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MOLECULAR INSIGHTS INTO VIRULENCE FACTORS OF URINARY TRACT INFECTION BACTERIA IN DIABETIC PATIENTS USING PCR

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ABSTRACT

Urinary tract infections (UTIs) represent a significant health burden, particularly in individuals with diabetes mellitus, who are predisposed to more frequent, severe, and complicated infections. The pathogenesis of UTIs is intricately linked to the expression of various bacterial virulence factors, which enable pathogens to adhere, invade, evade host defenses, and cause tissue damage. Traditional microbiological methods often focus on bacterial identification and antimicrobial susceptibility, but they may not fully elucidate the molecular mechanisms underlying infection severity and persistence. This article reviews the application of Polymerase Chain Reaction (PCR) as a powerful molecular tool for the precise detection and characterization of virulence genes in bacterial strains isolated from UTIs in diabetic patients. By targeting specific genes associated with adhesion, toxin production, biofilm formation, and antibiotic resistance, PCR offers enhanced sensitivity, specificity, and speed, providing critical insights into the molecular epidemiology and pathogenic potential of these isolates. Such molecular characterization is crucial for understanding disease progression, guiding targeted therapeutic strategies, and developing effective preventive measures in this vulnerable patient population.

Keywords: Urinary Tract Infections; Diabetes Mellitus; Virulence Genes; Polymerase Chain Reaction (PCR); Escherichia coli; Pseudomonas aeruginosa; Staphylococcus aureus; Enterococcus faecalis; Biofilm; Antibiotic Resistance.

INTRODUCTION

Urinary tract infections (UTIs) are among the most common bacterial infections affecting humans globally [16]. While UTIs can affect individuals of all ages and genders, certain populations, such as diabetic patients, exhibit a significantly higher susceptibility and often experience more complicated and recurrent infections [5, 27]. Diabetes mellitus, characterized by chronic hyperglycemia, leads to a cascade of physiological alterations that collectively predispose individuals to UTIs. These interconnected factors include significant changes in the immune system, impaired blood circulation, neuropathy affecting bladder function, and altered urinary glucose levels, all of which create a more permissive environment for bacterial growth and persistence in the urinary tract [5, 7].

Diabetic patients, particularly women, are noted to be more prone to frequent UTIs than their non-diabetic counterparts [5]. The heightened risk is primarily attributed to a compromised immune response, where elevated blood glucose levels (hyperglycemia) can directly impair the function of polymorphonuclear leukocytes, which are essential for effectively combating infections [7]. This leukocyte dysfunction can diminish the body's ability to clear invading bacteria. Furthermore, diabetes can lead to diabetic neuropathy, affecting bladder innervation and resulting in poor bladder contraction and incomplete emptying. This urinary retention creates a stagnant environment where bacteria can thrive and multiply, significantly increasing the risk of UTIs [7]. Diabetic women with UTIs are also more susceptible to severe complications such as pyelonephritis (kidney infection) and frequently require hospitalization when compared with non-diabetic women [28]. These combined factors—immune system impairment, anatomical susceptibility, and bladder dysfunction—render diabetic patients particularly vulnerable to recurrent and severe UTIs.

The ability of bacteria to cause UTIs is intricately linked to the expression of specific virulence factors. These factors are diverse and enable pathogens to successfully colonize, invade, evade host defenses, and cause tissue damage. They include adhesins that facilitate attachment to uroepithelial cells, toxins that directly damage host tissues, enzymes that degrade host components,

mechanisms for efficient iron acquisition from the host, and sophisticated systems for biofilm formation that promote bacterial survival, persistence, and resistance to antimicrobials and host immunity [18]. A comprehensive understanding of the specific virulence profiles of uropathogens is paramount for comprehending the mechanisms of infection, predicting disease severity, guiding targeted therapeutic strategies, and developing effective prevention measures.

Common bacterial culprits in UTIs include Escherichia coli (particularly uropathogenic E. coli or UPEC), Klebsiella pneumoniae, Pseudomonas aeruginosa, Proteus mirabilis, Enterococcus faecalis, and Staphylococcus aureus [1, 2, 12, 30]. Each of these pathogens possesses a unique repertoire of virulence genes that contribute to their pathogenic success. For instance, UPEC is well-known for its fimbrial adhesins, such as Type 1 fimbriae (encoded by genes like fimH) and P fimbriae (encoded by genes such as papC), which are crucial for initial colonization and adherence to the uroepithelium [2, 15, 18]. UPEC also produces potent toxins like Cytotoxic Necrotizing Factor 1 (CNF1), a 115-kDa chromosomally-encoded toxin that activates Rho GTPases and catalyzes the deamidation of small GTPases like RhoA, leading to cytopathic effects in host cells and contributing to tissue damage and inflammation [4, 13, 24]. Epidemiological studies have suggested a strong link between CNF1 production by E. coli and UTI severity, indicating its significance as a UPEC virulence factor [18, 24].

Pseudomonas aeruginosa is a significant opportunistic uropathogen and a major cause of complicated UTIs, especially in immunocompromised individuals such as those with diabetes [30]. Its prominence as a potent pathogen is attributed to several physiological factors, including minimal nutritional requirements, the ability to target immunocompromised hosts, a remarkable capacity to develop resistance through multiple mechanisms, and its potential to infect nearly any body site with extreme tolerance to a wide range of physical conditions. A key virulence factor is the secretion of alginate, an exopolysaccharide, which plays a crucial role in biofilm development [23, 25]. The alginate biosynthetic pathway, involving genes like algA, algC, and algD, is well-understood, with alginate production being first observed in mucoid strains from cystic fibrosis patients and later confirmed in in vivo biofilms in various infections, including UTIs [23, 25, 31]. P. aeruginosa also employs sophisticated cell-to-cell signaling systems, known as quorum sensing, to regulate the expression of its virulence factors. These include the las system (comprising lasI and lasR genes, controlling factors like LasB elastase and exotoxin A) and the rhl system (comprising rhlI and rhlR genes, regulating rhamnolipids and other extracellular factors like pyocyanin and alkaline protease) [6, 8, 14, 22, 31].

Staphylococcus aureus has shown an increasing incidence in UTIs, particularly in older patients and those with nosocomial infections, including diabetic patients [1]. A significant challenge in treating S. aureus infections is the emergence of Methicillin-Resistant S. aureus (MRSA), which is resistant to a broad group of beta-lactam antibiotics due to the presence of the mecA gene [11]. MRSA strains, especially community-associated MRSA (CA-MRSA), frequently carry the Panton-Valentine Leukocidin (PVL) virulence genes (lukS-PV and lukF-PV), which are encoded on a prophage and cause leukocyte destruction and tissue necrosis, contributing to severe infections [1, 17].

Enterococcus faecalis is a leading cause of bacteremia in nosocomial infections, including UTIs [12]. While much remains unknown about the specific factors E. faecalis utilizes to cause urinary tract infections, research has increasingly focused on identifying factors on both the host and pathogen sides that contribute to UTIs [12]. Biological markers, such as the efa gene (encoding E. faecalis endocarditis antigen), may play a critical role in modulating the expression of enterococcal virulence-associated genes, particularly in the urinary tract environment [26, 29]. A significant therapeutic challenge is the escalating resistance of E. faecalis to antimicrobials, leading to the emergence of vancomycin-resistant enterococci (VRE), often associated with the vanA or vanB glycopeptide resistance genes [3, 9, 10, 29].

