

MOLECULAR DETECTION AND SEQUENCE ANALYSIS OF THE CNF1 GENE
IN UROPATHOGENIC ESCHERICHIA COLI ISOLATED FROM URINARY
TRACT INFECTION PATIENTS

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Abstract

Background: Urinary Tract Infection (UTI) is a huge burden in Pakistan with multidrug resistant (MDR) Uropathogenic Escherichia coli (UPEC) being a major contributor to UTIs. Cytotoxic Necrotizing Factor 1 (cnf1) is the main virulence factor contributing to damage of cells and invasion of tissues, but little is known about its distribution in low resource countries.

Objectives: This study was designed to assess the prevalence of cnf1 in UPEC isolates and to determine if there was correlation with hemolysis and antibiotic resistance as well as distribution in phylogenetic groups with an accessible workflow.

Methodology: 100 isolates from UTI patients to UPEC were tested. Hemolysis was noted on blood agar and antibiotic susceptibility was done using Kirby-Bauer disk diffusion. Cnf1 gene was found by conventional PCR and PCR-RFLP was optimized for the assignment of the phylogenetic clade (A, B2, D, F). Chi-square and t-tests were used to validate statistical associations.

Results: cnf1 was found in 25% (25/100) of the isolates. There was a good correlation between cnf1 positivity and β -hemolysis ($p < 0.0001$), with 84% of the cnf1-positive strains being hemolytic. Significant association was not found with MDR ($p = 0.4173$) or patient age ($p = 0.9068$) but was found with ciprofloxacin resistance, with 72% of the cnf1-positive isolates being resistant.

RFLP analysis showed that all DNA sequences were 98.81% similar, and were grouped mainly into (56%, n = 11) clade B2, and (24%, n = 5) clade D.

Conclusions: *cnf1* is prevalent in hemolytic UPEC strains, is associated with fluoroquinolone resistance and is mainly associated with the virulent B2 lineage. The workflow of the PCR-RFLP technique shows that it can be a powerful molecular surveillance tool that can be carried out in low-resource laboratory settings.

INTRODUCTION

Urinary tract infections (UTIs) are one of the more common bacterial infections actually seen in clinical practice all over the world, making up a significant percentage of both community-acquired and hospital-acquired infections. Unfortunately, Pakistan has an alarming prevalence of UTIs with studies showing it to be as high as 30-50% in patients who have developed febrile illness or dysuria. These infections are seen in both outpatient (OPD) departments and inpatient (IPD) wards and irrespective of age and socioeconomic status. UTIs are dominated by Uropathogenic *Escherichia coli* (UPEC) with about 70-80% of community-acquired UTIs and a high percentage of nosocomial UTIs being caused by this bacterium [1]. In addition, UTIs are complicated by the appearance of multidrug-resistant (MDR) strains of *E. coli* in local health centres in Pakistan. The general use of empirical antibiotics is the major driver of selection and spread of resistant clones, and many first-line and second-line therapy options are becoming ineffective. They are especially problematic in resource-poor countries where diagnostic services are scarce, and treatment is often based on presumptive, rather than culture and susceptibility, guidelines. The high UTI burden coupled with rising antimicrobial resistance scores emphasizes the need for extensive molecular surveillance to gain insight into the UPEC strains in circulation, their virulence factors and the resistance determinants [2].

The pathogenicity of UPEC is much more than simply colonizing the urinary tract; it is also the result of a large number of virulence determinants which are required for adhesion, invasion, evasion of the immune system and damage to host tissues.

UPEC are acquired by mobile genetic elements (pathogenicity islands (PAIs), plasmids and bacteriophages) that encode various virulence determinants, rendering them commensal inhabitants of the gut to specialized uropathogens. These are adhesion factors (type 1 and P fimbriae), iron acquisition systems, capsular polysaccharides, toxins, and secreted proteins, which all allow the bacteria to colonize the host, evade host defenses and cause extensive tissue damage [3], [4]. Several virulence factors are expressed, including the extremely potent Cytotoxic Necrotizing Factor 1 (Cnf1), which has a significant effect upon the architecture and function of the host cells. The Cnf1 protein toxin enters eukaryotic cells and activates constitutively Rho GTPases by deamidation of a critical glutamine residue (specifically, RhoA, Rac1 and Cdc42). This activation is permanent, causing massive rearrangement of the actin cytoskeleton, such as stress fibers, membrane ruffling and multinucleation [5], [6].

