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Molecular Characterization and Mutational Analysis of Virulence Genes in Clinical Isolates of *Klebsiella pneumoniae* Obtained from Khyber Teaching Hospital, Peshawar

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Abstract: *Klebsiella pneumoniae* is a Gram-negative opportunistic pathogen implicated in various nosocomial and community-acquired infections. This study aimed to investigate key virulence factors contributing to the pathogenicity of K. pneumoniae. A total of 100 bacterial isolates were analyzed, of which K. pneumoniae was identified through 16S rRNA sequencing. The remaining isolates were classified as Escherichia coli, Citrobacter, and Shigella species. The virulence potential of K. pneumoniae was assessed by detecting the presence of the *cnf1* and *sfa* genes, which were identified in 7% and 17% of the isolates, respectively. Despite their low prevalence, these genes were sufficient to enhance the pathogenic capacity of the bacteria. Mutational analysis revealed genetic variations in the cnf1 gene, although these mutations were pre-existing within the analyzed strains.

Keywords: *Klebsiella pneumoniae*, virulence factors, 16S rRNA sequencing, *cnf1* gene, *sfa* gene, mutational analysis.

1. Introduction

Klebsiella pneumoniae, a member of the Enterobacteriaceae family, is a Gram-negative, non-flagellated, non-motile, facultatively anaerobic bacterium. It is commonly found in various environments, including water, soil, and plants, and can also establish a commensal relationship by colonizing the respiratory and gastrointestinal tracts of mammals. This bacterium poses a significant public health risk as a leading cause of hospital-acquired infections. K. pneumoniae can cause a wide range of infections, including bloodstream infections, wound infections, urinary tract infections, and respiratory tract infections [1]. Currently, *K. pneumoniae* is recognized as an important opportunistic pathogen responsible for community-acquired infections, particularly in immunocompromised individuals. The prevalence of infections is notably higher in hospital settings and is strongly associated with the use of antimicrobial agents [2]. Hypervirulent strains of *K. pneumoniae* demonstrate a remarkable capacity for rapid invasion, affecting even previously healthy individuals. These strains are associated with severe and often life-threatening infections, including meningitis, necrotizing fasciitis, pyogenic liver abscesses, severe pneumonia, and endophthalmitis [3,4]. The pathogenesis of *K. pneumoniae* is facilitated by an array of virulence factors that enable the bacterium to evade the host immune system and establish infections. Key virulence factors include the polysaccharide capsule, lipopolysaccharides, adhesins, and biofilm-forming abilities, all of which contribute to the

bacterium's ability to cause disease [5,6]. The diversity and expression of these virulence factors lead to variations in the clinical manifestations of infections caused by *K. pneumoniae* [7]. Additionally, this pathogen has demonstrated a significant ability to contaminate intensive care units and develop multidrug resistance, further complicating its management [8]. The presence of multiple virulence genes further enhances its pathogenic potential. Cytotoxic necrotizing factor 1 (CNF1) is a chromosomally encoded toxin that induces the deamidation of small GTPases, which are critical regulators of various cellular processes. CNF1 plays a significant role in pathogenesis by modulating host cell functions, including facilitating epithelial cell phagocytosis. Through the activation of Rho GTPases, CNF1 influences numerous cellular mechanisms in vitro, contributing to bacterial survival and infection dynamics. Fimbriae are another crucial virulence factor implicated in urinary tract infections (UTIs) and the progression to urosepsis. The initial step in UTI pathogenesis involves the adherence of bacteria to the urinary tract epithelium, which necessitates the expression of specific adhesins. Various adhesins have been identified in isolates associated with neonatal meningitis and UTIs. This adherence process relies on molecular recognition, where specific adhesins bind to complementary receptor molecules on the host cell surface [9]. Fimbrial adhesins, encoded by the *SFA* gene, enable *Klebsiella pneumoniae* to adhere to mammalian cells effectively. S fimbriae play a pivotal role in facilitating bacterial attachment to epithelial cells both in vitro and in vivo. Additionally, S fimbriae have been implicated in severe infections, including neonatal sepsis and meningitis [10]. Understanding the molecular mechanisms underpinning these virulence factors provides insight into the pathogenic potential of *K. pneumoniae* and highlights potential targets for therapeutic intervention.

