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Clinical and molecular analysis of ESBL, carbapenemase, and colistin-resistant bacteria in UTI patients

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Abstract

Uropathogens, particularly bacteria, can infect any part of the urinary tract and cause bacteriuria. Our study aimed to examine the antibiotic-resistant profile, associated risk factors, and phenotypic and genotypic features of ESBL, carbapenemase, and *mcr* resistance genes in multidrug-resistant bacteria. Samples were inoculated on culture media, identified using standard biochemical tests, and species confirmation was performed via 16S rRNA gene amplification. Furthermore, antibiotic susceptibilities were evaluated using the Kirby-Bauer disc diffusion method. The phenotypically confirmed resistant strains were further inspected for ESBL, carbapenemases, and *mcr* variants using PCR. Merely 57.24% (83/145) of the samples exhibited growth. Of these, 39.70% (33/83) were identified as *Klebsiella pneumoniae*, 27.70% (23/83) as *Escherichia coli*, 10.80% (9/83) as *Pseudomonas aeruginosa*, 9.60% (8/83) as *Staphylococcus aureus*, 7.20% (6/83) as *Proteus mirabilis,* and 4.80% (4/83) as *Staphylococcus saprophyticus*. Overall, 22.54% (16/71) of the gram-negative strains were confirmed molecularly to have resistant genes. The ESBL – producers accounted for 21.74% (5/23) of *E. coli*, 21.21% (7/33) of *K. pneumoniae*, and 22.22% (2/9) of *P. aeruginosa*. Likewise, carbapenemase-harboring strains included 6.06% (2/33) of *K. pneumoniae*, 4.35% (1/23) of *E. coli*, and 11.11% (1/9) of *P. aeruginosa*. Notably, 3.03% (1/33) of *K. pneumoniae*, 8.70% (2/23) of *E. coli*, and 11.11% (1/9) of *P. aeruginosa* strains tested positive for the *mcr*-1 gene. None of the *Proteus* strains showed any resistant genes. The most common variants were $bla_{\text{SHV-11}}$ (non-ESBL) and $bla_{\text{CTX-M-15}}$ (ESBL) accounted for 28.57% (4/14) each, $bla_{\text{TEM-116}}$ accounted for 14.29% (2/14), $bla_{\text{SHV-1}}$, $bla_{\text{SHV-75}}$, $bla_{\text{TEM-1}}$ and $bla_{\text{OXA-1}}$ accounted for 7.14% (1/14) each of the ESBL. Similarly, the carbapenemase variants included $bla_{\text{OXA-48}}$, $bla_{\text{NDM-1}}$, $bla_{\text{VIM-1}}$, and $bla_{\text{KPC-2}}$, each accounting for 25.0% (1/4), while 37.50% (6/16) of the strains exhibited co-existence of different gene variants. Based on our findings, it can be concluded that females, especially those in middle age, were more infected. These pathogens exhibited a wide range of ESBL, carbapenemase, and *mcr*-1 variants. Imipenem was suggested as the preferred medication.

Keywords: UTI, Uropathogens, Associated factors, Antimicrobial resistance, ESBL, Carbapenemase, *mcr*

1. Introduction

Pathogenic bacteria that commonly cause urinary tract infections (UTIs) can affect any part of the urinary tract, leading to bacteriuria [1]. This infection disrupts the regular operations of the normal kidneys and urinary tract, posing significant health risks to individuals with weakened immune systems. Additionally, it incurs substantial medical expenses and poses a significant threat to life [2]. The urinary tract is the second most common site of bacterial infection among humans, following the respiratory tract. The infection targets people of almost all ages, from neonates to aged people, but they are most frequently found in females between the ages of 16 and 35 years. According to a study, approximately 10.0% of women are infected yearly, while 60.0% experience at least one infection in their lifetime [3].

Bacteria cause UTIs more frequently in females than males due to differences in their urinary tract anatomy [4].

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However, pregnant females are at an even higher risk due to the composition of their urine, which contains excessive amounts of amino acids and glucose, making it easier for bacteria to grow and consequently facilitating bacterial growth in urine. As a result, there is a higher likelihood of developing bacteriuria after giving birth and increased chances of it becoming symptomatic or developing into a chronic infection [5, 6]. Pregnancy and sexual interaction are considerable risk factors in women that lead to bacteriuria because the pathogenic bacteria may pass through the female urethra during sexual contact [7].

Bacteriuria caused by gram-negative bacteria is a significant clinical and microbiological concern due to the presence of extended-spectrum beta-lactamase (ESBL), carbapenemase, and mcr resistance genes [8]. It is because numerous community-based research studies have reported the isolation of multidrug-resistant (MDR) strains, which have shown adverse effects due to the presence of ESBL, mcr, and carbapenemase genes [8, 9]. The unrestricted use and prescription of third-generation cephalosporin (3GC) and carbapenem antibiotics may contribute to the emergence of new classes of these beta-lactamase enzymes [10].

