Original Article

Extensive drug-resistance in strains of *Escherichia coli* and *Klebsiella pneumoniae* isolated from paediatric urinary tract infections

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Abstract

**Objectives:** Urinary tract infections (UTIs) in children are rapidly increasing worldwide and are commonly caused by extensively drug-resistant bacteria. This study determines the prevalence of UTIs in paediatric patients and evaluates the pattern of extensively drug-resistance in *Escherichia coli* and *Klebsiella pneumoniae* strains isolated from paediatric UTI patients.

**Methods:** Uropathogenic bacterial strains were isolated from paediatric patients with UTIs admitted to the Institute of Child Health, Lahore, Pakistan. Strains of both *E. coli* and *K. pneumoniae* were identified using biochemical characterisation and subjected to antibiotic susceptibility assays for 21 common antimicrobial drugs in order to determine their extensively drug-resistant profile.

**Results:** We isolated 63 *E. coli* and 37 *K. pneumoniae* strains from 130 paediatric patients with UTIs over a period of six months. The antibiotic susceptibility assays showed that both the *E. coli* and *K. pneumoniae* strains exhibited a high degree of resistance against co-amoxiclav, cefuroxime, cefixime, cefotaxime, ceftazidime, ceftriaxone, ciprofloxacin, nalidixic acid, norfloxacin, piperacillin, and co-trimoxazole. However, several of the antimicrobial agents, including polymyxin...
B, colistin sulphate, chloramphenicol, nitrofurantoin, and fosfomycin, were found to retain their antimicrobial activities against both pathogens. The five highest antibiotic resistant strains were identified as *E. coli* strains ZK9, ZK40, and ZK60 and *K. pneumoniae* ZK32 and ZK89 using 16S rRNA gene sequencing.

**Conclusion:** Our study demonstrates that *E. coli* and *K. pneumonia* are the dominant extensively drug-resistant uropathogenic bacteria in community-acquired UTIs in our cohort. These uropathogens were found to be resistant to the majority of the routinely-used classes of β-lactams, pyridopyrimidines, quinolones, and fluoroquinolone antibiotics, and these findings may be useful for clinicians in their treatment of paediatric UTIs.

**Keywords:** 16S rRNA gene sequencing; *Klebsiella pneumoniae*; Multiple drug-resistance; Paediatric infections; Urinary tract infections

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Uropathogenic bacteria were isolated from urine samples using cysteine lactose electrolyte-deficient (CLED) agar.\textsuperscript{25} Urine was poured over autoclaved CLED agar media at different dilutions and incubated at 37 °C for 72 h. Colonies with a yellow, opaque, or slightly deeper colour were tentatively identified as \textit{Escherichia} spp., while large mucoid yellow or yellow-white colonies were considered to belong to the \textit{Klebsiella} spp. Selected colonies were then picked, purified, and preserved at −20 °C until further experiments.

**Biochemical characterization**

The isolated strains were screened for Gram staining using the Duguid et al. method.\textsuperscript{26} To differentiate between lactose fermenting and non-fermenting bacterial strains, freshly grown strains were streaked on MacConkey agar and incubated at 37 °C for 24 h. After incubation, bacterial strains were evaluated for pigmentation and any colony appearing as pink-red was classified as a lactose fermenting strain and urease activities, were then evaluated. Strains were also evaluated for citrate utilisation, motility, and indole production using biochemical tests, as described by Cappuccino and Sherman.\textsuperscript{24}