2. Methodology

2.1 Sample Collection

A total of 100 bacterial isolates were obtained from various clinical specimens provided by the Pathology Department, Khyber Teaching Hospital, Peshawar. These samples were transported under sterile conditions to the Microbiology Research Laboratory at the Centre of Biotechnology and Microbiology (COBAM), University of Peshawar (UoP), for further analysis.

2.2 *Sub-Culturing and Bacterial Growth*

The bacterial samples were sub-cultured on Nutrient Agar and MacConkey agar for growth and isolation. Tryptone soy broth was utilized for the preservation of bacterial cultures [11].

2.3 Bacterial Identification

Preliminary identification of bacterial isolates was performed through Gram staining and microscopic analysis. Colony morphology, including shape, size, and structure, was also observed. For biochemical characterization, API 20E test strips were employed.

2.4 Antibiotic Susceptibility Testing

The antibiotic sensitivity of the bacterial isolates was evaluated using the disk diffusion method. Culture media were prepared and sterilized by autoclaving at 121°C for 15 minutes. Antibiotic disks were placed on bacterial lawns and incubated at 37°C for 24 hours. The susceptibility of the bacteria to antibiotics was determined by measuring the zones of inhibition and interpreting them as sensitive, intermediate, or resistant, following CLSI guidelines.

2.5 DNA Extraction and Molecular Identification

Bacterial genomic DNA was extracted from broth cultures using a GenJet Genomic DNA Extraction Kit (Catalogue #K0691). Molecular identification was conducted using universal primers for 16S rRNA gene amplification. The amplified products were sequenced at Macrogen, Korea. Sequence homology searches were performed using BLAST,

and closely related sequences were aligned and used to construct phylogenetic trees via MEGA 7.0 software.

2.6 Assay for Detection of Virulence Factors

The phenotypic activity of virulence factors was confirmed using a hemolysin tube assay [12]. Ten milliliters of blood from a healthy donor were centrifuged at 5000 rpm for 5 minutes to obtain an erythrocyte pellet. The pellet was washed three times with 0.9% NaCl to prepare a 3% erythrocyte solution. Isolated bacteria were cultured on MacConkey agar, and colonies were inoculated into Tryptone soy broth for 24 hours. After incubation, 2 mL of the bacterial culture was centrifuged at 1000 rpm for 10 minutes. The supernatant was mixed with an equal volume of the 3% erythrocyte solution and incubated at 37°C for 3 hours in a water bath. The mixture was centrifuged, and the supernatant, containing released hemoglobin, was measured at OD530nm. Triton X-100 served as the positive control, while the erythrocyte solution was used as the negative control. The hemolysis percentage was calculated using the following formula [13].

% Hemolysis = A (Samples)-A (Con)/ A (Max lysis)- A (Con) X 100

2.7 Molecular Detection of Virulence Genes

Polymerase Chain Reaction (PCR) was performed to amplify specific virulence genes using extracted DNA and gene-specific primers. Amplified products were analyzed via agarose gel electrophoresis (Bioron Life Sciences) following standard protocols. High-throughput sequencing of DNA and RNA was conducted, and selected PCR products were sequenced at Macrogen, Korea. The sequenced genes were subjected to BLAST analysis (https://blast.ncbi.nlm.nih.gov/), and homologous sequences were aligned and used for phylogenetic tree construction using MEGA 7.0 software (https://www.megasoftware.net/).