Traditional methods for identifying bacterial virulence factors often rely on phenotypic assays, such as enzyme production tests, adherence assays, or toxin neutralization tests. While valuable, these methods can be time-consuming, labor-intensive, and may lack the sensitivity or specificity required for comprehensive characterization, especially when virulence factors are not expressed under standard in vitro culture conditions. The advent of molecular techniques, particularly Polymerase Chain Reaction (PCR), has revolutionized the study of bacterial pathogenesis. PCR offers a highly sensitive, specific, and rapid method for detecting the presence of specific DNA sequences, including virulence genes, directly from bacterial isolates or even clinical samples [10]. This molecular approach provides a precise means to characterize the genetic potential of uropathogens, offering valuable insights beyond what phenotypic assays alone can provide. This article aims to provide a novel insight into the detection of various determinants of virulence in uropathogens, predominantly in the context of diabetic patients, that have not been fully elucidated by previous studies. It explores the utility of PCR in unraveling the molecular landscape of virulence genes in bacteria associated with UTIs in diabetic patients, emphasizing its role in enhancing our understanding of disease progression and informing clinical management.

METHODS

The molecular characterization of virulence genes in bacteria associated with UTIs in diabetic patients necessitates a robust methodological framework,

primarily centered on Polymerase Chain Reaction (PCR) techniques. This section provides a detailed outline of the general methodological approach for such investigations, encompassing sample collection, bacterial processing, molecular assays, and data analysis. The aim is to provide a comprehensive understanding of the experimental design and procedures employed to detect key virulence determinants in common uropathogens.

1. Study Design and Sample Collection

A comprehensive study design is crucial for obtaining representative and reliable data. In a typical study, a total of 1000 urine samples would be collected from both diabetic and non-diabetic patients. These samples would be sourced from various healthcare facilities, such as the Diabetology Department at Bangalore Hospital, India, and other diabetic centers across Bangalore city. This broad sampling ensures a diverse representation of patient demographics and infection profiles.

Patient recruitment would involve individuals from varying socioeconomic statuses (e.g., lower and higher socioeconomic groups) to assess potential correlations between socioeconomic factors and the prevalence of specific uropathogens or virulence gene profiles. Information regarding patient demographics, including age, history of urinary frequency, abdominal pain, and pre-existing conditions such as hypertension, cardiovascular diseases, lipidemia, or any previous history of infections, would be collected using self-prepared questionnaires administered to diabetic patients. For instance, a subset of 100 urine samples specifically from patients with Type 1 diabetes would be obtained from a dedicated Diabetic Center to allow for focused analysis within this specific diabetic cohort.

Urine samples would be collected as clean-voided midstream specimens in sterile containers to minimize contamination. Strict adherence to collection protocols is essential to ensure sample integrity. Following collection, urine samples would be processed within two hours to prevent bacterial overgrowth or degradation of bacterial components.

2. Bacterial Isolation and Identification

Upon arrival at the laboratory, 1 µl of each urine sample would be inoculated onto sterile Petri dishes using a calibrated inoculation loop. The samples would then be streaked onto HiChrome UTI agar (Himedia Labs India Pvt Ltd), a chromogenic medium designed for the presumptive identification of common urinary pathogens based on characteristic color reactions. The plates would be incubated overnight at 37∘C.

Bacterial colonies would be identified based on the color produced on the Hi-Chrome UTI Agar. A colony count of 100,000 cfu/ml (colony-forming units per milliliter) would be considered clinically significant for diagnosing a UTI. Non-lactose fermenters (NLFs) would be further identified using standard biochemical tests to confirm their identity. Once identified, isolated colonies of predominant UTI pathogens, such as Escherichia coli, Pseudomonas aeruginosa, Enterococcus faecalis, and Staphylococcus aureus, would be aseptically transferred into Eppendorf tubes containing semisolid media. These prepared tubes would serve as stock cultures, stored appropriately (e.g., at 4∘C for short-term or −80∘C for long-term storage with cryoprotectants) for subsequent analysis.

3. Antibiotic Susceptibility Testing

The antibiotic sensitivity of the isolated UTI pathogens would be assessed using the Kirby-Bauer disc diffusion method, strictly adhering to the Clinical and Laboratory Standards Institute (CLSI) guidelines. This standardized method ensures reproducible and comparable results.

The procedure would involve:

- Inoculum Preparation: A loopful of 3-4 colonies of the test organisms (S. aureus, E. coli, P. aeruginosa, and E. faecalis) would be selected from pure culture plates and transferred into 5 ml of sterilized Brain Heart Infusion (BHI) broth (Himedia Labs Pvt., Ltd.). The tubes would be incubated at 34∘C for 24 hours until the turbidity of the bacterial suspension matches a 0.5 McFarland standard, which corresponds to a bacterial concentration of approximately 1.5×108 cfu/ml.

- Plate Inoculation: A non-toxic sterile cotton swab would be dipped into the standardized inoculum and rotated to ensure complete saturation. Excess fluid would be expressed by pressing the swab firmly against the upper wall of the tube. The entire surface of a Mueller-Hinton agar plate would then be swabbed three to four times, rotating the plate approximately 60 degrees between each streak to ensure uniform bacterial growth. The inoculated plates would be allowed to dry for 5-15 minutes with the lid in place, ensuring the inoculum is absorbed into the agar.

- Disc Application: Following aseptic technique, commercially available antibiotic discs (Himedia Pvt., Ltd.), impregnated with specific antimicrobial agents, would be carefully placed on the surface of the inoculated agar using a sterile forcep. The discs would be spaced at a distance of at least 24 mm apart to prevent overlapping zones of inhibition.

- Incubation and Reading: The plates would be inverted and incubated at 37∘C for overnight incubation (typically 16-18 hours). After incubation, the diameter of the complete zone of inhibition around each antibiotic disc would be measured to the nearest millimeter. The results would be tabulated and interpreted as susceptible (S), intermediate (I), or resistant (R) according to the CLSI breakpoints for each antibiotic.

- Antibiotic Panel: Gram-positive and Gram-negative isolates would be tested against a panel of clinically relevant antibiotics, including cephalosporins, aminoglycosides, fluoroquinolones, and glycopeptides, as

recommended by CLSI standards.

- Selection for PCR: Isolates demonstrating resistance or specific profiles, such as vancomycin-resistant enterococci (VRE), multi-drug resistant (MDR) P. aeruginosa, and specific E. coli strains, would be selected for further molecular analysis to detect virulence genes by PCR.

4. MOLECULAR METHODS

The core of this study involves the application of PCR for the precise detection and characterization of virulence genes.

4.1. DNA Extraction and Purification

Both plasmid and genomic DNA would be extracted from the selected UTI isolates. Plasmid DNA purification would typically follow a modified alkaline lysis method, such as that described by P. Courvalin (Antibiotic Resistance Techniques, 5th edition, Pasteur Institute, 2008).

The detailed protocol for plasmid DNA extraction would involve:

- Bacterial Culture: A loopful of stock culture from each UTI isolate would be inoculated into 5 ml of Brain Heart Infusion (BHI) broth and incubated overnight at 37∘C under appropriate conditions (e.g., shaking for aerobic bacteria).

- Cell Harvesting: Two milliliters of the overnight cell suspension would be transferred to a microcentrifuge tube and centrifuged at 15,000 rpm for 5 minutes in a refrigerated centrifuge to pellet the bacterial cells.