Resistance mediated by enzymes belonging to the ESBL class can hydrolyze oxyimino beta-lactam and monobactam antibiotics, making it a major global health concern [11]. The most common ESBL variants commonly considered the leading cause of nosocomial and community-acquired infections comprise $bla_{\text{CTX-M}}$, bla_{SHV}, bla_{TEM}, and $bla_{\text{OXA-1}}$ [12]. Similarly, resistance mediated by carbapenemase variants, including $bla_{\text{NDM-1}}$, $bla_{\text{OXA-48}}$, bla_{VIM}, and $bla_{\text{KPC-2}}$, can hydrolyze all beta-lactam antibiotics except monobactam [11, 13]. These plasmid-mediated genes are primarily found in gram-negative bacteria, especially in *Enterobacteriaceae* isolates [14]. Importantly, these resistance genes can be easily transferred vertically to descendants and horizontally to other bacterial strains through gene transfer mechanisms [15]. The prevalence of both ESBL and carbapenemase-producing bacteria in the community poses a significant challenge to public health [16, 17]. The contemporary emergence of resistance to ESBL and carbapenemase has dramatically increased and propagated worldwide [18].

Being a plasmid-mediated resistant gene like carbapenemase and ESBLs, the mcr gene also diminishes bacterial sympathy towards colistin by translating an enzyme called phosphoryl ethanolamine transferase. The enzyme backs in decreasing the negative charge of the bacterial outer membrane, thereby promoting bacterial resistance [19]. The mcr gene was primarily documented in 2016 in bacterial strains belonging to *Enterobacteriaceae* [20, 21]. However, the resistance was rapidly propagated due to the high demand for colistin in clinical practices and its uncontrolled use in agriculture [22]. Many bacterial strains have been found to acquire colistin resistance, including Acinetobacter baumannii, *K. pneumoniae*, and *P. aeruginosa*. At the same time, others have been identified as naturally resistant in genera comprising Serratia, *Burkholderia*, and *Proteus* [23, 24].

The constant increase in plasmid-mediated resistance among bacteria carrying ESBL, carbapenemase, and mcr genes poses a major risk to public health. If left unaddressed, it could result in untreatable infections in the near future. Therefore, the primary objective of this study is to investigate the factors associated with UTI in the local population, with a particular focus on the prevalence of MDR Gram-negative bacteria. Specifically, the study aims to analyze the resistance patterns of uropathogenic strains and detect the phenotypic and genotypic presence of ESBL, Carbapenemase, and mcr resistance genes. By understanding the distribution of these resistance genes, this study seeks to contribute to improving the treatment strategies for UTIs and address the growing concern of antimicrobial resistance in the regions.

2. Materials and Methods

2.1. Study setting and specimen processing

The current study was conducted in the microbiology section of the Pathology laboratory at Ayub Medical College, Abbottabad, Pakistan. A total of 145 urine specimens were collected from patients with UTI (both male and female) who were referred by physicians during 7 months from June to December 2023. The inclusion criteria for sample collection were the entire gram-negative bacterial strains isolated from the urine samples while the exclusion criteria were non-urine samples or those collected outside the specified period. Midstream urine specimens, ranging from 20-30 ml, were collected from each patient using sterile, dried-up, wide-mouth, leakproof sterile urine containers. The containers were properly labelled for microbiological examination. Female patients suspected of having UTIs were advised to wash the urethral cavity area with fresh water, dry it, and then collect the urine in the container. Male participants were instructed to wash their hands with fresh water before collecting the specimen. In addition to collecting the urine specimens, we also recorded and considered various risk factors for UTIs in each patient. These factors included anatomical disorders, diabetes mellitus, kidney stones, pregnancy (excluding males), and the use of catheters.

The specimens were thoroughly mixed by gently shaking the container a few times. They were then carefully placed on pre-incubated MacConkey agar (CM0115-Oxoid) and Eosin Methylene Blue (CM0069-Oxoid) culture media designed explicitly for uropathogens. The labelled specimens were incubated under aerobic conditions at 37°C overnight. Plates were re-incubated for a further 24 hours in case of no growth. The grown colonies were differentiated as gram-negative and/or gram-positive bacteria using the gram-staining method. They were further identified via various biochemical tests, including catalase, coagulase, oxidase, Sulphur indole motility (SIM), and triple sugar iron (TSI) [7]. Each of the isolated bacteria was maintained in the Luria Bertani (LB) for further processing, i.e. molecular analysis and antibiotic sensitivity testing. Only gram-negative strains were subjected to molecular identification. In contrast, gram-positive strains were not included in the analysis, as gram-positive bacteria were not the primary focus of this study. Gram-positive strains were merely examined for susceptibility to ensure appropriate treatment for the patients in the study area.

2.2. Identification by 16S rRNA gene amplification

All the isolated bacterial strains were subjected to 16S rRNA identification. The genomic DNA was extracted from the selected strains, and the 16S rRNA genes were amplified by PCR using universal primers, 16S F (AAATTGAAGAGTTTGATCATGG) and 16S R (GCTTCTT-

TAAGGTAAGGAGGT) and sequenced at BGI (Qingdao, China) [25]. The EzTaxon-e server was used to identify phylogenetic neighbors (Figures S1, S2, and S3) and calculate pairwise 16S rRNA gene sequence similarities (http://eztaxon-e.ezbiocloud.net/) [26].

