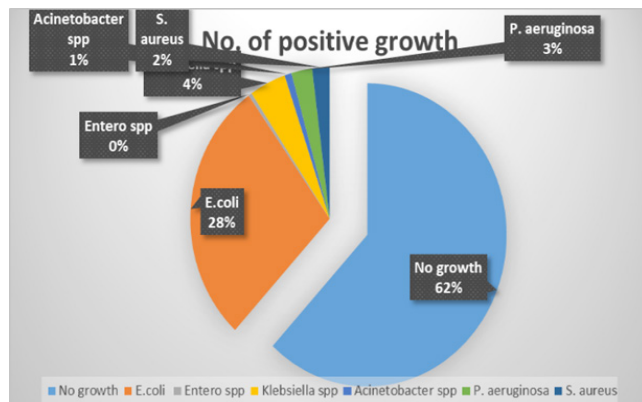**Figure-2. Percentage of total no. of samples collected from different collaborator labs**

Figure-3. Total no. of growth and non-growth samples

In the 500 urine samples, 193 samples tested positive for the isolation of organisms; of these, the culture positivity results showed that 140 (72.5%) of the samples were positive for E. coli and 114 (27.5%) were positive for other organisms, while 307 (61.4%) of the samples tested negative after culturing. Other uropathogens that are present in addition to E. coli include P. aeruginosa. (2.4%), Acinetobacter spp. (0.8%), Enterobacter spp. (0.4%), and Klebsiella spp. (4.2%). A significantly smaller number of Citrobacter and Staphylococcus spp. were also identified. When compared to acute cases, which were only 46 (23.84%), the number of the current study was conducted on 500 specimens from urine sample of urinary of suspected patients. The results were distributed according to the patient's age between 11-90 years old.

The lowest incidence was among 51-70 and 71-90 age group (4.0%), while the highest incidence was among 50-59 age group (43 %) as observed

in the Table f recurring UTIs was fairly substantial at 147 cases (76.16%).

Variable		Frequency	% Percentage
Gender	Male	177	35.4%
	Female	323	64.6%
Age (year)	11-30	8	4.0%
	31-50	45	22.5%
	51-70	86	43.0%
	71-90	29	14.5%

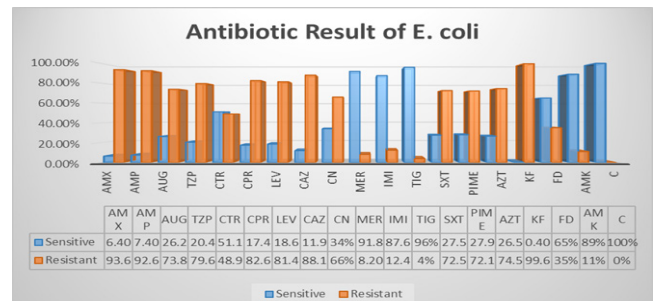**Table-II. Frequency distribution according to gender and age**

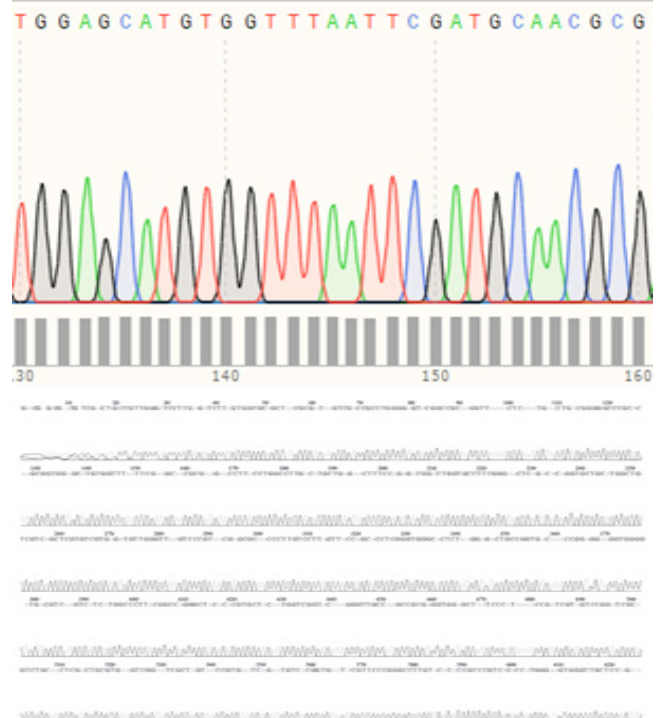**Figure-4. Different antibiotic drugs against E. Coli strain**