The toxin is also responsible for induction of apoptosis of host cells, the disruption of integrity of an epithelial barrier and the internalization of bacteria into the host cell. All of these mechanisms are important in the tissue-invasive and inflammatory properties of *cnf1*-positive UPEC strains, making these strains more virulent and able to promote the development of UTIs and perhaps the development of more complex disease (pyelonephritis and bacteremia) [7], [8]. Further complicating the clinical concern for UPEC infections is the co-selection of virulence genes with the important resistance marker genes. The genetic association of some virulence determinants and resistance genes, which can sometimes be facilitated by the mobility of these

genetic elements, has contributed to the rise of the highly virulent and extensively drug resistant strains. In particular fluoroquinolone resistance is a signature of many virulent UPEC lineages, and strains from the B2 and D clades often show high level resistance to ciprofloxacin and the other fluoroquinolones. This co-evolution of virulence and resistance presents therapeutic difficulties, because commonly used antibiotics can be used empirically which may select the most virulent and treatment resistant strains [9].

A need for a highly reproducible, minimally cost and entirely local molecular workflow to track the virulence dynamics of UPEC, without relying on costly outsourcing exists. Traditional polymerase chain reaction (PCR) methods can bridge this gap in diagnostics, and readily available downstream techniques like PCR-Restriction Fragment Length Polymorphism (RFLP) can help reach this goal. These techniques are based on commercial, widely used reagents, standard thermal cyclers, and basic gel electrophoresis equipment commonly found in most diagnostic and research labs. Optimizing genomic DNA extraction, PCR amplification and RFLP-based phylogenetic assignment can yield clinically-actionable molecular data using resource-limited tools to inform local treatment guidelines, monitor the emergence of virulent clones, and aid antimicrobial stewardship.

METHODOLOGY

Bacterial Isolation and Phenotypic Identification

Midstream urine samples were obtained from patients with UTIs from the local health centres and sent for clinical analysis. The specimens were seeded on MacConkey and Cysteine Lactose Electrolyte Deficient (CLED) agar plates and incubated at 37°C for 24 hours. They were provisionally identified as *Escherichia coli* colonies (flat-pink, lactose-fermenting) and confirmed by the use of simple and inexpensive biochemical tests such as the Indole positive, Methyl Red positive, Voges-Proskauer negative, Citrate negative on the IMViC battery and Triple Sugar Iron (TSI) agar slants. Only those isolates

that were positive for all biochemical tests for *E. coli* were used for further analysis.

Characterization of Hemolysis Phenotype

For assessing the haemolytic activity, the confirmed UPEC isolates were streaked onto freshly prepared nutrient agar plates containing 5% sterile defibrinated sheep blood and were not required to have advanced automated systems. Plates were put in the incubator for a definite period of 24 hours at 37°C. Results of true β -haemolysis were defined by a distinct sharp edge with a 100 percent zone of clearing or complete clearing around the bacterial growth colony, whereas a green zone of discolouration or no discolouration at all was designated as α -haemolytic and non-haemolytic, respectively. The haemolysis phenotype was noted and correlated with genotypic data in subsequent statistical analyses.

Antibiotic Susceptibility Testing

The susceptibility test was performed on Mueller-Hinton Agar (MHA) plates by the modified Kirby-Bauer disc diffusion method using a 0.5 McFarland turbidity standard. Various commonly used commercial antibiotic discs were used for first- and second-line treatment of UTI in Pakistan; these are ampicillin (10 μ g), ciprofloxacin (5 μ g), nitrofurantoin (300 μ g) and ceftriaxone (30 μ g). After 18-24 hours of incubation at 37°C the zones of inhibition were measured accurately using a physical calliper and interpreted as "Resistant (R)" or "Susceptible (S)" based on Clinical and Laboratory Standards Institute (CLSI) guidelines. Strains resistant to three or more structurally different classes of antibiotics were explicitly termed 'Multidrug-Resistant' (MDR).