2.3. Antimicrobial susceptibility testing (AST)

The antimicrobial susceptibility pattern of all isolated uropathogens was tested on Mueller Hinton agar (MHA) (CM0337-Oxoid) [4] using commercially available selected antibiotics (Oxoid) given in Figure 1, as recommended for gram-positive and gram-negative uropathogenic bacteria [27]. Prior to the test, the colonies of each bacterial isolate were inoculated into pre-incubated capped tubes containing 5ml tryptic soy broth (CM129-Oxoid) and allowed to incubate for 18 h at 35±2°C. The turbidity of each tube was standardized to a 0.5 McFarland index and inoculated on pre-incubated MHA against antibiotic discs following the Kirby-Bauer disc diffusion method [28]. The plates were then incubated at 37°C for 18 h. The obtained results of each strain were examined, measured, and compared with a standard chart for uropathogens [7].

2.4. Phenotypic detection of ESBL, carbapenemase, and *mcr*, bearing strains.

All the isolated strains of gram-negative bacteria that exhibited high resistance to 3GC (ceftriaxone, cefotaxime) and carbapenem antibiotics were subjected to a double disc synergy test and Modified Hodge test (Figure S4) for the phenotypic detection of ESBL [17] and carbapenemase-producing strains, respectively [29]. Before the test, the strains were inoculated in capped test tubes containing 5ml of tryptic soy broth each. After reaching a 0.5 McFarland index, the culture was streaked on MHA medium plates as a lawn.

In the case of ESBL detection, the 3GC antibiotics were placed 24mm apart from each other with a central amoxicillin-clavulanic acid (20µg, 10µg) antibiotic disc, as previously described [16]. The enhancement of the zone of inhibition of any antibiotic disc of 3GC towards the central disc after the incubation period at 36±2°C was detected as ESBL producers. For carbapenemase detection, a disc of meropenem was placed at the center of the testing area, and the tested strain was streaked out in a straight line from the edge of the plate to the disc. After inoculation, the plates were incubated for 18 hours at 35±2°C. The strain was considered positive for carbapenemase enzymes when the culture appeared as a cloverleaf pattern. Meanwhile, broth micro-dilution methods (Figure S5) were used to detect colistin-resistant isolate phenotypically using colistin sulfate powder (Oxoid) [30]. The achieved results of the minimum inhibitory concentration (MIC) were interpreted by EUCAST (the European Committee on Antimicrobial Susceptibility Testing) guidelines as followed in the previous study [24]. The *E. coli* ATCC25922 strain was used as a negative control for susceptibility testing of all antibiotics, and the *K. pneumoniae* ATCC1795 strain was used as a positive control for susceptibility testing.

2.5. PCR amplification of antibiotic resistance genes

The resistant strains detected phenotypically as ESBL, carbapenemase producers, and/or colistin-resistant were selected for antibiotic-resistant genes (ARG) identification. The current study used previously described primers (Table S1) and well-optimized conditions for conventional and multiplex PCR (Polymerase Chain Reaction) to inspect the presence of the aforementioned genes [8]. The genomic DNA of the selected bacterial strains was extracted using the boiling method, as described previously [31]. A 100bps DNA marker (ladder) was used to identify gene sizes. Finally, the resulting gene product was visualized using a gel documentation system (Figure S6). The PCR products were sequenced, and gene confirmation was performed by aligning the sequencing result on NCBI.

2.6. Data Analysis

The analysis was conducted using IBM SPSS version 22.0 and GraphPad Prism 8.0. A Chi-square test was conducted to analyze risk factors comparatively between gender and age groups. The p-value <0.05 was considered statistically significant. Binary logistic regression was examined using dichotomous and continuous variables, while logistic regression was examined with multichotomous variables. The phylogenetic analysis was conducted using MEGA11.

3. Results

For this study, we analyzed a total of 145 urine specimens from patients who were referred by their physician due to suspected urinary tract infections. The age of the patients ranged from 1 to 90 years, with a mean age of 37.34±20.414 years. Among the specimens, only 57.24% (83/145) showed colonies of different strains of bacteria when they were inoculated on appropriate culture media. The majority of positive cases were reported in the female population, with a higher percentage than the male population. Among different age groups, the 16-30 age group had the highest number of positive cases, with a total of 22 cases. This was followed by the 46-60 age group, which had 21 cases. The lowest number of positive cases was found in individuals under the age of 15 (≤15 years). The correlation between gender and age group of UTI patients was found to be statistically insignificant (Table 1).