Table 1: Specific Antibiotic used for the determination of Resistance Pattern of *Klebsiella pneumoniae*

Antibiotic	Concentration (µg/ml)	Sensitive (S) Range (µg/ml)	Resistant (R) Range (µg/ml)
Cefepime	30	≤2	≥8
Ertapenem	10	≤0.5	≥1
Fosfomycin	200	≤32	≥128
Imipenem	10	≤1	≥4
Meropenem	10	≤1	≥4
Tazobactam	64	S ≤16/4	R ≥64/4
Piperacillin	100	≤16	≥128
Tigecycline	15	≤1	≥2
Amikacin	30	≤16	≥64
Aztreonam	30	≤8	≥16

Table 2. List of primers designed for Virulence gene

Genes	**Primers sequence**	**Size (bp)**
cnf-1	F: AAGATGGAGTTTCCTATGCAGGAG R: CATTCAGAGTCCTGCCCTCATTATT	498
Sfa	F: CTCCGGAGAACTGGGTGCATCTTAC R: CGGAGGAGTAATTACAAACCTGGCA	328

3. Results

Out of the 100 bacterial isolates analyzed, 70 were identified as Klebsiella pneumoniae, while the remaining isolates included Escherichia coli (12), Citrobacter spp. (10), and Shigella spp. (8). Antibiotic susceptibility testing of the K. pneumoniae isolates revealed varying levels of sensitivity and resistance to the antibiotics tested. The disc diffusion method was employed to determine the antibiotic susceptibility patterns of the isolates. Among the *K. pneumoniae* isolates, 49% were sensitive to cefepime, 41% to tigecycline, and 51% to fosfomycin. Resistance to ceftazidime was observed in 35% of the isolates, while the majority of the isolates exhibited complete or intermediate susceptibility to amikacin, ertapenem, and meropenem. Additionally, some *Enterobacter cloacae* isolates demonstrated resistance to cefepime (7%) and cefpirome, while 32% were sensitive to newer-generation beta-lactams. The remaining 33 isolates displayed resistance to beta-lactam antibiotics. The quality of the DNA extracted from the isolates was assessed through gel electrophoresis, confirming its suitability for further molecular analyses. No mutations were identified in the sequence of the *sfa* gene, which corresponds to accession number NC_006375.1. However, next-generation sequencing (NGS) analysis of the *cnf1* gene revealed mutations present in all isolates, resulting in amino acid changes at positions 109 and 497. At nucleotide position 301, the codon GGG was altered to CCC, leading to a substitution of glycine with proline. Additionally, at nucleotide positions 316 and 329, the codon TCA was changed to TGC, resulting in a substitution of serine with cysteine.

Table 3: Antibiogram of K. pneumoniae obtained from different types of samples

	Antibiotics	Abbreviation	Sensitive (n)	Percentage (%)	Resistant (n)	Percentage (%)
1	Cefepime	CFP	49	58.3	35	41.6
2	Ertapenem	CTP	59	70.2	25	29.7
3	Fosfomycin	FOS	51	60.7	33	39.2
4	Imipenem	IMI	40	57.1	44	52.3
5	Meropenem	MER	45	53.5	39	46.4
6	Tazobactam	TZB	40	47.61	44	52.3
7	Piperacillin	PIP	37	44	47	55.9
8	Tigecycline	TGC	41	48.8	43	51.19
9	Amikacin	AK	54	64.2	30	35.71
10	Aztreonam	AZE	47	55.9	37	44.0

Table 3: The result of specific antibiotic used against *S. aureus*

	Nucleotide Position	Reference Amino Acid	Altered Amino Acid	Protein Position	Mutation
1	301	G(GGG)	P(CCC)	109	Missense mutation
2	316 329	S(TCA)	C(TGC)	497	Missense mutation