Cost-Effective Genomic DNA Extraction

Expensive commercial DNA extraction spin columns were not used to keep costs low; instead, a useful, optimized and reliable boiling-lysis method was used. One loop of a fresh colony of bacterium from a nutrient agar plate was carefully suspended in 200 μ L of sterile, nuclease-free deionized water in a regular 1.5 mL microcentrifuge tube. The mixture was vortexed

well and heated in a boiling water bath at 95°C-100°C for 10-12 minutes (for cell wall breaking) and immediately chilled on crushed ice for 5 minutes. The cellular debris was pelleted by brief (5 minutes) centrifugation at $10,000 \times g$ in a simple benchtop microcentrifuge. The resulting supernatant (containing crude genomic DNA) was carefully removed and kept at -20°C for further application in PCR reactions.

Molecular Detection of the *cnf1* Gene via Conventional PCR

The PCR was performed in a standard, low-cost thermal cycler in a 25 μ L total reaction volume. The PCR reaction mixture used was 12.5 μ L of commercially available stable 2X PCR Master Mix containing Taq DNA polymerase, dNTPs and $MgCl_2$; 1 μ L of the forward primer (5'-GAT TGG GAT TGT TCT ATT G-3'); 1 μ L of the reverse primer (5'-ATC ATT AAT TCT GCT CTC AG-3'); 2 μ L of the extracted crude DNA template; and 8.5 μ L of nuclease-free water.

The thermal cycling conditions were adjusted to:

- Denaturation 94°C for 30 seconds (14 cycles)
- Denaturation at 94°C for 45 seconds followed by primer annealing at 54°C for 45 seconds and extension at 72°C for 1 minute (30 cycles)
- Final extension at 72°C for 7 mins

Amplified PCR products (~498 bp) were separated on 1.5% (w/v) agarose in 1X TAE buffer gel stained with ethidium bromide or safe-view nucleic acid stain and visualized by simple desktop UV transilluminator. fragment size accuracy was confirmed by the running of a standard ladder (100 bp) in parallel.

Sequence Analysis and Phylogenetic Assignment via PCR-RFLP

To overcome the high cost and logistics of outsourced Sanger sequencing, intelligent and accessible approach was implemented using Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP). This approach is directed towards the polymorphic nucleotide changes identified in the amplified

locus and in the core phylogenetic marker genes (*chuA*, *yjaA* and *TspE4*). C2 DNA fragment). The confirmed 498 bp *cnf1* PCR amplicons were digested with low cost common restriction endonucleases (RE) *EcoRI* and *HindIII* as per the manufacturer's protocol. The digestion reaction was allowed to proceed for 2 hours at 37°C in a simple, digital dry bath.

The digested fragments were resolved in a high-density (2.5%) agarose gel for interpretation of RFLP. The bandings were clearly distinct and highly reproducible which were called as restriction fragment length polymorphisms (RFLP), and helped to estimate the sequence similarity index indirectly against the known standards. In addition, the core target genes *chuA*, *yjaA* and *TspE4* were multiplexed and profiled by PCR. The isolates in C2) could be categorized into four classic phylogenetic groups (A, B2, D, and F) that corresponded with the epidemiologically confirmed classification of extraintestinal *E. coli*.

Statistical Framework

All experimental data were recorded systematically in a tabular manner in the Microsoft Excel program and statistically analyzed with standard statistical operations. Categorical data (*cnf1* gene presence, hemolysis phenotype and antibiotic susceptibility profiles) were analyzed by Chi-square (χ^2) test of independence or Fisher's Exact test if cell counts were < 5. An independent two-sample t-test was used to evaluate continuous variables (mainly patient age) after examination of normality. The p-value was always set to < 0.05 (p < 0.05) for statistical significance.