The logistic values obtained from the analysis showed no statistically significant associations. None of the variables examined, such as being female, having an anatomical disorder of UTIs, diabetes mellitus, kidney stone, pregnancy, catheter, urology, surgical, or gynae conditions, demonstrated any significant effects. The odds ratios and confidence intervals for each variable fell within a range that did not reach statistical significance (p>0.05). Only pregnancy showed slightly significant results, while the other variables were highly insignificant (Table 2).

Of the 145 specimens, 83 (57.24%) tested positive for bacteria. Among positive specimens, 71 (85.54%) exhibited growth as gram-negative bacteria, including *K. pneumoniae, E. coli*, *P. aeruginosa*, and *P. mirabilis*. The remaining 12 specimens (14.46%) showed growth as gram-positive bacteria, specifically *S. aureus* and *S. saprophyticus*. The most prevalent uropathogenic bacteria isolated was *K. pneumoniae*, accounting for 39.70% of all cases. This was followed by *E. coli* (27.70%), *P. aeruginosa* (10.80%), *S. aureus* (9.60%), and *P. mirabilis* (7.20%). *S. saprophyticus* was the least prevalent bacteria, accounting for only 4.80% of cases. Interestingly, in contrast to the male population, the prevalence rate for each bacterial strain was higher in the female population. However, no significant was found between genders regarding bacterial isolates (Table 3).The

Table 1. Frequency distribution of positive samples in the age groups and statistical relationship between age and gender variables.

Age group (Year)	Total frequency n (%)		Male n (%)		Female n (%)		p-value
	Total	Positive	Total	Positive	Total	Positive	
1-15	18 (12.41)	11 (13.25)	07 (12.96)	03 (11.11)	11 (12.09)	08 (14.29)	0.205
16-30	48 (33.10)	22 (26.51)	15 (27.77)	04 (14.81)	33 (36.26)	18 (32.14)	0.072
31-45	31 (21.38)	15 (18.07)	13 (24.07)	07 (25.93)	18 (19.78)	08 (14.29)	0.605
46-60	30 (20.70)	21 (25.30)	11 (20.37)	06 (22.22)	19 (20.88)	15 (26.78)	0.160
≥ 61	18 (12.41)	14 (16.87)	08 (14.81)	07 (25.93)	10 (10.99)	07 (12.50)	0.375
Total	145 (100)	83 (57.24)	54 (37.24)	27 (32.53)	91 (62.76)	56 (67.47)	

Table 2. Logistic regression analysis of UTI, associated factors, gender, and wards.

Variable	Level	OR	95% CI	*p*-value
Gender	Female	0.906	0.373-2.202	0.828
	Male	1		
Associated factors	Anatomical disorder of UTIs	0.717	0.045-11.492	0.815
	Diabetes mellitus	0.253	0.017-3.764	0.319
	Kidney Stone	0.377	0.027-5.226	0.467
	Pregnancy	0.056	0.003-1.126	**0.060**
	Catheter	0.271	0.024-3.102	0.271
	Other	1		
Wards	Urology	1.042	0.160-6.787	0.966
	Surgical	1.323	0.202-8.684	0.770
	Gynae	6.957	0.628-77.042	0.114
	Others	1		

Table 3. Frequency distribution of uropathogenic bacteria isolated from UTI patients.

Bacterial isolate	Total positive n (%)	Male n (%)	Female n (%)	p-value
Klebsiella pneumoniae	33 (39.76)	11 (13.25)	22 (26.51)	0.890
Escherichia coli	23 (27.71)	06 (07.23)	17 (20.48)	0.430
Pseudomonas aeruginosa	09 (10.84)	04 (04.82)	05 (06.02)	0.410
Proteus mirabilis	06 (07.23)	02 (02.41)	04 (04.82)	0.960
Staphylococcus aureus	08 (09.64)	03 (03.62)	05 (06.02)	0.750
Staphylococcus saprophyticus	04 (04.82)	01 (01.20)	03 (03.62)	0.740
Total	83 (57.24)	27 (32.53)	56 (67.47)	

bacterial strains exhibited different resistance patterns to the antibiotics under evaluation. Figure 1 highlights the resistance patterns of both gram-negative and gram-positive strains of isolated bacteria. Gram-negative bacteria, such as *P. aeruginosa*, demonstrated significant resistance to ceftriaxone, cefotaxime, nitrofurantoin, gentamicin, ciprofloxacin, and fosfomycin. However, it displayed lower resistance to imipenem and amikacin. It was discovered that *P. mirabilis* exhibited a relatively prominent level of resistance. It showed complete resistance to ceftriaxone, gentamicin, and cefotaxime and high resistance to ciprofloxacin (77.80%) and nitrofurantoin (67.60%). However, it did not exhibit any resistance to fosfomycin and imipenem. In the current study, the most prevalent *K. pneumoniae* exhibited the lowest resistance pattern among gram-negative bacteria. The results indicated complete resistance to ceftriaxone, with ciprofloxacin showing 94.0% resistance and gentamicin showing 91.0% resistance. The strains showed high sensitivity to imipenem, meropenem, and fosfomycin.