- Cell Lysis: The supernatant would be discarded, and the resulting bacterial pellet would be re-suspended thoroughly in 100 µl of TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA). For Gram-positive bacteria like Enterococcus species, an additional step would be included: the pellet would be further re-suspended in 100 µl of TGE-lysozyme solution and incubated at 37∘C for a specified period (e.g., 30 minutes) to facilitate cell wall digestion. Subsequently, 200 µl of a freshly prepared NaOH-SDS solution (e.g., 0.2 M NaOH, 1% SDS) would be added to the mixture. The tube would be gently inverted 4-5 times to mix the contents without vigorous vortexing, which could shear chromosomal DNA. The mixture would then be allowed to stand on ice for 5 minutes to complete the alkaline denaturation of DNA.

- Neutralization and Precipitation: To neutralize the solution and precipitate chromosomal DNA and cellular debris, 150 µl of cold potassium acetate solution (e.g., 3 M potassium acetate, pH 4.8) would be added. The tube would be immediately inverted several times and placed on ice for an additional 10 minutes.

- Phenol-Chloroform Extraction: Following neutralization, a phase separation step would be performed. 200 µl of Tris-saturated phenol (Bangalore Genei India Pvt., Ltd.) and 200 µl of chloroform would be added to the mixture. The tube would be vigorously vortexed to mix the phases thoroughly and then centrifuged for 3 minutes (e.g., at 12,000 rpm) to separate the aqueous (top), interphase, and organic (bottom) layers. Approximately 400 µl of the upper aqueous layer, containing the plasmid DNA, would be carefully transferred to a fresh microcentrifuge tube, avoiding any white residues at the interphase.

- Ethanol Precipitation: The transferred supernatant would be washed with 800 µl of absolute ethanol to precipitate the DNA. The mixture would be inverted several times and then centrifuged (e.g., at 12,000 rpm for 10 minutes). The supernatant would be discarded, and the DNA pellet would be washed with 70% ethanol (e.g., 500 µl) to remove residual salts. This wash step would be followed by another centrifugation for an additional 5 minutes.

- Resuspension: The 70% ethanol would be carefully removed, and the pellet would be air-dried for 10-15 minutes to evaporate any remaining ethanol. Finally, the purified plasmid DNA pellet would be re-suspended in 100 µl of sterile TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) containing 1 µl of RNAse (Chromous Biotech Pvt Ltd) to degrade any contaminating RNA. This method ensures the isolation of high-quality plasmid DNA suitable for subsequent PCR analysis.

4.2. Primer Design and Synthesis

Specific oligonucleotide primers are the cornerstone of PCR assays, dictating the specificity and efficiency of amplification. Primers would be commercially synthesized based on sequences of specific virulence genes described in previous validated studies. These sequences would be rigorously compared with gene sequences available in public databases like GenBank to ensure their accuracy and specificity for the target genes (Table 2).

For example, primers would be designed to amplify:

- For Escherichia coli: The cnf1 gene, encoding cytotoxic necrotizing factor type 1.

- For Enterococcus faecalis: The vanA gene, associated with vancomycin resistance, and the efa gene, encoding the E. faecalis endocarditis antigen.

- For Pseudomonas aeruginosa: The algD gene (GDP mannose dehydrogenase) involved in alginate synthesis, and the quorum sensing genes las-R and rhl-R.

- For Staphylococcus aureus: The mecA gene (methicillin resistance) and the pvl gene (Panton-Valentine Leukocidin).

4.3. PCR Amplification Protocols

PCR amplification would be performed using a thermocycler (e.g., MJ research- INC, USA) with specific cycling conditions optimized for each target gene to

ensure efficient and specific amplification. The general PCR reaction mixture (e.g., 44 µL total volume) would typically contain:

- Template DNA (e.g., 1 µL of extracted bacterial DNA)
- Deoxynucleotide triphosphates (dNTPs) (e.g., 1.5 µL of 2.5 mM dNTPs)
- Taq buffer (e.g., 5 µL of 10X Taq Assay buffer)
- Taq polymerase (e.g., 0.5-1 µL)
- Forward and reverse primers (e.g., 1 µL of each primer)
- Nuclease-free water (to bring the reaction volume to the desired total)

Specific cycling conditions for each gene would be as follows:

- Detection of cnf1 gene in E. coli:
  - Initial denaturation: Not specified, but typically 94-95°C for 2-5 minutes.
  - 30 consecutive cycles:
    - Denaturation: 1 minute at 95◦C
    - Annealing: 1 minute at 50◦C
    - Extension: 3 minutes at 72◦C
  - Final extension: 10 minutes at 72◦C
  - Expected product length: 543-600 bp.
- Detection of vanA gene in Enterococcus faecalis:
  - Initial denaturation: 10 minutes at 95◦C
  - 40 cycles:
    - Denaturation: 1 minute at 94◦C
    - Annealing: 1 minute at 50◦C
    - Extension: 1 minute at 72◦C
  - Final extension: 5 minutes at 72◦C
  - Expected product length: 231 bp.
- Detection of efa gene in Enterococcus faecalis:
  - Initial enzyme activation: 15 minutes (temperature not specified, typically 94-95°C).
  - Denaturation step: 95◦C (duration not specified).
  - 35 consecutive cycles:
    - Denaturation: 20 seconds at 94◦C
    - Annealing: 45 seconds at 68◦C
    - Extension: 15 seconds at 72◦C
  - Expected product length: 688 bp.
- Detection of algD gene in Pseudomonas aeruginosa:
  - Initial denaturation: 5 minutes at 94◦C
  - Cycling conditions (number of cycles not specified, typically 25-35):
    - Annealing: 45 seconds at 50◦C
    - Extension: 1 minute at 72◦C
  - Single final extension: 5 minutes at 72◦C
  - Expected product length: 1310 bp (1 kb).
- Detection of quorum sensing genes (las-R and rhl-R) in P. aeruginosa:
  - Initial denaturation: 1 minute at 94◦C
  - 30 cycles:
    - Annealing: 1 minute at 52◦C
    - Primer extension: 1.5 minutes at 72◦C
  - Expected product lengths: 706 bp for las-R and 675 bp for rhl-R.
- Detection of mecA gene in Staphylococcus aureus:
  - Initial denaturation: 45 seconds at 95◦C
  - Cycling conditions (number of cycles not specified, typically 25-35):
    - Extension: 1 minute at 60◦C (this implies annealing and extension are combined)
  - Final extension: 2 minutes at 72◦C
  - Expected product length: 400 bp.
- Detection of pvl gene in Staphylococcus aureus:
  - Denaturation: 30 seconds at 94◦C
  - Cycling conditions (number of cycles not specified, typically 25-35):
    - Annealing: 1 minute at 55◦C
    - Elongation: 1 minute at 72◦C
  - Final elongation: 1 minute at 72◦C
  - Expected product length: 450 bp.

4.4. Agarose Gel Electrophoresis

All PCR products and purified plasmid DNA would be analyzed using agarose gel electrophoresis to confirm successful amplification and determine product size.

- Plasmid DNA Visualization: Five microliters of the purified plasmid DNA would be loaded directly onto a 1% agarose gel. A Lambda DNA/Hind III digest molecular marker (Chromous Biotech Pvt Ltd), generating eight fragments ranging from 1215 bp to 23,130 bp (at a concentration of 200 ng/µl), would be run alongside the samples to estimate plasmid sizes. This step ensures the isolation of high-quality plasmid DNA suitable for further

analysis.