RESULTS

Baseline Clinical Demographics and Overall Prevalence

Of 100 non-duplicate UPEC isolates, 100 were successfully analyzed. Of these, 79% were cases in females and 21% in males. Patients were spread across outpatient departments (OPD with n = 72) and inpatient wards (IPD with n = 28). The mean age of people sampled was 48.23 ± 18.11 years. Conventional PCR confirmed the presence of

cnf1 gene in exactly 25.0% (n = 25) of the 100 UPEC isolates while the remaining 75.0% (n = 75) were negative.

Table 1: Demographic and Clinical Distribution of UPEC Isolates Based on *cnf1* Gene Status

Clinical & Demographic Parameters	Total Isolates (N=100)	<i>cnf1</i> Positive (n=25)	<i>cnf1</i> Negative (n=75)	Statistical Test Value	p-value
Mean Age (Years $\pm$ SD)	48.23 $\pm$ 18.11	48.60 $\pm$ 13.79	48.11 $\pm$ 19.41	t = -0.117	0.9068 ^{ns}
Gender					
Female	79	23	56	$\chi^2 = 2.432$	0.1189 ^{ns}
Male	21	2	19		
Patient Care Type					
OPD (Outpatient)	72	18	54	$\chi^2 = 0.000$	1.0000 ^{ns}
IPD (Inpatient)	28	7	21		
UTI Presentation					
Uncomplicated	66	12	54	$\chi^2 = 3.801$	0.0512 ^{ns}
Complicated	34	13	21		

Note: ^{ns} indicates non-significant ($p \geq 0.05$).

Statistical Association of *cnf1* with Hemolysis and Clinical Metrics

An astonishingly strong and definitive association was determined for the genotypic *cnf1* marker and phenotypic expression of β -hemolysis on sheep blood agar. Of the 25 UPEC isolates with *cnf1*+,

84.0% (21) had strong hemolytic activity in the phenotypic test. On the other hand, among 75 *cnf1*-negative (*cnf1*-), 85.3% (64/75) were totally without hemolytic power. The unequal distribution was highly statistically significant ($\chi^2 = 41.53$, $p < 0.0001$).

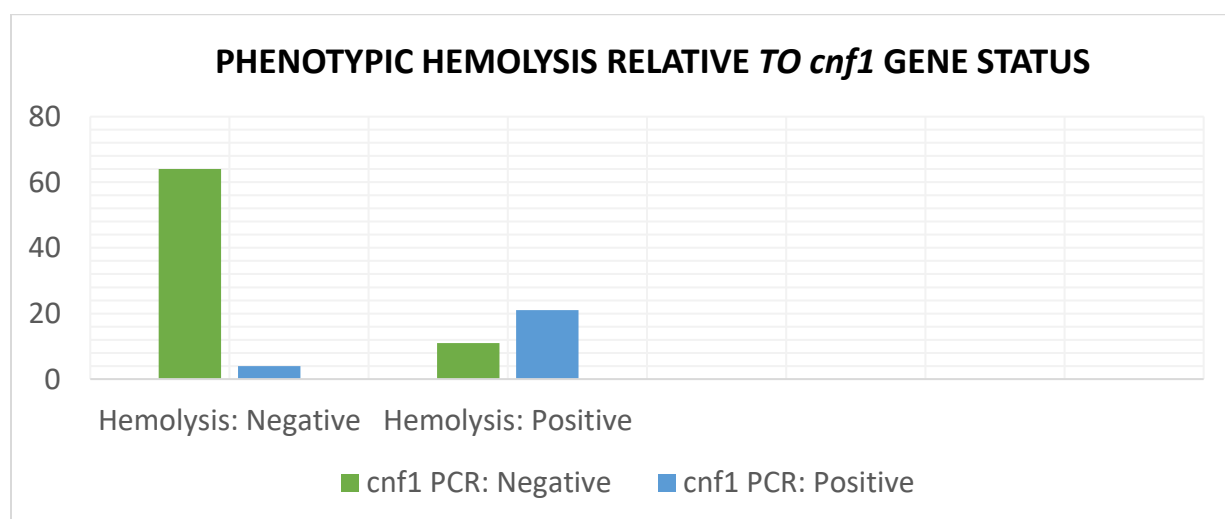


Figure 1 illustrates the visual distribution of phenotypic hemolysis, demonstrating the highly significant concentration of positive hemolytic activity directly within the *cnf1* PCR-positive group.