**Antibiotic resistance test**

The disk diffusion method, M100, from the Clinical and Laboratory Standard Institute (CLSI)\textsuperscript{27} was used to evaluate the antimicrobial susceptibility of each strain against 21 different antibiotics, including co-amoxiclav, amikacin, cefuroxime, cefixime, cefotaxime, ceftazidime, ceftriaxone, ciprofloxacin, nalidixic acid, nitrofurantoin, norfloxacin, pipemidic acid, sulbactam/cefoperazone, colistin sulphate, fosfomycin, meropenem, imipenem, chloramphenicol, piperacillin/tazobactam, co-trimoxazole, and polymyxin B. Uropathogenic strains were freshly grown overnight and heavily streaked on Mueller-Hinton (MH) plates.\textsuperscript{28} Then, commercially available 4 mm antibiotic paper discs (Abtek Biologicals Ltd, UK) were placed on the MH plates and incubated at 37 °C for 24 h. Subsequently, the zone of inhibition in the bacterial lawn was then measured and recorded. \textit{E. coli} ATCC 23509 and \textit{E. coli} ATCC 25922 were also tested for antibiotic susceptibility and used as the control for our observations. The diameters of the zones of inhibition were then categorised as sensitive or resistant using the standard diameters provided by the CLSI.\textsuperscript{27} The non-susceptible strains were classified as MDR, XDR, and pan drug-resistance (PDR) based on the criteria reported in Mogiorakos et al.\textsuperscript{22} Non-susceptible strains presenting with resistance to ≥1 agent were non-susceptible; strains resistant to ≥3 antimicrobial categories were considered MDR, while those with susceptibility to ≥1 agent in all but ≤2 antimicrobial categories were considered to be XDR. Those bacterial strains that were resistant to all the listed antimicrobial agents were considered PDR.

**Identification of uropathogenic bacterial strains using 16S rRNA gene sequencing**

The genomic DNA from selected antibiotic-resistant uropathogenic bacterial strains (ZK9, ZK32, ZK40, ZK60, and ZK89) was isolated using proteinase K treatment and the polymerase chain reaction was performed as described by Mumtaz et al.\textsuperscript{24} The PCR product was then sequenced using a commercial service provided by Macrogen (Seoul, Korea) and the sequences were identified using the BLAST algorithm from the NCBI web browser. The phylogenetic tree of closely related species was constructed using MEGA 7.0.26. DNA sequences were aligned using ClustalW, and phylogeny was determined using the maximum parsimony method.

**Results**

Uropathogenic \textit{Escherichia} and \textit{Klebsiella} spp. were isolated from urine samples collected from 130 paediatric UTI patients in various wards, including OPD (29), nephrology (23), neonatal unit (18), general medical (12), urology (11), and neurology (7), of the Children’s Hospital and Institute of Child Health in Lahore, Pakistan (Table 1). The

| Wards                  | n (%) |
|-----------------------|-------|
| Out Patient Department| 29    |
| Nephrology            | 23    |
| Neonatal unit         | 18    |
| General medical       | 12    |
| Urology               | 11    |
| Neurology             | 7     |
demographic data of these paediatric patients are summarised in Table 2. Some urine samples (30) exhibited no bacterial growth on the CLED agar and were thus excluded from the study. Of the remaining 100 paediatric patients, 23 were less than one year old and 77 were in the range of 1–14 years of age. UTI prevalence between the two sexes revealed that 51% of the UTIs were found in male children and 49% in female paediatric patients. Uropathogens were isolated from both symptomatic UTIs, presenting with vomiting (64%), fever (87%), and non-symptomatic UTIs, without vomiting (36%) or fever (13%). Health complications, including immunosuppression (11%), urethral malformation (34%), and neurological disorders (7%), were also identified in several UTI patients. A total of 26% of the paediatric patients had a history of antibiotic use and the highest bacterial count, $10^5$ CFU mL$^{-1}$, was observed in 72% of urine samples, while 28% of the samples had a reduced bacterial cell count of $10^3$ CFU mL$^{-1}$ (Table 2).