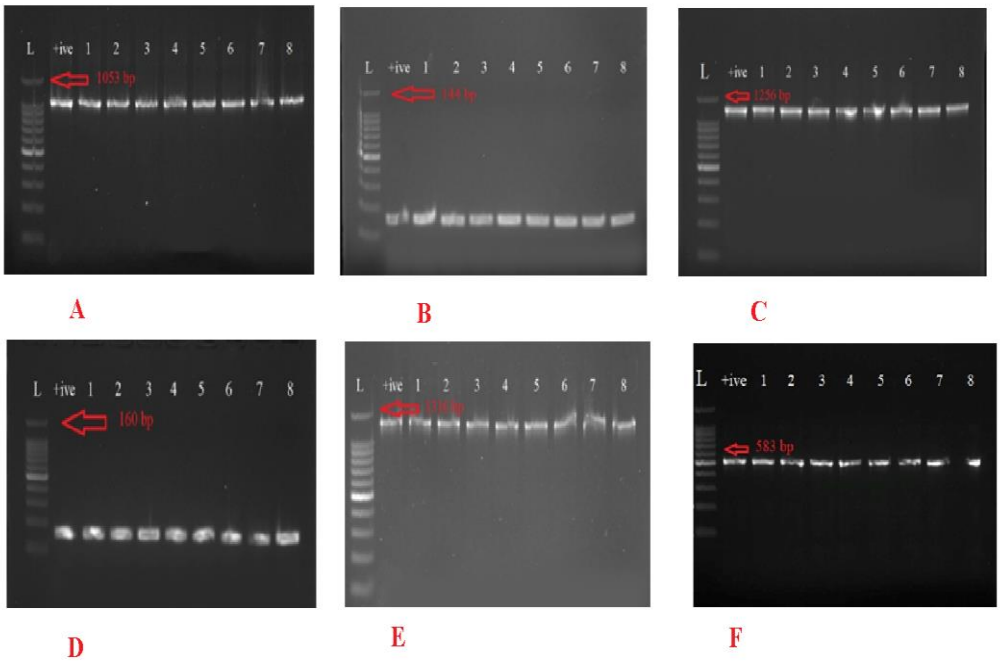

Figure 1: Electrophoretogram showing amplicons of *S. aureus* efflux resistance gene. L: 100bp molecular marker, Lane 1 to 8: positive isolates of the amplified gene **(A)** *Nor*A gene **(B)** *mgr*A gene **(C)** *mep*A gene **(D)** *mep*R gene **(E)** *mde*A gene **(F)** *mec*A gene

4. Discussion

Klebsiella pneumoniae has emerged as a significant pathogen due to its ability to evade host defenses and its association with a wide range of infections. This study investigated clinical isolates of K. pneumoniae from Khyber Teaching Hospital (KTH), Peshawar, focusing on antibiotic susceptibility patterns, molecular identification, and virulence gene characterization. The findings were compared with previous studies conducted globally to highlight similarities and differences in antibiotic resistance, molecular profiling, and virulence. A total of 70 isolates were confirmed as K. pneumoniae, while the remaining 30 isolates were identified as Escherichia coli (12), Citrobacter spp. (10), and Shigella spp. (8). These proportions align with studies conducted in Iraq, where API 20E strips were also employed for bacterial identification, demonstrating the utility of this method for distinguishing K. pneumoniae from other Gram-negative bacteria [4]. However, a study from California in 1981 reported a higher prevalence of K. pneumoniae, potentially reflecting regional differences in bacterial incidence or changes in diagnostic techniques over time [3]. The antibiotic susceptibility testing revealed significant resistance among K. pneumoniae isolates. In the current study, 49% of isolates were sensitive to cefepime, 41% to tigecycline, and 51% to Fosfomycin. This is consistent with findings from Kuala Lumpur, where a similar disc diffusion method revealed moderate sensitivity to cefepime and Fosfomycin among K. pneumoniae isolates [5]. However, resistance to ceftazidime was observed in 35% of isolates in our study, a figure slightly lower than the 42% resistance reported in a study conducted in Denmark [7]. Such discrepancies may stem from differences in antibiotic usage policies and local patterns of antimicrobial resistance. Molecular identification through 16S rRNA sequencing confirmed the isolates as K. pneumoniae. Phylogenetic analysis using MEGA 7.0 software provided further validation, aligning closely with findings from Denmark, where 16S rRNA sequencing was similarly