Out of the gram-positive bacteria, *S. saprophyticus* exhibited the highest resistance to evaluated antibiotics. Both *S. aureus* and *S. saprophyticus* exhibited a prominent level of resistance to lincomycin and clindamycin, with a complete resistance rate of 100%. These strains also demonstrated considerable resistance to cephradine, with rates of 87.50% and 50.0% respectively. Additionally, ciprofloxacin showed 75.0% and 50.0% resistance rates for *S. saprophyticus* and *S. aureus*, respectively. It is important to note that these strains did not exhibit any zone of inhibition when exposed to imipenem. On the other hand, when assessing the effectiveness of antibiotics against all bacterial strains, imipenem emerged as the most potent, with meropenem and fosfomycin closely following (Figure 1).

Of the gram-negative bacteria analyzed, the percentages of ESBL producers were as follows: 21.74% (5/23) for *E. coli*, 21.21% (7/33) for *K. pneumoniae*, and 22.22% (2/9) for *P. aeruginosa* strains. Additionally, carbapenemase was found in 6.06% (2/33) of *K. pneumoniae* strains, 4.35% (1/23) of *E. coli* strains, and 14.28% (1/7) of *P. aeruginosa* strains. Furthermore, the *mcr*-1 gene was detected in 3.03% (1/33) of *K. pneumoniae*, 8.70% (2/23) of

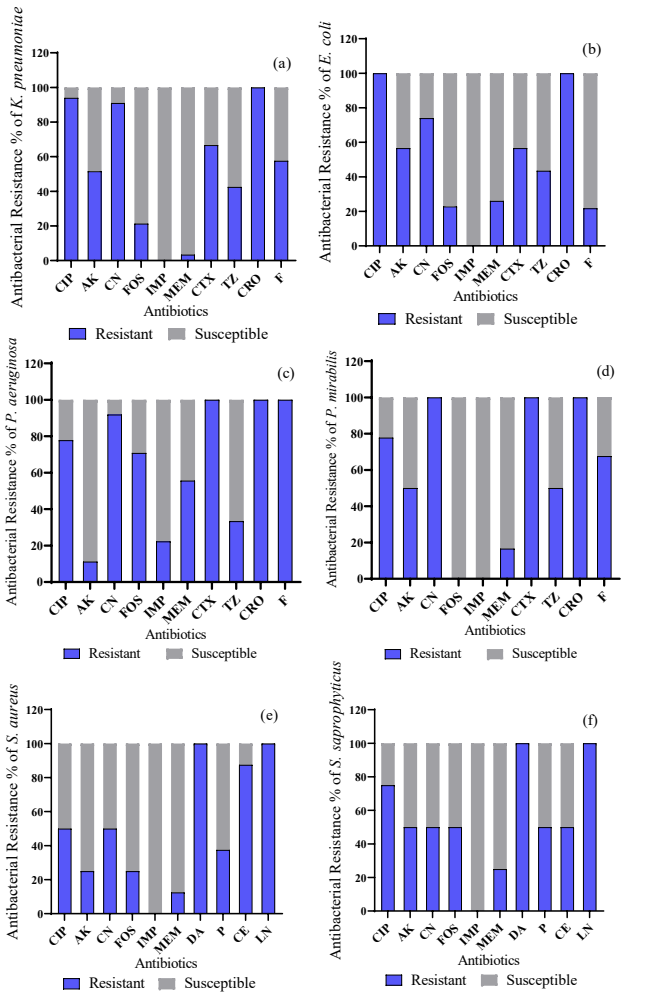

Fig. 1. Antimicrobial resistance patterns posed by bacteria isolated from UTI patients, including *K. pneumoniae* (2a), *E. coli* (2b), *P. aeruginosa* (2c), *P. mirabilis* (2d), *S. aureus* (2e), and *S. saprophyticus* (2f) respectively. CN - ciprofloxacin; AK - gentamicin; FOS - fosfomycin; IMP - imipenem; MEM - meropenem; CTX - cefotaxime; TZ - tazobactam; CRO - ceftriaxone; F - nitrofurantoin; DA - clindamycin; P - penicillin G; CE - cephradine; LN – lincomycin.

E. coli strains, and 11.11% (1/9) of *P. aeruginosa* strains. None of the *P. mirabilis* strains tested were found to be ESBL or carbapenemase producers. However, it is important to note that *Proteus* strains naturally resist colistin, as confirmed phenotypically (Figure 2).

From the analysis of gene variants, it was found that bla_{SHV-11} (non-ESBL) and $bla_{CTX-M-15}$ accounted for 28.57% (4/14) each of the ESBL gene variants, $bla_{TEM-116}$ accounted for 14.29% (2/14), bla_{SHV-1}, bla_{SHV-75}, bla_{TEM-1} and bla_{OXA-1} accounted for 7.14% (1/14) each. Additionally, each strain included carbapenemase-producing genes, with bla_{OXA-48}, bla_{NDM-1}, bla_{VIM-1}, and bla_{KPC-2}, each accounting for 25.0% (1/4) of the strains. It is worth noting that all strains (4/4) expressed *mcr*-1. Furthermore, 37.50% (6/16) of the strains exhibited the co-existence of different gene variants (Table 4).