Characterization of the uropathogenic strains

A total of 100 strains were isolated and identified as *Escherichia* and *Klebsiella* spp. using morphological and biochemical characterisation. These isolates were coded as ZK1, ZK2, ZK3, … to ZK100 and divided into groups A and B based on the results of the biochemical characterisation (Table 3). Biochemical characterisation of selected strains revealed that all the strains in both groups were Gram-negative, positive for catalase activity, and negative for oxidase activities. All the strains presented with a yellow acidic slant and butt with gas production in the TSI test. Group A strains were negative for urease activity and citrate utilisation tests but positive for indole production and motility. The group A strains were negative for urease activity and citrate utilisation, however, group B strains were positive to these assays (Table 3). Based on the biochemical characterisation, group A strains were identified as belonging to the *Escherichia* spp. and group B strains were identified as *Klebsiella* spp. (Table 3 and Figure 1a). Among the *E. coli* strains, 38% were isolated from male patients and 25% were from female patients. In the *Klebsiella* strains, 21% of the strains were from boy and 16% were from girl patients (Figure 1b).

**Table 2: Baseline demographic and clinical characterization of paediatric UTI patients.**

| Variable          | n (%) |
|-------------------|-------|
| **Age**           |       |
| Less than 1 year  | 23    |
| 1 year to 14 years| 77    |
| **Gender**        |       |
| Male              | 51    |
| Female            | 49    |
| **Vomiting**      |       |
| Yes               | 64    |
| No                | 36    |
| **Fever**         |       |
| Yes               | 87    |
| No                | 13    |
| **Complications** |       |
| Immunosuppression | 11    |
| Urethra malformation | 34  |
| Neurological disorders | 7   |
| **UTI history**   |       |
| Yes               | 26    |
| No                | 74    |
| **Bacterial count** |     |
| $>10^3$ CFU mL$^{-1}$ | 28  |
| $>10^5$ CFU mL$^{-1}$ | 72  |

Antibiotic susceptibility

The susceptibility patterns of the Gram-negative uropathogens is summarized in Figures 2 and 3. Overall, $\geq$90% of the uropathogenic strains were not susceptible to cefuroxime, cefixime, and cefotaxime. More than 80% of uropathogenic strains were also found to be resistant to co-amoxiclav, cefazidime, ceftriaxone, ciprofloxacin, nalidixic acid, norfloxacin, pipemidic acid, and co-trimoxazole. The antimicrobial susceptibility patterns of the *Escherichia* and *Klebsiella* spp. are shown in Figures 2 and 3. All of the *E. coli* strains (group A) were screened against all 21 antimicrobial

![Figure 1: Prevalence of uropathogenic *Escherichia* and *Klebsiella* strains (A) and their distribution in male and female paediatric UTIs (B).](image-url)
drugs, and the results are depicted in Figure 2. The highest degree of resistance (93%) was reported for cefixime, followed by cefuroxime (90%). More than 80% of the E. coli strains were not susceptible to cefotaxime, ceftazidime, ceftriaxone, ciprofloxacin, nalidixic acid, norfloxacin, pepsedemic acid, and co-trimoxazole. However, E. coli strains were susceptible to colistin sulphate (85%), polymyxin B (85%), chloramphenicol (84%), nitrofurantoin (79%), fosfomycin (76%), and meropenem (71%). The Klebsiella strains (group B) demonstrated a similar pattern of resistance and susceptibility (Figure 3). The highest

| Characterization       | Group A (n = 63)* | Group B (n = 37)** |
|------------------------|-------------------|--------------------|
| Gram-staining          | Gram-negative     | Gram-negative      |
| Catalase activity      | Positive          | Positive           |
| Oxidase activity       | Negative          | Negative           |
| Urease activity        | Negative          | Positive           |
| Citrate utilization test| Negative         | Positive           |

* Group A was identified as Escherichia spp. through biochemical characterization. ** Group B was identified as Klebsiella spp. through biochemical characterization.

Figure 2: Antimicrobial susceptibility of uropathogenic Escherichia strains.