- PCR Product Visualization: All PCR products would be examined on 1.5% to 2% agarose gels, depending on the expected size of the PCR product. Gels would be prepared with appropriate concentrations of agarose in Tris-Acetate-EDTA (TAE) or Tris-Borate-EDTA (TBE) buffer. After electrophoresis, the gels would be stained with a DNA-binding dye (e.g., ethidium bromide or a safer alternative) and visualized under UV light. The presence of a band of the expected size for each target gene would confirm successful amplification.

4.5. 16S rDNA Sequencing for Identification

For precise identification of bacterial isolates, particularly for confirming species identity of strains like Pseudomonas aeruginosa, 16S ribosomal DNA (rDNA) sequencing would be performed.

- Genomic DNA Isolation: Genomic DNA would be isolated from pure cultures of the test organisms using standard methods.

- PCR Amplification: The 16S rDNA fragment would be amplified using a high-fidelity polymerase chain reaction to ensure accuracy.

- Sequencing and Analysis: The PCR product would be sequenced bidirectionally using both forward and reverse primers (e.g., by Chromous Biotech Pvt Ltd India). The obtained sequence data would then be aligned and analyzed for homology with sequences available in public databases, such as GenBank (e.g., NCBI Accession No. for P. aeruginosa AL98; AJ249451, 42A2 NCBI 40045; AJ309500 0.996 Pseudomonas sp.; OLB-1, AJ387904), to confirm the species identification of the isolates.

4.6. Growing and Analyzing Static Biofilms in P. aeruginosa Isolates

To assess the biofilm-forming capacity of P. aeruginosa isolates, a colony biofilm assay would be performed using a previously described method [21]. Five isolates of P. aeruginosa would be selected for this study.

The detailed procedure would be:

- Culture Preparation: A loopful of the P. aeruginosa culture would be inoculated into 5 ml of Luria-Bertani (LB) broth and grown overnight at 37∘C with shaking.

- Membrane Preparation: Using sterile forceps, a 25 mm nitrocellulose membrane with a pore size of 0.22 µm would be positioned in a sterile Petri plate. This setup would be performed approximately 30 cm apart from a UV light source in a sterile environment (e.g., a laminar flow hood) to maintain sterility.

- Inoculation: Diluted stationary-phase cultures (from the overnight growth) would be prepared in the appropriate medium to an optical density at 600 nm (OD600) of 0.05, which corresponds to approximately 1.64×108 cfu/ml. Five microliters of this diluted culture would be inoculated onto the shiny side of the nitrocellulose membrane, which has been placed on an agar medium plate not containing antibiotics. This procedure would be repeated for all remaining membranes, inoculating up to six membranes on the same agar plate.

- Incubation for Biofilm Formation: Once the liquid on the membranes had dried, the plates would be incubated upright at the appropriate temperature (e.g., 37∘C) for 24 hours to allow biofilm development. After the initial 24 hours, each membrane would be gently lifted off its agar plate using sterile forceps and transferred to a fresh agar plate (up to six membranes per plate) to provide fresh nutrients. These plates would then be incubated for an additional 24 hours at the appropriate temperature (e.g., 37∘C).

- Biofilm Growth Analysis: Biofilm growth would be quantified by aseptically transferring the membranes into 15 ml tubes containing 10 ml of sterile phosphate-buffered saline (PBS). The tubes would be capped, and the samples would be vigorously vortexed to detach all the bacteria from the biofilm. A dilution series of the vortexed samples would be prepared (e.g., 10-fold serial dilutions), and each dilution would be plated onto separate agar plates (e.g., LB agar). The plates would be incubated overnight, and the colony count (cfu) would be determined. The average number of colony-forming units per membrane would then be calculated.

- Antibiotic Treatment of Biofilms: A similar method would be followed for membranes supplemented with and without antibiotics (e.g., Ceftriaxone and Tobramycin) to assess the impact of these antibiotics on biofilm viability and resistance. This would involve incorporating the antibiotics into the agar medium on which the membranes are incubated.

5. DATA ANALYSIS

Quantitative data, such as colony counts and gene prevalence, would be tabulated in percentages (frequencies). Statistical analysis would be performed using statistical software such as SPSS Version 26.0 (SPSS Inc., Chicago, IL). Continuous variables (e.g., age, colony counts) would be summarized in the form of means and standard deviations. Categorical variables (e.g., presence/absence of a gene, prevalence of uropathogens) would be summarized as percentages. The Chi-square test or Fisher's exact test, whichever is appropriate based on sample size and expected cell counts, would be applied for comparing categorical variables between different groups (e.g., diabetic vs. non-diabetic, different socioeconomic groups). A p-value of less than 0.05 ($p<0.05$) would be considered statistically significant, indicating a 95% confidence interval.

6. ETHICAL CONSIDERATIONS

Ethical approval for the study would be addressed in accordance with institutional guidelines. In cases where

urine samples are collected directly from the microbiology departments of various hospitals after obtaining the required institutional approvals for anonymized or de-identified samples, no additional ethical approval from a separate research ethics committee might be mandatory, as decided by an advisory committee. However, strict adherence to patient privacy and data anonymization protocols would always be maintained.

RESULTS

The application of Polymerase Chain Reaction (PCR) for the molecular characterization of virulence genes in bacterial isolates from urinary tract infections (UTIs), particularly in diabetic patients, has yielded comprehensive and significant insights into the pathogenic landscape of these infections. This section details the key findings regarding uropathogen prevalence, antibiotic susceptibility, and the molecular detection of specific virulence genes across various bacterial species, along with observations on biofilm formation.

1. Prevalence of Uropathogens in Diabetic Cohorts

Analysis of the collected urine samples revealed distinct patterns in the prevalence of uropathogens among diabetic and non-diabetic patient groups.

- Overall Diabetic Patients: Among the diabetic patients, a total of 250 individuals were included (140 males and 110 females). The prevalence of uropathogens in males was Escherichia coli 46 (32.9%), Enterococcus faecalis 44 (31.4%), Pseudomonas aeruginosa 2 (1.4%), and Staphylococcus aureus 26 (18.6%). In diabetic females, the prevalence was E. coli 28 (25.5%), E. faecalis 32 (29.1%), P. aeruginosa 10 (9.1%), and S. aureus 22 (20%). Notably, 22 males (15.7%) and 18 females (16.4%) were negative for UTI.

- Higher Socioeconomic Diabetic Group: In the higher socioeconomic group with diabetes, the prevalence was observed as E. coli 18 (13.8%) in males and 13 (10.8%) in females, E. faecalis 32 (24.6%) in males and 36 (30.0%) in females, and P. aeruginosa 30 (23.1%) (gender distribution for P. aeruginosa not specified in the provided data for this subgroup).

- Type 1 Diabetic Group: For patients with Type 1 diabetes (total 100 samples), the incidence of uropathogens was E. coli 22 (44%) in males and 15 (30%) in females, E. faecalis 10 (20%) in males and 20 (40%) in females, and S. aureus 14 (28%) in males and 8 (16%) in females. A small proportion, 8% of males and 14% of females, showed no evidence of UTI in this group.

- Non-Diabetic Patients: The prevalence of uropathogens in non-diabetic patients was significantly lower compared to diabetic patients. E. coli was found in 8.4% and E. faecalis in 20% of non-diabetic samples. Importantly, there was no prevalence of P. aeruginosa observed in the non-diabetic group. S. aureus was detected in 10.8% of non-diabetic patients. A substantial majority, 60.88% of non-diabetic patients, were culture-negative for UTI. These findings on the prevalence of UTI among diabetic patients align with previously published data [27].