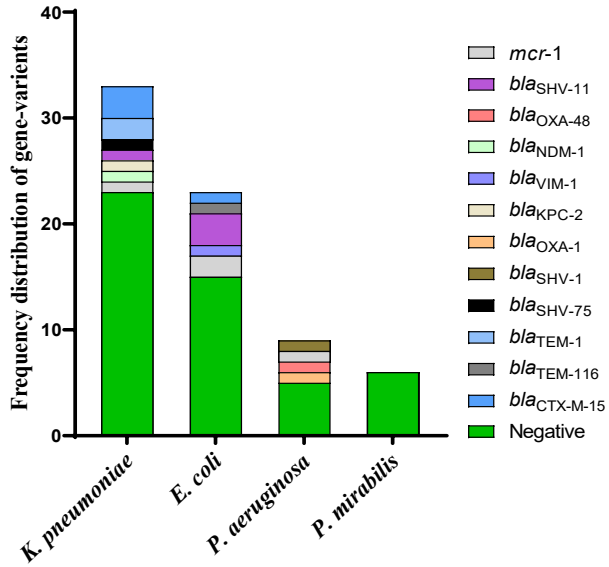

Fig. 2. Frequency distribution of ESBL-, carbapenemase-, and *mcr*-1-variants harboring gram-negative bacteria.

Table 4. Demographic existence of ESBL, carbapenemase, and *mcr*-1 variants in gram-negative bacteria.

Isolates	Sex	Age (y)	Ward	Organism	Detected gene-variants
Sam04KP	F	70	Surgical	*K. pneumoniae*	bla_{TEM-1}
Sam17PA	F	63	Urology	*P. aeruginosa*	bla_{OXA-1}, *mcr*-1
Sam40KS	F	75	Surgical	*K. pneumoniae*	bla_{NDM-1}
Sam43KS	F	50	Urology	*K. pneumoniae*	bla_{TEM-1}, *mcr*-1
Sam53KS	M	40	Urology	*K. pneumoniae*	$bla_{CTX-M-15}$
Sam59PA	M	51	Surgical	*P. aeruginosa*	bla_{SHV-1}, bla_{OXA-48}
Sam74KS	F	44	Gynae	*K. pneumoniae*	$bla_{CTX-M-15}$, bla_{SHV-75}
Sam77EC	F	15	Urology	*E. coli*	$bla_{TEM-116}$
Sam85KS	F	25	Gynae	*K. pneumoniae*	bla_{KPC-2}
Sam86EC	F	55	Surgical	*E. coli*	bla_{SHV-11}, *mcr*-1
Sam91EC	M	25	Surgical	*E. coli*	bla_{SHV-11}
Sam107EC	F	30	Gynae	*E. coli*	$bla_{CTX-M-15}$, *mcr*-1
Sam116KS	M	65	Urology	*K. pneumoniae*	$bla_{CTX-M-15}$
Sam120KS	F	35	Surgical	*K. pneumoniae*	bla_{SHV-11}
Sam122EC	F	60	Urology	*E. coli*	bla_{VIM-1}
Sam133EC	F	26	Gynae	*E. coli*	bla_{SHV-11}

4. Discussion

Pathogenic strains, particularly gram-negative bacteria, found in urine samples have received significant attention due to their possessing a wide range of plasmid-mediated resistance genes. This is sparked not only of interest because of their role in causing UTIs, but also due to their potential implications for antibiotic resistance [14]. These genes enable bacteria to defend themselves against a wide range of potent antibiotics commonly found in the community. This study examined various pathogenic bacteria strains, including *E. coli*, *P. aeruginosa*, *K. pneumoniae*, *P. mirabilis*, *S. aureus*, and *S. saprophyticus*. These strains were identified as potential culprits behind urinary tract infections in both males and females within the study area. Previous studies consistently reported that certain bacterial strains may be responsible for causing UTIs in the population [32-34]. Our study revealed that these uropathogens were quite common among females, with an infection rate of 67.47% (56/83), compared to males, with an infection rate of 32.53% (12/83), and an average age of 37.34±20.414 years. These results align with previous research findings, which observed a higher infection rate among females (73.0%) compared to males (27.0%), with an average age of 35.82±15.3 years [35]. Females are more susceptible to urinary tract infections compared to males. In this study, we have identified several risk factors associated with UTIs, including anatomical disorders, diabetes mellitus, kidney stones, pregnancy, and the use of catheters. However, none of these factors demonstrated any significant correlation with the infection. This aspect of the study is backed by a recently published research that also takes these related factors into account [7].