Figure-5. Chromatograph generated by DNA sequence analyzer (Forward and Reverse Sequence)

DISCUSSION

A similar study was conducted by Abbas et al., in 2019, UTI incidence among recipients of kidney

transplants ranged from 11.7% to 67.5%. The overall UTI prevalence was 32.6%. E. coli was the most prevalent among Gram-negative organisms that cause UTI, followed by Klebsiella pneumonia with prevalence rates of 41.3% and 11.9%, respectively. Additionally, coagulase-negative Staphylococci (9.4%) and Enterococcus spp. (9.8%) had the highest incidence among Gram-positive bacteria. Ampicillin had the highest rate of resistance (91.2%) among Gram-negative organisms, followed by ceftazidime (89.5%). The frequency of the lowest level of resistance, against imipenem, was 14.3%.[14] Results that males more infected than females, may be justified by the fact that in Pakistan, the men are more exposed to the factors of the outside environment. There were 140 E. coli positive urine samples out of 500 total. Women between the ages of 30 and 50 had the greatest isolation rates (22.5%). The age bracket of 11 to 30 years (4%), meanwhile, came next. Of the isolates, just 16 percent of those in the 0–25 age range were found. The age group from 51 to 75 years old likewise had the greatest isolation rate in males (10% n = 30). The age bracket of 26 to 50 years (n = 21) was next (7% of the total). In the 0–25 years age range, only 4% of the isolates (n = 12) were found. Age group 76–100 years had the lowest isolation rate (n = 7) with a 2 percent incidence. Males (36.6%) had a higher rate of UTI than females (64.4%) (2 = 4.8, P 0.02). E. coli was identified in the sample at a frequency of 28%, whereas other species were present at a prevalence of just 10.6%. E. coli prevalence was found to be 63% in Iran by Mortazavi and Shahin (2009) and 88% in Bangladesh.[14]

The age range of 51–75 years had the largest percentage of these 140 E. coli strains in both the female and male samples in the current study. Comparable findings from a research conducted in Punjab and Khyber Pakhtunkhwa, Pakistan, indicated that the prevalence of urinary tract infections was greater among people aged 41 and older.[15] The age group of 41 to 60 years represented the largest proportion of patients in western Nepal. Both of these studies had findings that agree with ours, which might be due to a variety of things, including the fact that the majority of cases are recurrent ones.

In addition, it implies that postmenopausal symptoms are present in the female patients in this age range. According to the results of the study[16], non-secretor status, a history of UTIs, along with other inherited propensities, as well as urodynamic factors, particularly incontinence, the presence of a cystocele, and residual urine volume, are the factors that have the greatest impact on the occurrence of recurrent UTI among postmenopausal women. However, there was a significantly lower isolation rate in women between the ages of 26 and 50, or 25%, despite the fact that this is the age group in the Lahore region where women are more likely to have frequent coitus, become pregnant, use antibiotics, and be exposed to spermicides, all of which are significant risk factors for both acute and recurrent UTIs. In patients hospitalized to or receiving outpatient care at both hospitals in Gujranwala, the bacterial etiology of urinary tract infections (UTIs) was identified. Escherichia coli was also tested for its resistance pattern against a variety of drugs. 500 samples were successfully used to isolate bacteria. Female samples (87.5%) had a non-significantly greater rate of bacterial etiological agent isolation than did male individuals (71.3%). Out of 193 microorganisms isolated from patients, Staphylococcus aureus (38.94%), Proteus species (22.54%), and Pseudomonas spp. (21.52%) had the greatest rates of isolation, followed by E. coli.