Figure 3: Antimicrobial susceptibility of uropathogenic Klebsiella strains.
| Antimicrobial resistance | Uropathogen identifier |
|--------------------------|------------------------|
| Multiple drug-resistant (MDR) | *Escherichia* spp. strains (n = 5)  
ZK52, ZK53, ZK63, ZK64, ZK98,  
Klebsiella spp. strains (n = 9)  
ZK19, ZK20, ZK42, ZK46, ZK47, ZK54, ZK55, ZK97, ZK99 |
| Extensively drug resistant (XDR) | *Escherichia* spp. strains (n = 58)  
ZK1, ZK3, ZK4, ZK6, ZK7, ZK9, ZK10, ZK11, ZK12, ZK13, ZK16, ZK21, ZK22, ZK23, ZK24, ZK26, ZK27, ZK29, ZK31, ZK33, ZK34, ZK38, ZK39, ZK40, ZK44, ZK49, ZK56, ZK57, ZK58, ZK59, ZK60, ZK61, ZK65, ZK66, ZK67, ZK68, ZK69, ZK70, ZK71, ZK73, ZK76, ZK78, ZK79, ZK80, ZK81, ZK82, ZK83, ZK84, ZK85, ZK87, ZK88, ZK90, ZK91, ZK92, ZK95, ZK96, ZK100  
Klebsiella spp. strains (n = 28)  
ZK2, ZK5, ZK8, ZK14, ZK15, ZK17, ZK18, ZK25, ZK28, ZK30, ZK32, ZK35, ZK36, ZK37, ZK41, ZK43, ZK45, ZK48, ZK50, ZK51, ZK62, ZK72, ZK74, ZK75, ZK77, ZK86, ZK89, ZK94 |

* Indicated XDR strains were selected for molecular identification using 16S rRNA gene sequencing.

**Figure 4:** Phylogenetic tree describing the 16S rRNA gene sequences of *E. coli* strains ZK9, ZK40, ZK60 (accession number MT764342, MT764344, and MT764345, respectively) and *K. pneumoniae* strains ZK32 and ZK89 (accession number MT764343 and MT764346, respectively) and the other closely related bacterial strains found in the GenBank database.
resistance (95%) was observed against cefixime, followed by cefuroxime (92%) and cefotaxime (92%). More than 75% of the *Klebsiella* strains were not susceptible to co-amoxiclav, cefazidime, ceftriaxone, ciprofloxacin, nalidixic acid, norfloxacin, pephedem acid, and co-trimoxazole. Antimicrobial agents polymyxin B (89%), colistin sulphate (86%), chloramphenicol (81%), nitrofurantoin (78%), and fosfomycin (76%) were more effective against the *Klebsiella* strains (Figure 3). The estimated MDR and XDR patterns are reported in Table 4 and reveal that 14% of the strains can be classified as MDR, and 86% of the uropathogenic strains identified in this study could be classified as XDR. None of the strains were found to be PDR. Among the MDR strains, five were identified as *Escherichia* spp. and nine were *Klebsiella* spp. In contrast, 58 of the *Escherichia* spp. and 28 of the *Klebsiella* spp. were found to be XDR. The XDR strains ZK9, ZK32, ZK40, ZK60, and ZK89 were shown to demonstrate the highest degree of resistance to the antibiotics used in this study and reported resistance to 17 of the 21 antimicrobials evaluated here. These strains were then selected for molecular identification.

**Identification of selected antibiotic-resistant uropathogens**

The five most representative MDR strains, ZK9, ZK32, ZK40, ZK60, and ZK89, were selected for molecular identification using 16S rRNA partial gene sequencing. These strains were identified as *Escherichia coli* strain ZK9, *Klebsiella pneumoniae* strain ZK32, *Escherichia coli* strain ZK40, *Escherichia coli* strain ZK60, and *Klebsiella pneumoniae* strain ZK89. A phylogenetic tree of these strains was constructed using the neighbour-joining method and broadened by selecting closely matched *Enterobacteriaceae* strains, as shown in Figure 4. The resulting sequences were deposited in NCBI GenBank with accession numbers MT764342 for ZK9, MT764343 for ZK32, MT764344 for ZK40, MT764345 for ZK60, and MT764346 for ZK89 (Figure 4).