2. Antibiotic Susceptibility Patterns

A total of seventy isolates (twenty each of E. coli, P. aeruginosa, E. faecalis, and ten of S. aureus) were subjected to antibiotic sensitivity testing using the Kirby-Bauer disc diffusion method. The results, summarized in Table 4, indicated varying resistance and sensitivity patterns among the predominant uropathogens.

- E. coli: Showed sensitivity to Fosfomycin (FO), Levofloxacin (LE), Ciprofloxacin (CIP), Amikacin (AK), Gentamicin (G), Piperacillin-Tazobactam (PIT), Nitrofurantoin (NIT), and Tobramycin (TOB). Resistance was observed against Cefotaxime (CTX), Amoxicillin-Clavulanate (AMC), Co-trimoxazole (COT), Ceftazidime (CAZ), and Nalidixic Acid (NA).

- E. faecalis: Exhibited sensitivity to Streptomycin (STR), Amikacin (AK), Fosfomycin (FO), Gentamicin (G), Levofloxacin (LE), Ofloxacin (OF), Tetracycline (TE), and Vancomycin (VA). Resistance was noted for Nitrofurantoin (NIT), Tobramycin (TOB), Ciprofloxacin (CIP), Cefotaxime (CTX), Co-trimoxazole (COT), Ampicillin-Sulbactam (AMP/S), and Amoxicillin-Clavulanate (AMC).

- P. aeruginosa: Demonstrated sensitivity to Imipenem (IMP), Meropenem (MRP), Amikacin (AK), Tobramycin (TOB), and Ticarcillin (TIC). Resistance was observed against Amoxicillin-Clavulanate (AMC), Ceftazidime (CAZ), Levofloxacin (LE), and Aztreonam (AZ).

- S. aureus: Was sensitive to Gentamicin (G), Linezolid (LZ), Teicoplanin (TEI), Vancomycin (VA), and Amikacin (AK). Resistance was found against Amoxicillin-Clavulanate (AMC), Clindamycin (CD), Methicillin (METH), Ciprofloxacin (CIP), and Co-trimoxazole (COT).

3. Molecular Detection of Virulence Genes

PCR amplification was successfully performed for various virulence genes across the selected uropathogens, providing direct molecular evidence of their pathogenic potential. The product sizes were confirmed by agarose gel electrophoresis.

3.1. Escherichia coli Virulence Factors

Fifteen E. coli isolates were investigated for the presence of the cnf1 gene, which encodes Cytotoxic Necrotizing Factor 1 (CNF1). Amplification products of the expected size (approximately 600 bp) for cnf1 were detected in seven isolates, accounting for 46% of the tested E. coli strains (Fig. 1). The oligonucleotide primers designed for cnf1 demonstrated 100% specificity and sensitivity, allowing for rapid and accurate detection of this virulence gene in E. coli strains isolated from diabetic urine samples.

3.2. Enterococcus faecalis Virulence Factors

Molecular analysis of Enterococcus faecalis isolates focused on the vanA gene, associated with vancomycin resistance, and the efa gene, encoding a cell wall adhesion factor.

- VanA Gene: Fourteen E. faecalis isolates were tested for the presence of the vanA gene. Clear PCR products of the expected size (300 bp) for vanA were obtained in four isolates, representing 28% of the tested strains. There was 100% agreement between the PCR results and previously published genotypes and phenotypes for vancomycin resistance (Fig. 3).

- Efa Gene: Out of ten E. faecalis isolates tested for the efa gene, eight (80%) were found to be positive in isolates from diabetic urine samples. The product size for the efa gene was 600 bp (Fig. 2).

3.3. Pseudomonas aeruginosa Virulence Factors and Biofilm Characteristics

Pseudomonas aeruginosa isolates were characterized for the presence of the algD gene and quorum sensing genes (las-R and rhl-R), as well as their biofilm-forming capabilities.

- 16S rDNA Sequencing: A 1.5-kb 16S rDNA fragment was successfully amplified from P. aeruginosa isolates using high-fidelity PCR. Sequence data alignment and analysis identified one multi-drug resistant (MDR) P. aeruginosa strain, which showed high similarity to P. aeruginosa GenBank entry AJ249451.

- AlgD Gene: The prevalence of the virulence gene encoding alginate (algD) was determined by PCR. A 1 kb PCR product was obtained following successful amplification and compared with a 5 kb ladder (Chromous Biotech Pvt India Ltd) (Fig. 4). While only one isolate of MDR P. aeruginosa was investigated for this specific gene, its sequence was submitted to GenBank with accession number MT916847.

- Quorum Sensing Genes (las-R and rhl-R): PCR amplification of the P. aeruginosa quorum sensing genes, las-R and rhl-R, was performed on thirty-three isolates. Twenty isolates (60%) were found positive for these genes. The product sizes were determined to be 700 bp for las-R and 600 bp for rhl-R, respectively, in each positive isolate (Fig. 5). It was noted that these product sizes were slightly less than the expected sizes mentioned in some earlier studies.

- Biofilm Formation: P. aeruginosa isolates exhibited complete biofilm formation in all tested isolates (Fig. 6). A detailed analysis of colony counts from biofilm assays is presented in Table 3.

  - Effect of Antibiotics on Biofilms: The colony count of P. aeruginosa isolates treated with ceftriaxone indicated a higher colony count compared to untreated membranes in some instances, reflecting resistance to cephalosporins within the biofilm. Similarly, resistant mutants may be indicated in biofilm producers of P. aeruginosa isolates when treated with Tobramycin, although a more significant colony count was consistently observed in untreated membranes.

  - Biofilm Producer Prevalence: Out of 20 isolates, 16 were identified as biofilm producers (Table 3), indicating a high prevalence of this virulence mechanism.

3.4. Staphylococcus aureus Virulence Genes

Ten S. aureus isolates were investigated for the presence of the mecA gene (methicillin resistance) and the pvl gene (Panton-Valentine Leukocidin). Six of these MRSA strains (60%) were found to be positive for both the mecA and pvl genes (Table 5, Fig. 7 and Fig. 8). The product sizes were 400 bp for mecA and 450 bp for pvl.

In summary, the results from PCR-based molecular characterization provided a detailed genetic profile of virulence factors in various bacterial species causing UTIs in diabetic patients. The findings highlight the significant prevalence of genes associated with adhesion, toxin production, biofilm formation, and antimicrobial resistance, offering crucial insights into the molecular epidemiology and pathogenesis of these infections in a vulnerable patient population.

DISCUSSION

The molecular characterization of virulence genes in bacteria associated with urinary tract infections (UTIs) in diabetic patients using Polymerase Chain Reaction (PCR) represents a profound advancement in our understanding and management of these complex infections. Diabetes mellitus profoundly impacts host immunity and physiology, creating an environment conducive to more persistent, severe, and recurrent UTIs [5, 7, 27]. The chronic hyperglycemia, compromised immune responses, and bladder dysfunction observed in diabetic individuals collectively contribute to their increased susceptibility and the often-complicated nature of these infections [5, 7]. Consequently, a detailed understanding of the specific bacterial factors that contribute to pathogenesis in this vulnerable patient group is not merely beneficial but crucial for effective clinical intervention.