The overall prevalence rate of uropathogens in the current study was 57.24% (83/145). The most common uropathogens were *K. pneumoniae* (39.76%), *E. coli* (27.71%), *P. aeruginosa* (10.84%), and *P. mirabilis* (7.23%). In a previous research study conducted in Argentina, the prevalence rate was 25.5%, with *E. coli* being the most prevalent uropathogens (47.30%), followed by *Enterococcus faecalis* (13.60%) and *K. pneumoniae* (11.90%). The prevalence rate of gram-negative uropathogens was 78.80%, which is maximal compared to gram-positive uropathogens [34]. Our results also found that 85.50% of the bacterial isolates were gram-negative (*E. coli*, *P. aeruginosa*, *K. pneumoniae*, *P. mirabilis*) in which *K. pneumoniae* (39.76%) was the most prevalent uropathogens, followed by *E. coli* (27.71%). It is important to note that previous studies have shown variations in the prevalence rate of uropathogens [4, 9, 15]. The reason could be poor infection control practices, variations in detection methodologies, or other hidden factors.

The pathogenic strains are increasingly becoming resistant to recently used broad-spectrum antibiotics, which poses a challenge to public health [8]. Our study found that gram-negative bacterial isolates were more resistant to the evaluated antibiotics compared to gram-positive bacteria. This is consistent with previous research, which also showed that gram-negative isolates had a higher resistance potency than gram-positive isolates [7]. Among the gram-negative pathogens, we found that *P. aeruginosa* exhibited the highest resistance to a wide range of evaluated antibiotics, followed by *P. mirabilis*, *E. coli*, and *K. pneumoniae*. This is in line with the findings of a previous study that also reported high resistance in *P. aeruginosa* [36]. The results of our study showed that *P. aeruginosa* had a 100% resistance rate to ceftriaxone, cefotaxime, and nitrofurantoin. It also showed relatively lower resistance rates to fosfomycin (78.80%), ciprofloxacin (78.80%), and gentamicin (74.0%). *P. aeruginosa* exhibited sensitivity to amikacin (88.80%), followed by imipenem (77.70%) and tazobactam (66.60%). In a previous study, it was found that *P. aeruginosa* had a high sensitivity to amikacin (74.70%) and imipenem (89.40%) but demonstrated significant resistance to other antibiotics such as ceftriaxone (99.20%), cephradine (99.20%), and fosfomycin (63.90%) [37].

In this study, *P. mirabilis* exhibited complete resistance to ceftriaxone, gentamicin, and cefotaxime. It showed a resistance rate of 77.80% to ciprofloxacin and 66.70% to nitrofurantoin. However, it demonstrated full sensitivity to fosfomycin and imipenem and an 83.40% sensitivity to meropenem. The *E. coli* bacteria showed complete resistance to ciprofloxacin and ceftriaxone and a significant resistance of 74.0% to gentamicin. However, imipenem (100%) and meropenem (74.0%) demonstrated strong effectiveness against all antibiotics. The resistance rates of *K. pneumoniae* to ceftriaxone, ciprofloxacin, gentamicin, and cefotaxime were high, ranging from 66.70% to 100%. However, it showed sensitivity to imipenem, with a 100% sensitivity rate, as well as moderate sensitivity to fosfomycin (78.70%) and meropenem (69.60%). Previous studies have found comparable results to ours, indicating that *P. mirabilis*, *E. coli*, and *K. pneumoniae* are extremely sensitive to imipenem, meropenem, and fosfomycin. However, these bacteria have shown high resistance to ceftriaxone, gentamycin, ciprofloxacin, and cefotaxime [38, 39].

Among the gram-positive bacteria in our study, *S. saprophyticus* exhibited the highest resistance level, with *S. aureus* following closely behind. The resistance rates of *S. saprophyticus* to clindamycin and lincomycin were both 100%, while ciprofloxacin showed a resistance rate of 75.0%. However, it showed sensitivity to imipenem at 100% and meropenem at 75.0%. The bacterium *S. aureus* showed high resistance to clindamycin (100%), lincomycin (100%), and cephradine (87.50%). On the other hand, imipenem (100%), meropenem (87.50%), fosfomycin (75.0%), and penicillin G (62.50%) demonstrated strong effectiveness against this bacterium. Another study supports our findings that certain gram-positive bacteria, such as *S. aureus* and *S. saprophyticus*, exhibited high resistance to clindamycin and ciprofloxacin but were sensitive to imipenem, meropenem, penicillin G, and fosfomycin [40].

The expanded resistance towards commercially available antibiotics such as carbapenems, beta-lactams, and other potential drugs poses a global clinical concern [8]. Our investigation concluded that the isolates containing resistance genes, such as ESBL, carbapenemase, and *mcr*-1, had a remarkable level of multidrug resistance, particularly against 3GC and carbapenemase. This increase in resistance to these antibiotics can be explained, which is consistent with previous research [41]. According to our current study, *P. aeruginosa* had the highest prevalence of ESBLs (22.22%), followed by *E. coli* (21.74%) and *K. pneumoniae* (21.21%). In a previous study, these infections were also identified as ESBL producers, which yielded similar results [9]. Another earlier study reported similar findings for these gram-negative bacteria [18]. Our study also found that 6.06% (2/33) of *K. pneumoniae*,

4.35% (1/23) of *E. coli*, and 11.11% (1/9) of *P. aeruginosa* expressed carbapenemase. Similarly, in our current study, *K. pneumoniae* expressed 3.03% *mcr*-1, *E. coli* 8.70% (2/23), and *P. aeruginosa* 11.11% (1/9). This aspect of our study aligns with previous findings [8]. However, none of the targeted resistant genes were found in *P. mirabilis* in our study. It should be noted that *P. mirabilis* has been reported in the past for the expression of these genes [42]. This lack of presence could be due to the low prevalence of *P. mirabilis* in the study area.