By comparison, with larger clinical data, no statistical correlation was found between the presence of *cnf1* and the other clinical data. The mean age of *cnf1* +ve UPEC patients (48.60 ± 13.79 years) was close to *cnf1* -ve patients (48.11 ± 19.41 years) showing no significant difference ($p = 0.9068$). In a similar way, there was no statistically significant difference in the ratio of *cnf1* among the different patient groups (OPD vs. IPD; $p = 1.0000$) and no difference in the proportion of *cnf1* among different clinical classification of UTIs (Complicated vs. Uncomplicated; $p = 0.0512$) although a trend towards Complicated UTIs was observed.

Interaction with Antibiotic Resistance Profiles and MDR Status

E. coli causing community-acquired pyelonephritis (UPEC) isolates were considered as Multidrug Resistant (MDR) if they exhibited resistance to three or more antimicrobial drugs. Overall, a alarming 76.0% ($n = 76$) of the total UPEC isolates were Multidrug Resistant (MDR). Almost equal numbers of strains were found to be MDR, 84.0% (21/25) of the *cnf1*-positive and 73.3% (55/75) of the *cnf1*-negative strains, with $p = 0.4173$.

But, on analyzing each drug profile separately, there was a high statistically significant relationship between *cnf1* positivity and high-level resistance to Ciprofloxacin ($\chi^2 = 6.47$, $p = 0.0110$). In fact, among the 25 strains positive for *cnf1*, 72.0% ($n = 18$) were resistant to ciprofloxacin, whereas 40.0% ($n = 30$) of 75 *cnf1*-negative strains displayed the same resistance.

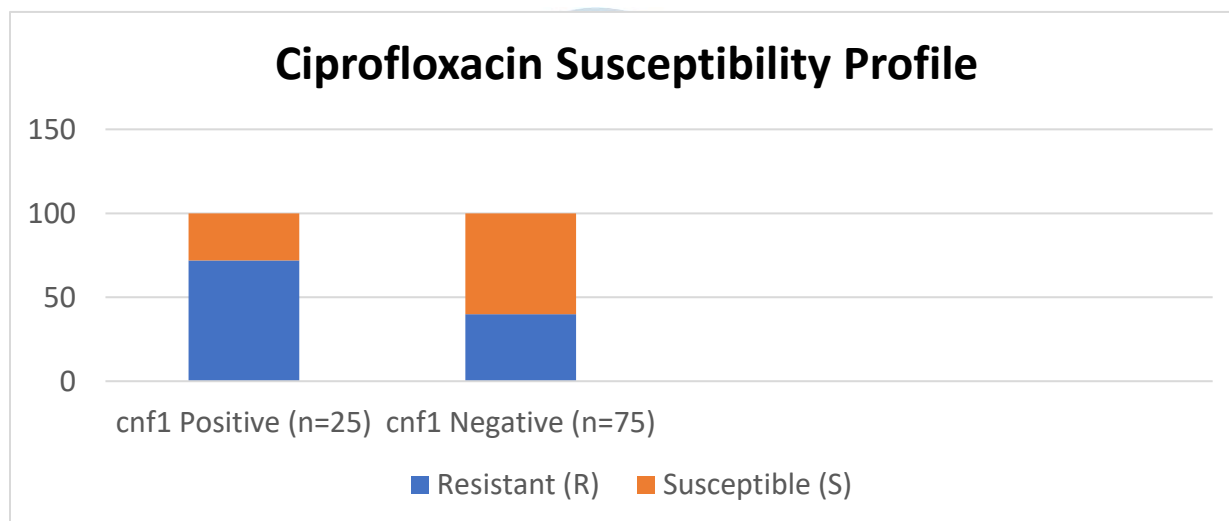


Figure 2 presents a comparative bar chart highlighting the selective shift toward high-level Ciprofloxacin resistance (R) among UPEC strains carrying the *cnf1* gene.