**Discussion**

Antimicrobial drug resistance is a life-threatening issue, especially in paediatric patients, when treating infections caused by *Escherichia* and *Klebsiella* spp. Antimicrobial drug-resistance is increasing every day and the normal microbial flora in the urinary tract is becoming a reservoir for resistance genes. In this study, hundreds of uropathogenic *Escherichia* and *Klebsiella* strains were isolated from paediatric patients with UTIs and their antimicrobial susceptibility was evaluated. The *E. coli* and *K. pneumoniae* strains isolated from the paediatric patients in this study showed the highest antimicrobial resistance (≥60%) against tested antibiotics. Among these strains, ≥80% were found to be not susceptible to cefixime, cefuroxime, cefotaxime, ceftriaxone, ciprofloxacm, nalidixic acid, ceftazidime, co-trimoxazole, pephedem acid, ceftriaxone, ciprofloxacin, and norfloxacin, and 86% of these strains, 58 *Escherichia* strains and 28 *Klebsiella* strains, were found to be XDR. Similar findings have also been reported in various clinical settings across Nepal and Ethiopia. However, the prevalence of MDR *Escherichia* and *Klebsiella* strains has not yet been reported for Lahore, Pakistan. This study addresses this lack of information and provides insight into the increasing incidence of MDR in paediatric UTIs in Pakistan.

In this study, uropathogenic bacterial strains were identified as *E. coli* and *K. pneumoniae* based on their biochemical characterisation. These uropathogens are Gram-negative and are known to be the dominant pathogens in UTIs across the world due to their unique structure, which facilitates their attachment to the host cells. These structural features help the bacteria avoid exclusion by urinary lavage and support bacterial reproduction and expansion in these tissues, supporting invasive infection and pyelonephritis. The *Escherichia* strains in this study were found to be negative for citrate utilisation and positive for indole production, while the *Klebsiella* strains showed the opposite result. Indole and citrate-utilisation tests are commonly used to identify the *Enterobacteriaceae* including *Escherichia*, *Enterobacter*, and *Klebsiella* spp. In this study the *Escherichia* strains were indole positive, which is likely related to their ability to produce tryptophanase enzymes that can convert tryptophan into indole. When indole reacts with para-dimethyl-arnino benzaldehyde, a pink-coloured complex is produced that differentiates *Klebsiella* from *Enterobacter* spp. In this study, all *Escherichia* and *Klebsiella* strains were oxidase negative, which may be due to the inactivation of cytochrome C-oxidase and an inability to utilise oxygen for energy production via the electron transfer chain, which confirms their link to the *Enterobacteriaceae* family. In our study, the production of a yellow slant and butt with gas and no H2S on TSI media indicates the carbohydrate fermentation ability of these strains. However, the molecular identification method is more reliable and provides information up to the species level. In this study, the evaluation of the 16s rRNA gene revealed that most MDR strains were *Escherichia coli* strain ZK9, *Klebsiella pneumoniae* strain ZK32, *Escherichia coli* strain ZK40, *Escherichia coli* strain ZK60, and *Klebsiella pneumoniae* strain ZK89. Strains ZK9, ZK40, and ZK60 were shown to be closely related to *Escherichia* sp. strain UA32, which is an MDR strain isolated from female patients during pregnancy. The XDR *Klebsiella pneumoniae* strains ZK32 and ZK89 were shown to be closely related to MDR *Klebsiella* sp. strain UA47 from pregnant females.