PCR has emerged as an indispensable tool in this context due to its inherent advantages of high sensitivity, specificity, and rapidity compared to traditional phenotypic assays. The ability to directly detect specific DNA sequences encoding virulence factors allows for a more precise assessment of a bacterial isolate's pathogenic potential, even if these factors are not expressed under standard in vitro laboratory conditions. This genetic information provides insights into the inherent capabilities of the pathogen that phenotypic tests might miss.

1. Interpretation of Key Findings

1.1. Escherichia coli and Cytotoxic Necrotizing Factor 1 (CNF1)

Our findings, demonstrating the presence of the cnf1 gene

in 46% of tested E. coli isolates from diabetic patients, underscore the potential role of this cytotoxin in UTI pathogenesis within this population. Uropathogenic E. coli (UPEC) is the major causative agent of UTIs, and while UTIs are often considered acute, self-limiting infections, the prevalence of recurrent infections is high, especially in diabetic patients [16, 18]. CNF1 is known to activate Rho GTPases, leading to deamidation of small GTPases like RhoA, which can result in significant cytopathic effects on host cells and tissue damage [4, 13]. The detection of cnf1 by PCR directly indicates the bacterium's genetic capacity to produce this potent toxin, suggesting a potential for more severe disease outcomes and a more aggressive course of ascending UTIs in diabetic patients [24]. While limited studies have identified the role of the cnf gene in UPEC in vivo, our study's findings, based on bacterial isolates grown in vitro, propose an intriguing new line of study for investigators interested in the pathogenesis of UTI, specifically highlighting the direct involvement of the cnf gene in the etiology of ascending UTIs in patients with diabetes. This simple yet powerful method of PCR detection provides a molecular marker that can be used to identify strains with higher pathogenic potential.

1.2. Enterococcus faecalis and Antibiotic Resistance/Adherence

Our study revealed the presence of vancomycin-resistant enterococci (VRE) urinary isolates in diabetic patients with a history of UTI, with 28% of tested E. faecalis isolates being positive for the vanA gene. This finding is significant given the increasing challenge posed by VRE in nosocomial infections [3, 10, 29]. Our data indicates that despite the predominance and widespread distribution of the vanA resistance determinant, this genotype has become well-established and shows remarkable stability in the diabetic population [9]. The rapid identification of these resistance determinants through PCR is paramount for guiding appropriate antimicrobial therapy and implementing effective infection control measures, especially in diabetic patients who are at higher risk for complicated and recurrent infections.

Furthermore, the high prevalence of the efa gene (80%) in E. faecalis isolates from diabetic urine samples suggests a potential role for this gene in the pathophysiology of UTI. While efa has primarily been associated with the virulence of endocarditis, our study indicates its potential involvement in bacterial adhesion within the urinary tract, despite the fact that only a few UTI isolates were found positive for carrying this gene in some previous studies [12, 26]. This finding warrants further investigation into the specific mechanisms by which efa contributes to E. faecalis pathogenesis in UTIs.

1.3. Pseudomonas aeruginosa: Biofilms, Quorum Sensing, and Multidrug Resistance

Pseudomonas aeruginosa is a formidable pathogen in complicated UTIs, particularly in immunocompromised individuals like diabetic patients [30]. Our study's findings regarding the prevalence of quorum sensing genes (las-R and rhl-R) and the algD gene, coupled with observed biofilm formation, are highly significant. The detection of las-R and rhl-R in 60% of P. aeruginosa isolates confirms the widespread genetic capacity for these sophisticated cell-to-cell signaling systems, which regulate the expression of numerous virulence factors and are central to biofilm formation [6, 8, 14, 22, 31]. While the observed product sizes were slightly less than expected in some instances, their consistent detection highlights the importance of these systems.

The high prevalence of biofilm formation among P. aeruginosa isolates (16 out of 20 tested were biofilm producers) is a critical finding. Biofilms contribute significantly to chronic infections and confer substantial resistance to antibiotics and host defenses [14, 21, 31]. Our experiments, which included analysis of biofilms among the P. aeruginosa isolates, significantly reflected high antimicrobial resistance to beta-lactams (e.g., ceftriaxone) and aminoglycosides (e.g., tobramycin). The observation of higher colony counts in treated membranes compared to untreated ones in some instances further supports the enhanced resistance of biofilm-embedded bacteria. This underscores the challenge of treating biofilm-associated UTIs in diabetic patients.

Our study also revealed a significant difference in the proportion of MDR P. aeruginosa strains isolated from diabetic patients belonging to the lower socioeconomic status, expressing key virulence genes. While the relationship between MDR phenotype and virulence remains controversial, our findings align with reports documenting the rise in MDR nosocomial P. aeruginosa strains that continue to threaten hospitalized patients despite various control measures. The characterization of these strains, found to harbor the algD gene and display high swimming motility and adhesiveness, further supports their pathogenic potential. The observed higher frequency of spread of quorum sensing and biofilm-associated genes among MDR P. aeruginosa isolates indicates a strong correlation between these virulence mechanisms and multidrug resistance.

1.4. Staphylococcus aureus and Methicillin Resistance/Leukocidin Production

The finding that 60% of tested S. aureus isolates from UTIs in diabetic patients were positive for both the mecA (methicillin resistance) and pvl (Panton-Valentine Leukocidin) genes is alarming. S. aureus is increasingly recognized as a cause of UTIs, and its misidentification as a contaminant can lead to undertreatment and life-threatening illness [1]. The prevalence of MRSA, particularly in older patients and those with nosocomial infections, is a growing concern [1, 11]. The mecA gene confers resistance to a large group of beta-lactam antibiotics, making treatment challenging [11]. Furthermore, the presence of PVL genes, which encode a

potent cytotoxin causing leukocyte destruction and tissue necrosis, indicates the potential for more severe and invasive infections. While PVL genes were historically infrequent, their high prevalence in newly emerging community-associated MRSA (CA-MRSA) strains, often associated with smaller and more easily transferable SCCmec genetic elements, highlights a significant public health threat [17]. Our study demonstrates that these genes are involved in community-acquired UTIs in diabetic patients, necessitating vigilant surveillance and appropriate therapeutic strategies.

2. Implications for Diabetic UTI Pathogenesis and Clinical Management

The comprehensive molecular characterization of virulence genes in uropathogens from diabetic patients provides several critical implications for understanding disease pathogenesis and guiding clinical management:

- Enhanced Diagnostic Markers: The detection of specific virulence genes (e.g., cnf1, algD, las-R, rhl-R, mecA, pvl, vanA, efa) can serve as valuable diagnostic markers. Rapid molecular assays can identify strains with higher pathogenic potential or specific resistance mechanisms, allowing for more targeted and timely antimicrobial therapy. This is particularly important in diabetic patients where empirical treatment might be less effective due to the prevalence of resistant or highly virulent strains.

- Understanding Disease Progression: The presence of multiple virulence factors in a single isolate, as noted in our study, reflects the aggressive nature of UTI pathogens in diabetic patients. For instance, a P. aeruginosa strain capable of both extensive biofilm formation and robust quorum sensing is likely to cause a more persistent and difficult-to-treat infection. Understanding these combinations can help clinicians predict disease severity and tailor management strategies.

- Infection Control and Surveillance: Molecular characterization provides a powerful platform for epidemiological surveillance programs. By tracking the prevalence and distribution of specific virulence gene profiles and resistance determinants, public health authorities can monitor the emergence and spread of highly virulent or drug-resistant clones within healthcare settings and the community. This information is invaluable for implementing effective infection prevention strategies, such as enhanced hygiene protocols or isolation measures for patients colonized with VRE or MRSA.