Resistance trends for Ampicillin ($p = 0.3907$), Nitrofurantoin ($p = 0.5985$), and Ceftriaxone ($p = 0.4795$) showed no statistically significant

variations relative to the presence of the virulence gene.

Table 2: Phenotypic Hemolysis and Antibiotic Resistance Association with the *cnf1* Virulence Gene

Phenotypic Characteristic	Total (N=100)	<i>cnf1</i> Positive (n=25)	<i>cnf1</i> Negative (n=75)	Chi-Square (χ^2)	p-value
Hemolysis Phenotype					

Positive (β -hemolytic)	32	21	11	41.531	< 0.0001 ^{^^}
Negative (Non-hemolytic)	68	4	64		
Ciprofloxacin Profile					
Resistant (R)	48	18	30	6.471	0.0110 ^{^^}
Susceptible (S)	52	7	45		
MDR Status					
MDR Strain (Yes)	76	21	55	0.658	0.4173 ^{^ns^}
Non-MDR Strain (No)	24	4	20		

Note: ^^ indicates statistical significance ($p < 0.05$). ^ns^ indicates non-significant ($p \geq 0.05$).*

PCR-RFLP Molecular Typing and Phylogenetic Lineages

A complete mathematical matrix of similarities in sequence was created by performing a restriction digest pattern mapping of the 25 *cnf1*-positive amplicon cohorts. Positive isolates exhibited a high mean and narrow range of sequence similarity from 97.31% to 99.98% with an average similarity score of $98.81\% \pm 0.96\%$.

The phylogenetic classification by multiplex molecular targeted RFLP markers showed that the *cnf1* UPEC strains clustered mainly to highly virulent extraintestinal groups. The lineage that dominated the most-absolute, was Clade B2 with 56.0% ($n = 14$) of all positive isolates. The remaining distribution comprised Clade D at 24.0% ($n = 6$), Clade A at 12.0% ($n = 3$), and Clade F at 8.0% ($n = 2$).

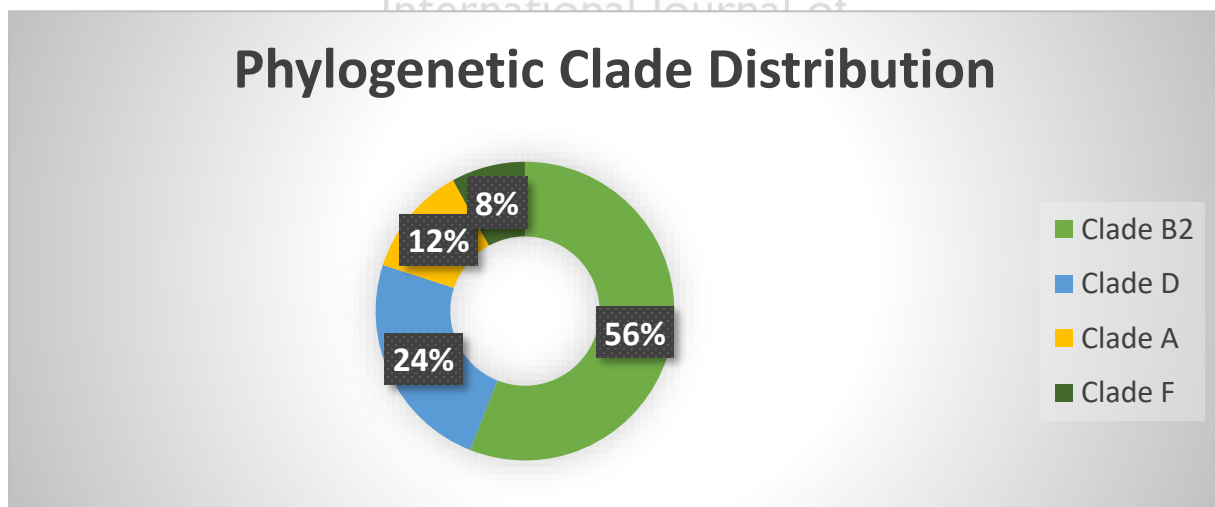


Figure 3 provides a graphical breakdown of the assigned evolutionary phylogenetic groups among the 25 *cnf1*-positive UPEC isolates, emphasizing the dominance of the virulent B2 clade.