In one of the similar study, Amoxicillin and Meropenem had the largest and lowest prevalence's of resistance, with 80.8% and 13%, respectively. Antibiotic resistance and biofilm were shown to be positively correlated in 14 of 17 studies (82.35%).[17] In this study, the different kind of antibiotics account as 20, were utilized against E. coli isolates, which showed that most of the antibiotics showed resistance while some showed sensitive. The results showed that resistant percentage of different antibiotics such as ampicillin 92.6%, amoxicillin 93.6%, augment 73.8%, Tzp 79.6%, ceftriaxone 88.9%, ciprofloxacin 82.4%, gentamycin 66%, meropenem 8.2%, tigecycline 4%, imipenem 12.4% and colistin 0.0%. In the current studies, most of the isolate were resistant to fluoroquinolones class of

antibiotic, resistant to ciprofloxacin 82.4% and resistant to levofloxacin 81.4% respectively. While list the high level of resistant to amoxicillin and ampicillin, 93.6% and 92.6%. In this study the 4th generation drug cefepime showed the 50% resistant and sensitivity, which means due to overused of high potential antibiotic drug practice in Pakistan, there is a modification or alteration of gene structure may occur, which developed a protein such as beta-lactamase and carbapenemase protein base enzyme.

In this study, the cephalosporins drug also showed a variety of resistance which included ceftriaxone showed 88.9% resistant, which were commonly used in practice as a first line in injectable form for every kind of bacterial Infections but our study showed high resistant against this drug, which mean over used of ceftriaxone developed a resistant property in a pathogen bacterium. The isolated strains of E. coli showed high level of resistant to amoxicillin, ampicillin, ciprofloxacin, levofloxacin, ceftriaxone then showed intermediate resistance and sensitivity to certain drugs which includes gentamycin, azeterom, nitro and Trimethoprim-sulfamethoxazole while the other drugs showed low level of resistance including amikacin, imipenem, meropenem, tigecycline and colistin The colistin drug showed 100% sensitive against E. coli strain in our study and also showed that colistin is the only drug of choice against gram negative bacteria. Meropenem worked well against germs collected from people of various ages and genders. On the other hand, isolates from persons older than 40 years had reduced imipenem effectiveness.[18] In order to evaluate the efficacy of the medicines given for the treatment of illnesses in Gujranwala, the susceptibility pattern for E. coli strains was examined. Additionally, our research showed that 90% of the E. coli isolates were amikacin-sensitive. In a recent study, it was observed that catechin, isolated from Canarium patentinervium Miq, exhibited significant antibacterial activity against E. coli strains, including clinical isolates from UTI patients, and showed strong synergistic effects when combined with tetracycline. Catechin also effectively reduced E. coli biofilm formation by down regulating the acrA gene.

In a study conducted at Timurgara District Hospital in Pakistan, 200 women aged 18-65 years with symptoms of uncomplicated urinary tract infection (UTI) were examined. Out of these, 109 were diagnosed with pyelonephritis and 91 with cystitis. Microbiological testing revealed that 21.5% of the samples were culture-positive, with E. coli, Enterococcus spp., and Klebsiella pneumoniae being the most common bacteria isolated. The study found high resistance rates to ciprofloxacin and ceftriaxone in E. coli isolates, while resistance to fosfomycin was low.[20] Antibiotics alternatives should be used like in a study, it is noticed that vitamin C at a concentration of 1.25 mg/ml effectively inhibited the growth of most bacteria studied and showed a positive effect when combined with antibiotics. Vitamin C also effectively reduced the formation of biofilms by the bacteria. The expression of certain genes in the bacteria was down-regulated after treatment with vitamin C. In experiments with rats, vitamin C showed a rapid curative effect for urinary tract infections (UTIs) similar to antibiotics, and combining vitamin C with a lower dose of nitrofurantoin had a better therapeutic effect. Therefore, the study concludes that vitamin C can be a beneficial treatment for UTIs, both as an antibacterial and anti-biofilm agent, and either alone or in combination with antibiotics.[22]

Improvements in virtual consultations could provide an opportunity to enhance patient care in the long term.

CONCLUSION
The epidemiology of ESBL-producing isolates can be studied using multilocus sequence typing (MLST). To increase the success of multidrug resistant E. coli infections, clinical investigations are also required to develop the most efficient antimicrobial therapies and duration of therapy. These investigations are also required to determine the economic consequences of these infections as well as the epidemiology and risk factors for the emergence of MDR E. coli.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

SOURCE OF FUNDING

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2	Beenish Haleem	Acquisition, Data Analysis & interpretation.	
3	Sana Akhlaq	Drafting the article for intellectual content & Final Approval of the version. Diagram design & formatting proof reading.	
4	Manal Tariq	Data acquisition & Proof reading.	
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