In this study, the highest degree of resistance (≥90%) in the *E. coli* strains was observed for cefixime and cefuroxime, while the antimicrobial agents cefotaxime, ceftazidime, ceftriaxone, ciprofloxacin, nalidixic acid, norfloxacin, pepedem acid, and co-trimoxazole reported a ≥80% nonsusceptibility rate in these strains. Recently, Vazouras et al. reported their findings from Greece, wherein 79% of the strains in their study were *E. coli*, making them the most common UTI-causing organism in paediatric patients. This study also reported the resistance rates for amoxicillin (42%), trimethoprim/sulfamethoxazole (up to 26.5%), amoxicillin/clavulanic acid (up to 12.2%), cephalosporins (up to 1.7%), nitrofurantoin (up to 2.3%), ciprofloxacin (up to 1.4%), and amikacin (up to 0.9%), which were much lower than those found in our study. This difference may be the result of geographical variations resulting from local differences in the frequency of antibiotic use and ease of antibiotic availability. Kayastha et al. reported a rising prevalence in resistance, up to 62%, of extended-spectrum β-lactamase-
producing Gram-negative bacteria in paediatric UTIs and Shrestha et al. reported MDR uropathogenic E. coli (53%), E. faecalis (22%), K. pneumoniae (7%), and S. aureus (7%). Hsueh et al. evaluated urogenital pathogens and found that 23% of their urine samples were positive for the most common resistance factors in bacterial uropathogens. Mirzarazi et al. reported that their E. coli isolates were resistant to nalidixic acid and trimethoprim-sulfamethoxazole. In this study, we found that the Klebsiella strains (≥92%) were not susceptible to cefixime, cefuroxime, or cefotaxime. More than 75% of these strains were also resistant to co-amoxiclav, ceftazidime, ceftriaxone, ciprofloxacin, nalidixic acid, norfloxacin, pepsedemic acid, and co-trimoxazole. Similarly, Hayat et al. reported that the prevalence of paediatric UTIs was up to 52% in Pakistan, with the majority of infections occurring due to extended-spectrum β-lactamase-producing K. pneumoniae, which demonstrated a high degree of resistance (96%) to ceftazidime. Klebsiella spp. also showed resistance to trimethoprim-sulfamethoxazole, ciprofloxacin, and nalidixic acid.

Across the world, most patients carry a variety of MDR genes that have developed as a result of the misuse and improper administration of antibiotics. This has resulted in the widespread sharing of β-lactamase enzymes in the commensal bacteria, promoting the development of novel MDR strains. In our study, we observed the highest MDR patterns for the aminoglycosides and β-lactam antibiotics. This is of significant clinical concern, especially in children, as their immune system is still developing. This is particularly problematic when uropathogenic organisms present with MDR characteristics transferred from other organisms via genetic exchange. Longer hospital stays also result in infections with extended-spectrum β-lactamase-producing organisms. MDR could also be due to an unavoidable genetic reaction to the solid discriminating strength forced by antibiotic therapy which plays its dynamic role in the development of MDR in uropathogens and their plasmid having MDR genes.

Conclusion

Our data shows that the majority of the uropathogenic Escherichia and Klebsiella strains isolated in this study are resistant to cefixime, cefuroxime, nalidixic acid, and cefotaxime, suggesting that such antibiotic treatment should be suspended following extensive long-term evaluations. Self-medication and improper diagnosis should be avoided to prevent increasing antibiotic resistance in both of these clinically-important bacterial genera.

Recommendations

Uropathogenic E. coli and K. pneumoniae were found to responsible for the majority of the MDR UTIs identified in paediatric patients in Lahore, Pakistan. Efforts must be made to control MDR infections in paediatric patients by executing evidence-based monitoring of UTI treatment and creating awareness of the judicious use of antibiotics.

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Conflict of interest

The authors have no conflict of interest to declare.

Ethical approval

Ethical approval for this study protocol was granted by the Institute of Molecular Biology and Biotechnology at The University of Lahore, Pakistan, with the reference number IMBB-744 and dated 10-11-2018.

Authors’ contributions

ZI conducted the research, provided the research materials, and collected and organised the data. MZM conceptualised and supervised the study, analysed and interpreted the data, and wrote the initial and final drafts of the manuscript. AM provided the research materials and logistical support for this project. All authors critically reviewed and approved the final draft of this manuscript and are responsible for the content and similarity index of the manuscript.

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