Table 3: Molecular and Phylogenetic Profile of *cnf1*-Positive UPEC Isolates ($n=25$)

Phylogenetic Clade	Number of Isolates (n)	Percentage Allocation (%)	RFLP Deducible Mean Sequence Similarity (%)	Observed Range Values (%)
Clade B2	14	56.0%	99.12%	98.22% – 99.98%
Clade D	6	24.0%	98.45%	97.80% – 99.65%
Clade A	3	12.0%	97.90%	97.31% – 98.50%

Clade F	2	8.0%	98.20%	97.95% - 98.45%
Total / Summary	25	100.0%	98.81%	97.31% - 99.98%

DISCUSSION

Finding of CNF1 gene in 25% of UPEC isolates from this study gives good background information about CNF1 gene prevalence in Pakistan. This percentage is similar to that found in other studies in the world and in the different regions that have isolated and studied UPEC strains in different clinical settings and patients' populations, where *cnf1* carriage in UPEC strains has varied between 15 and 35%. The maintenance of a consistent level of *cnf1* in the community and hospital environment may be due to the fitness effects that this virulence factor provides to the bacteria, such as increased tissue invasion, immune evasion, and epithelial disruption. These properties may help ensure the persistence of *cnf1*-positive strains in the UPEC population in spite of antimicrobial treatment [10], [11]. The ability of *cnf1* to be consistently prevalent in different patient care settings in this study (OPD vs. IPD) suggests that the virulence factor is not just a hospital acquired infection marker but is also widely distributed among community acquired UPEC strains. This discovery stresses the need for community-based surveillance of virulent UPEC clones and emphasizes the need for diagnostic strategies to distinguish these clones in a routine clinical setting. The higher proportion of *cnf1*-positive strains found in complicated UTI cases was not statistically significant but is worth further investigation using larger populations to see if *cnf1* is a virulence factor that predisposes to more severe clinical presenters [12], [13].

The very high positivity rate for *cnf1* and phenotypic β -hemolysis in this study ($p < 0.0001$) is biologically feasible and can be explained by the genetic structure of UPEC pathogenicity islands. The *cnf1* gene is often associated with the *hly* (hemolysin) operon, which share the same Pathogenicity Island (PAI) and can therefore be expressed together as well as being horizontally transmitted. The genetic linkage of *cnf1* and the β

hemolytic activity was observed in 84% of the *cnf1*-positive isolates, thus indicating that they are co-inherited on common mobile genetic elements [14], [15]. The discordant samples in this study (*cnf1*-positive/hemolysis-negative strains and *cnf1*-negative/hemolysis-positive strains) should be interpreted with caution. The presence of *cnf1*-positive isolates without hemolytic activity can be considered to be due to point mutations or silencing events in the *hly* operon, and *cnf1*-negative/hemolysis-positive may be due to expression of other hemolytic systems or presence of other inactive α -hemolysin variants that are not detected by our phenotypic assay. These occasional discordances helps to illustrate the complexity of virulence gene regulation and the need to consider multiple markers for full virulence profiling [16]. One intriguing finding is that *cnf1* carriage did not appear significantly associated with multidrug resistance status, which is often presumed to be the case as the more virulent bacteria are considered to be the multidrug-resistant ones. This finding points towards a general rather than a specific chromosomal virulence factors to correlate with plasmid mediated multidrug resistance in all UPEC. The high baseline MDR prevalence of 76% observed in this study is an indication of the widespread antibiotic pressure in the local health care setting and also indicates that determinants of resistance have spread widely within different UPEC lineages, likely via horizontal gene transfer [17].