- Targeted Therapeutic Strategies: Insights into specific virulence pathways can inform the development of novel therapeutic strategies. For example, understanding the mechanisms of quorum sensing in P. aeruginosa could lead to the development of anti-virulence drugs that disrupt bacterial communication rather than directly killing the bacteria, potentially reducing selective pressure for resistance [20]. Similarly, targeting biofilm formation could enhance the efficacy of existing antibiotics.

- Personalized Medicine: In the long term, a detailed molecular understanding of uropathogen virulence in diabetic patients could pave the way for more personalized treatment approaches. By knowing the specific virulence profile of an infecting strain, clinicians could select the most effective antimicrobial agents or adjunctive therapies.

3. Comparison with Existing Literature

Our findings align with and expand upon existing literature regarding the role of virulence factors in UTIs. The high prevalence of UPEC and its associated virulence factors is well-documented [2, 18]. Similarly, the increasing importance of P. aeruginosa and the challenges posed by its biofilm-forming and quorum-sensing capabilities are consistent with previous reports [14, 21, 25, 31]. The emergence of MRSA and VRE in UTIs, particularly in vulnerable populations, is a global concern, and our study contributes to the epidemiological data on their prevalence and associated genetic determinants [1, 3, 10, 11, 17, 29].

However, our study provides novel insights by specifically focusing on the interplay of these factors within the diabetic patient population, where the host environment significantly influences pathogenesis. The observation that certain virulence genes, such as efa in E. faecalis, might play a more prominent role in UTI pathogenesis than previously emphasized, particularly in diabetic patients, warrants further investigation. Furthermore, the correlation between MDR P. aeruginosa strains from lower socioeconomic status and the expression of key virulence genes highlights a potentially important epidemiological link that requires broader validation.

4. Limitations of the Study

While our study provides valuable molecular insights, it is important to acknowledge certain limitations:

- Gene Presence vs. Expression: PCR detects the presence of a gene, not necessarily its expression or the functional activity of the encoded virulence factor. A gene might be present but not actively transcribed or translated under the conditions of infection. Phenotypic assays remain important for confirming gene expression and assessing the actual impact of virulence factors in vivo.

- Single-Center Data: Although samples were collected from various hospitals and centers within Bangalore city, the study is essentially confined to a single geographical region. This might introduce some bias, and the findings may not be fully generalizable to other populations or regions with different epidemiological patterns of uropathogens. Larger studies using alternate typing methods and UTI isolates from widely different areas and types of UTIs would be beneficial for a more

comprehensive interpretation of the data.

- Limited AlgD Investigation: The algD gene in P. aeruginosa was investigated in only one MDR isolate. While this provided initial molecular evidence, a broader analysis across more P. aeruginosa strains would be necessary to establish its overall prevalence and significance in diabetic UTI isolates.

- Socioeconomic Status and Virulence: While a correlation between lower socioeconomic status and the expression of certain virulence genes in MDR P. aeruginosa was noted, further in-depth studies are needed to understand the underlying socioeconomic and clinical factors contributing to this observation.

5. Future Research Directions

Based on the findings and limitations of this study, several future research directions are suggested:

- Transcriptomic and Proteomic Studies: To complement PCR-based detection, future studies should incorporate transcriptomic (e.g., RNA sequencing) and proteomic analyses to assess the actual expression levels of virulence genes and the production of virulence factors in vivo or under conditions mimicking the host environment.

- Functional Assays: More extensive functional assays (e.g., adherence assays, cytotoxicity assays, biofilm quantification assays) should be performed on isolates with specific virulence gene profiles to correlate genetic potential with phenotypic virulence.

- Longitudinal Studies: Longitudinal studies are needed to track the persistence of specific virulent or resistant strains in diabetic patients and to understand the impact of these virulence factors on long-term clinical outcomes, including recurrence rates and complications.

- Host-Pathogen Interactions: Further research should explore the intricate interplay between diabetes-induced host immune dysfunction and bacterial virulence factors. This could involve in vitro and in vivo models to elucidate how the diabetic environment influences bacterial virulence gene expression and host susceptibility.

- Novel Therapeutic Targets: The insights gained from molecular characterization can be leveraged to identify and validate novel therapeutic targets that specifically disrupt virulence mechanisms (e.g., quorum sensing inhibitors, anti-adhesin compounds) rather than solely focusing on antimicrobial killing.

- Broader Epidemiological Studies: Conducting larger, multi-center studies across diverse geographical regions would provide a more comprehensive epidemiological picture of virulence gene prevalence and distribution in uropathogens from diabetic patients. This would also allow for the assessment of regional variations and the identification of emerging virulent clones.

- Genomic Sequencing: Whole-genome sequencing of representative isolates could provide a more complete understanding of their genetic makeup, including the presence of novel virulence genes, mobile genetic elements, and resistance determinants, offering a holistic view of their pathogenic evolution.

CONCLUSION

Virulence genes play a pivotal role in the pathogenesis of urinary tract infections, contributing to antimicrobial resistance, adhesion, and biofilm formation. While previous studies have primarily focused on Uropathogenic E. coli (UPEC), this study expands the scope by investigating a broader range of virulence factors in other notable uropathogens responsible for UTIs, particularly in patients with diabetes mellitus who are at increased risk for recurrent and complicated infections.

Our study unequivocally demonstrates that uropathogenic strains isolated from diabetic patients carry a diverse array of virulence genes that significantly contribute to the pathogenesis of UTI. Specifically, we observed the prevalence of cnf1 in E. coli, vanA and efa in E. faecalis, and algD, las-R, and rhl-R in P. aeruginosa, alongside mecA and pvl in S. aureus. These findings collectively indicate that UTIs in diabetic patients are often complicated by a combination of antimicrobial resistance, potent toxin production, robust adhesion mechanisms, and extensive biofilm formation.

Molecular studies, such as those employing PCR, are invaluable in understanding the genetic variations within a pathogen and its inherent pathogenic potential. While they may not directly assist in immediately adjusting existing treatment strategies at the point of care, they provide a crucial platform for understanding the circulating strains, identifying emerging threats, and developing better surveillance programs and infection prevention strategies. Our study specifically demonstrated the utility of virulence genes as precise diagnostic markers in multidrug-resistant (MDR) uropathogens, offering a molecular approach to characterize the aggressiveness of these infections in a vulnerable patient population. Continued research leveraging advanced molecular techniques will be essential for developing more effective diagnostic tools, targeted therapeutic interventions, and comprehensive strategies to combat UTIs in diabetic individuals.

REFERENCES

1. Baba-Moussa, L., Anani, L., Scheftel, J.M., Couturier, M., Riegel, P., Haikou, N., Hounsou, F., Monteil, H., Sanni, A. and Prévost, G. 2008. Virulence factors produced by strains of Staphylococcus aureus isolated from urinary tract infections. J. Hosp. Infect. 68, 32–38.

2. Behzadi, P. 2018. Uropathogenic Escherichia coli and fimbrial adhesins virulome. Urinary tract infection: result of pathogen strength or weakness

virulence and host factors in diabetic patients. Diabet. Med. 10, 550–554.

3. Bell, J.M., Paton, J.C. and Turnidge, J. 1998. Emergence of vancomycin-resistant enterococci in Australia: phenotypic and genotypic characteristics of isolates. J. Clin. Microbiol. 36, 2187–2190.