The uropathogens in our study expressed different variants of ESBL (bla_{SHV-1}, bla_{SHV-75}, $bla_{CTX-M-15}$, bla_{TEM-1}, $bla_{TEM-116}$, and bla_{OXA-1}), non-ESBL (bla_{SHV-11}), carbapenemase genes (bla_{OXA-48}, bla_{NDM-1}, bla_{VIM-1}, bla_{KPC-2}), and *mcr*-1. Similarly, previous studies have also reported the expression of these gene variants in similar isolates [8, 14]. We found that the prevalence rate of ESBL variants $bla_{CTX-M-15}$ and bla_{SHV-11} (non-ESBL) accounted for 28.57% (4/14) each, $bla_{TEM-116}$ accounted for 14.29% (2/14), bla_{SHV-1}, bla_{SHV-75}, bla_{TEM-1} and bla_{OXA-1} accounted for 7.14% (1/14) each. Comparably, the existence rate of carbapenemase variants bla_{OXA-48}, bla_{NDM-1}, bla_{VIM-1}, and bla_{KPC-2} was 25.0% (1/4) each, while *mcr*-1 was expressed in all cases (100%, 4/4). Additionally, 37.50% (6/16) of the strains exhibited co-existence of different gene variants. These findings are consistent with a previous study [8].

Our study also emphasizes the importance of identifying alternative treatments or combination therapies for UTIs caused by resistant strains. Empirical therapy with broad-spectrum antibiotics like imipenem should be complemented by laboratory testing to guide more targeted treatment, thereby enhancing the success of therapy while minimizing the risk of resistance development.

5. Conclusion

Based on the findings of the study, it was observed that the female population in the study area had a higher prevalence compared to the male population, particularly within the middle age group. The infection was primarily associated with specific age groups. The study findings identified various resistant variants of ESBL (bla_{SHV-1}, bla_{SHV-75}, $bla_{CTX-M-15}$, bla_{TEM-1}, $bla_{TEM-116}$, bla_{OXA-1}), non-ESBL (bla_{SHV-11}), and carbapenemase (bla_{OXA-48}, bla_{NDM-1}, bla_{VIM-1}, bla_{KPC-2}) in multiple gram-negative MDR strains, including *K. pneumoniae*, *E. coli*, and *P. aeruginosa*. Molecular analysis revealed that certain gene variants coexist within a small number of strains. Treating MDR strains with such a wide range of genes can be quite challenging. In the study area, imipenem was suggested as the preferred drug among orally administered antibiotics, followed by meropenem and fosfomycin. Administering antibiotics with the appropriate dose and duration under medical expertise is crucial in preventing or reducing the development of antibiotic resistance. Before prescribing antibiotics, it is important to evaluate MDR strains of gram-negative bacteria for the presence of resistance genes such as ESBL-, carbapenemase-variants, and *mcr*-1.

Study limitations

The study has several limitations, including a small sample size of 145 urine samples, which may not represent the broader population and limit the generalizability of the findings. Potential risk factors for UTIs, such as anatomical disorders, diabetes, and pregnancy, show no significant correlation, possibly due to sample size or unaccounted confounding factors, the study geographic limitations may also affect the relevance of the results to other regions with different bacterial resistance profiles, additionally, the focus on ESBL and carbapenemase resistance left other resistance mechanisms, like efflux pumps or alter targets, under explored, liming a comprehensive understanding of antibiotic resistance in the study population.

Conflict of interest

All the authors declare no conflict of interest.

Ethical approval and consent to participate

The research study was approved by the institutional bioethical committee (IBC) of Hazara University, Pakistan (F. No. 23/HU/IBC/2023/31) and found in accordance with the ethical principles and policies followed by this university. Furthermore, the proposed procedure and methodology of this research are in accordance with the ethical principles.

Availability of data and material

Data is contained within the article and supplement materials.

Author contributions

All authors contributed to the intellectual input and assisted in this study and manuscript preparation. Conceptualization, F.A.; Methodology, F.A., Q.S. and S.A.; formal analysis, P.T.S. and A.A.; investigation, F.A. and J.J.; data curation, R.U.K. and H.B.; visualization, F.A. and T.A.; writing-original draft preparation, F.A., P.T.S and H.B.; writing-review editing & editing, F.A., S.R. and R.U.K; Supervision, S.A. and Q.S.; project administration, Q.S. and S.A.; funding acquisition, S.R., T.A., A.A. All authors have read and agreed to the published version of the manuscript.

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