In contrast, the highly significant relationship between the positivity for *cnf1* and the resistance to ciprofloxacin ($p = 0.0110$) is of clinical importance. Selective linkage indicates that the fluoroquinolone pressure could actually directly favor the maintenance of *cnf1*-positive strains of *E. coli*, either because these genes are linked on the chromosome or because when *E. coli* are subjected to fluoroquinolones, *cnf1* is also co-selected. The clinical impact of this co-selection pressure is

significant since fluoroquinolones could be used as empiric treatment UTI and this could inadvertently select highly cytotoxic, tissue-invasive B2 and D strains. This discovery highlights the importance of prudent use of fluoroquinolones in the management of UTIs and the potential for the addition of virulence testing in addition to antimicrobial susceptibility testing to help inform treatment [18], [19]. The high proportion of phylogenetic clade B2 (56%) and the comparatively high proportion of phylogenetic clade D (24%) among *cnf1*-positive isolates are good confirmations that the strains found locally are true, highly adapted extraintestinal pathogens and not opportunistic commensals. In fact, the over-representation of *cnf1*-positive isolates in clades B2 and D indicates conservation of this virulence factor in highly pathogenic clades, and it is well established that clades B2 and D harbour the majority of the ExPEC virulence genes [20]. Based on the PCR-RFLP mapping, the average similarity score of 98.81% was obtained between the *cnf1*-positive isolates, suggesting a high genetic conservation of the *cnf1* gene between different phylogenetic backgrounds. This conservation implies that the *cnf1* gene product should be under strong purifying selection, as it can keep its functional integrity because it brings a fitness benefit for strains of UPEC. Our results clearly illustrate the utility of PCR-RFLP profiling, because the simple digestion of enzymes can give high-fidelity epidemiological information without relying on outsourcing to a commercial sequencing facility [21].

Limitations of the Study

The study has a number of drawbacks. First, the sample sizes in this study, 100 UPEC isolates from a geographically concentrated capture zone, may not fully represent the multi-regional epidemiological differences in Pakistan and may lead to a reduced power for subgroup analyses. Second, the antibiotic resistance testing was done purely with disc diffusion; the incorporation of minimum inhibitory concentration (MIC) data by using microdilution would give valuable

information regarding the level of antibiotic resistance and distinguish between low-level and high-level resistance phenotypes. Third, although PCR-RFLP was very successful for sorting the major clades and for establishing the similarity indexes, it is incapable of identifying the minor single nucleotide polymorphisms (SNPs) or new variants of the entire gene body that can be discovered only by full-gene high-throughput Sanger and next-generation platforms. Lastly, these in vitro biochemical profiles and molecular endpoint screens were not quantitative, and dynamic expression levels of the *cnf1* protein during active human tissue pathogenesis were not quantified. Prospective analyses with larger and more heterogeneous patient populations, functional assays, and clinical outcome analyses would further validate our understanding of the role of *cnf* in UTI pathogenesis.

CONCLUSION

In the current study, the prevalence of the *cnf1* virulence gene is found to be 25% in the UPEC isolates of Pakistan, which is similar to the findings from other parts of the world and highlights its clinical significance. An exceptionally high correlation of *cnf1* carriage with blood agar activity supports the genetic co-localisation of these virulence factors and allows blood agar testing to be used as a sentinel marker of virulent strains. Importantly, there is a high association between *cnf1* positivity and ciprofloxacin resistance (72%) with implications for patient outcome, as this empirical therapy may select more tissue-invasive clones. The preponderance of phylogenetic clade B2 (56%) and clade D (24%) suggest that these strains are bona fide extraintestinal pathogens and not just commensal opportunists. The optimised workflow for the boiling-lysis DNA extraction and PCR-RFLP proves that strong molecular surveillance is possible in low-resource areas. Overall, this affordable strategy provides a viable and sustainable way to monitor virulence dynamics and create evidence-based clinical guidelines to contribute significantly to improving

UTI management and antimicrobial stewardship in Pakistan.

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