4. Flataue, P. and Boquet, P. 1997. Cytotoxic necrotizing factor 1 from Escherichia coli: a toxin with a new intracellular activity for eukaryotic cells. Folia Microbiol. 43, 285–289.

5. Boyko, E.J., Fihn, S.D., Scholes, D., Abraham, L. and Monsey, B. 2005. Risk of urinary tract infection and asymptomatic bacteriuria among diabetic and nondiabetic postmenopausal women. Am. J. Epidemiol. 161, 557–564.

6. Bratu, S., Gupta, J. and Quale, J. 2006. The expression of the las and rhl quorum sensing systems in clinical isolates of P. aeruginosa is not correlated with efflux pump expression or antimicrobial resistance. J. Antimicrob. Chemother. 58(6), 1250–1253.

7. Brauner, A., Flodin, U., Hylander, B. and Ostenson, C.G. 1993. Bacteriuria, bacterial of the host. Croatia: InTechOpen, pp: 65–83.

8. Cotar, A.I., Dinu, S., Chifiriuc, M.C., Banu, O., Iordache, C., Larion, C., Bucur, M., Dracea, O. and Lazar, V. 2008. Screening of molecular markers of quorum sensing in Pseudomonas aeruginosa strains isolated from clinical infections. Roum. Biotech. Lett. 13(3), 3765–3770.

9. Dahl, K.H., South Simonsen, G., Olsvik, O. and Sundsfjord, A. 1999. Heterogeneity of the vanB gene cluster of genomically diverse clinical strains of the vancomycin-resistant enterococci. Antimicrob. Agents Chemother. 43, 1105–1110.

10. Dutka-Malen, S., Evers, S. and Courvalin, P. 1995. Detection of glycopeptide resistance genotypes and identification to the species level of clinically relevant enterococci by PCR. J. Clin. Microbiol. 33, 24–27.

11. Gomroki, F., Mohammed, H.B. and Malla, S. 2015. Amplification of methicillin resistant gene (mecA) gene from the MRSA strains. Int. J. Pharm. Clin. Res. 7(3), 198–203.

12. Guzmàn, C.A., Pruzzo, C., LiPira, G. and Calegari, L. 1989. Role of adherence in pathogenesis of Enterococcus faecalis urinary tract infection and endocarditis. Infect. Immun. 57(6), 1834–1838.

13. Hassan, M., Flayyih, M. and Yaseen, N. 2015. Isolation and purification of CNF1 (Cytotoxic necrosis factor 1) produced from bacteria Escherichia coli and study its role against apoptosis in vitro. Iraqi J. Cancer Med. Genetics 8(2), 157–163.

14. Hemmati, J., Nazari, M., Abolhasani, F.S. and Asghari, B. 2024. In vitro investigation of relationship between quorum-sensing system genes, biofilm forming ability, and drug resistance in clinical isolates of Pseudomonas aeruginosa. BMC Microbiol. 24, 99.

15. Holden, N.J., Totsika, M., Mahler, E., Roe, A.J., Catherwood, K. and Lindner, K. 2006. Demonstration of regulatory cross-talk between P. fimbriae and type 1 fimbriae in uropathogenic Escherichia coli. Microbiology 152(4), 1143–1153.

16. Hooton, T.M. and Stamm, W.E. 1997. Diagnosis and treatment of uncomplicated urinary tract infection. Infect. Dis. Clin. North Am. 11(3), 551–581.

17. Jo-Ann, M., John, M.C., Vicky, L., Sameer, E., Thomas, L., Wendy, H. and Kunyan, Z. 2006. Novel multiplex PCR assay for detection of the staphylococcal virulence marker panton-valentine leukocidin genes and simultaneous discrimination of methicillin-susceptible from resistant staph. J. Clin. Microbiol. 44(3), 1141–1144.

18. Johnson, J.R. 1991. Virulence factors in Escherichia coli urinary tract infection. Clin. Microbiol. Rev. 4, 80–128.

19. Lanotte, P., Watt, S., Merghitti, L., Dartiguelongue, N., Rastegar-Lari, A., Goudeau, A. and Quentin, R. 2004. Genetic features of P. aeruginosa isolates from cystic fibrosis patients compared with those of isolates from other origins J. Med. Microbiol. 53(1), 73–81.

20. López-Jácom, L.E., Garza-Ramos, G., Hernández-Durán, M., Franco-Cendejas, R., Loarca, D., Romero-Martínez, D., Nguyen, P.T.D., Maeda, T., González-Pedrajo, B., Díaz-Guerrero, M., Sánchez-Reyes, J.L., Díaz-Ramírez, D. and García-Contreras, R. 2019. AiiM lactonase strongly reduces quorum sensing controlled virulence factors in clinical strains of Pseudomonas aeruginosa isolated from burned patients. Front. Microbiol. 10, 2657.

21. Merritt, J.H., Kadouri, D.E. and O'Toole Analyzing, G.A. 2005. Growing and analyzing static biofilms. Curr. Protoc. Microbiol. 1, 1–25.

22. Miranda, S.W., Asfahl, K.L., Dandekar, A.A. and Greenberg, E.P. 2022. Pseudomonas aeruginosa quorum sensing. Adv. Exp. Med. Biol. 1386, 95–115.

23. Ramsey, D.M. and Wozniak, D.J. 2005. Understanding the control of P. aeruginosa alginate synthesis and prospectus for management of chronic infections in cystic fibrosis. Mol. Microbiol. 56(2), 309–322.

24. Rippere-Lampe, K.E., O'Brien, A.D., Conran, R. and

Lockman, H.A. 2001. Mutation of the gene encoding cytotoxic necrotizing factor type 1 (cnf1) attenuates the virulence of uropathogenic Escherichia coli. Infect. Immun. 69, 3954–3964.

25. Sabharwal, N., Dhall, S., Chhibber, S. and Harjai, K. 2014. Molecular detection of virulence genes as markers in Pseudomonas aeruginosa isolated from urinary tract infections. Int. J. Mol. Epidemiol. Genet. 5(3), 125–134.

26. Salah, R., Dar-Odeh, N., Abu Hammad, O. and Shehabi, A.A. 2008. Prevalence of putative virulence factors and antimicrobial susceptibility of Enterococcus faecalis isolates from patients with dental diseases. BMC Oral Health 8, 17.

27. Saleem, M. and Daniel, B. 2011. Prevalence of urinary tract infection among patients with diabetes in Bangalore City. Int. J. Emerg. Sci. 1(2), 133–142.

28. Scholes, D., Hooton, T.M., Roberts, P.L., Stapleton, A.E., Gupta, K. and Stamssin, W.E. 2000. Risk factors for recurrent UTI in young women. J. Infect. 182, 1177.

29. Shepard, B.D. and Gilmore, M.S. 2002. Antibiotic-resistant enterococci: the mechanisms and dynamics of drug introduction and resistance. Microbes Infect. 4(2), 215–224.

30. Shigemura, K., Arakawa, S., Sakai, Y., Kinoshita, S., Tanaka, K. and Fujisawa, M. 2006. Complicated urinary tract infection caused by Pseudomonas aeruginosa in a single institution (1999-2003). Int. J. Urol. 13(5), 538–542.

31. Singh, P.K., Parsek, M.R. and Welsh, M.J. 2000. Quorum sensing signals indicate that lungs with cystic fibrosis are infected with bacterial biofilm. Nature 407, 762–764.