employed to construct phylogenetic trees of Klebsiella strains [7]. This underscores the universal application of 16S rRNA as a reliable marker for bacterial identification. Virulence gene detection revealed the presence of sfa and cnf1 genes, which are critical for pathogenicity. Comparative analysis showed that the sfa gene sequence exhibited no mutations, aligning with previous studies from Kyoto, Japan, where no significant variations were reported in sfa gene sequences from clinical isolates [38]. However, mutations were identified in the cnf1 gene at nucleotide positions 301, 316, and 329, resulting in amino acid changes from glycine to proline and serine to cysteine. These mutations differ from findings in a study at Hopkins University, where targeted analysis of cnf1 revealed no mutations but highlighted its potential as a target for controlling meningitis caused by K. pneumoniae [8]. The identification of such mutations in our study highlights the evolving genetic landscape of K. pneumoniae and the need for region-specific research to monitor these changes. The multidrug-resistant nature of K. pneumoniae observed in this study aligns with the growing global concern. Studies from Malaysia and Iraq similarly reported resistance to newer beta-lactams, such as cefepime and cefpirome, in K. pneumoniae and related species [5, 6]. This resistance underscores the pressing need for novel therapeutic strategies and highlights the pathogen's adaptability in response to antibiotic pressure. Finally, the virulence of K. pneumoniae, driven by factors such as sfa and cnf1 genes, emphasizes its role in causing severe infections like urinary tract infections, sepsis, and meningitis. Our findings contribute to the understanding of these genes' functions in local isolates, complementing studies from Kyoto and Hopkins that have explored similar gene targets for therapeutic interventions [8, 38]. The observed mutations in the cnf1 gene open new avenues for investigating their impact on bacterial virulence and their potential as targets for antimicrobial therapy.

5. Conclusions

Klebsiella pneumoniae is a significant opportunistic pathogen associated with a wide range of infections, including those affecting the brain, lungs, liver, bladder, and bloodstream. The alarming rise in infections caused by multidrug-resistant strains of *K. pneumoniae* poses a substantial challenge to treatment with conventional therapeutic methods. Following *Escherichia coli*, *K. pneumoniae* has emerged as the second most prevalent causative agent of urinary tract infections (UTIs), underscoring its role as a major contributor to this clinical condition. The exploration and characterization of virulence factors in *K. pneumoniae* using molecular approaches have significantly advanced our understanding of its pathogenic mechanisms. In the current study, the detection of the *sfa* and *cnf1* virulence genes provided critical insights into the pathogenicity of the isolates. These findings highlight the importance of continued research to fully elucidate the roles of these and other virulence factors in emerging strains of *K. pneumoniae*.

Author Contributions

The authors have contributed significantly to the study. Sadaf Gohar played a key role in the design and conceptualization of the study, as well as in the collection of bacterial isolates and data analysis. She also conducted various molecular and biochemical experiments. Sajeela Akbar was responsible for performing microbiological assays and antibiotic susceptibility testing, while also assisting in data interpretation and contributing to the drafting and revision of the manuscript. Faiza Mazhar, as the corresponding author, supervised the overall study, guided the experimental design, and coordinated laboratory activities. She also took the lead in drafting and finalizing the manuscript for publication. Syed Sohail Shah and Asmat Ullah provided technical expertise for molecular identification and phylogenetic analysis, assisting with troubleshooting and the interpretation of sequencing results. Muhammad Taha contributed to bioinformatics analyses, including sequence alignment and the construction of phylogenetic trees, and participated in the

critical review and editing of the manuscript. All authors have reviewed and approved the final version of the manuscript and accept responsibility for its content.

Conflicts of Interest

The authors declare no conflicts of interest.

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