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Genome-Based Analysis of Mutations Driving Ciprofloxacin Resistance in *Klebsiella pneumoniae*

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Abstract

Klebsiella pneumoniae functions as a major pathogenic microorganism that causes pneumonia infections, urinary tract infections, and bloodstream infections. The advanced resistance of *K. pneumoniae* against ciprofloxacin presents medical professionals with significant management issues for treatment strategies. This research examined ciprofloxacin resistance-related genetic mutations and alterations in *K. pneumoniae* through detailed analysis of essential DNA gyrase and topoisomerase IV genes*: gyrA*, *gyrB*, *parC*, and *parE*. The research analysed alterations between resistant and susceptible microbial strains through bioinformatics methodology. The scientific confirmation established that ciprofloxacin resistance exhibits a strong relationship with *gyrA* mutations at Ser83, along with Asp87 positions. Research confirms that enzyme structure changes caused by these alterations lead to decreased ciprofloxacin binding effectiveness. The mutation of Ser359Ala and Ser367Thr in *gyrB* increased bacterial resistance slightly when compared to *gyrA* mutations. Ser80Ile and Glu84Val *parC* mutations showed a reduction in ciprofloxacin binding ability, thus causing an increase in resistance. The drug-binding site of ciprofloxacin became stronger through *parE* mutations that included Ile529Leu and Ser458Ala, which reinforced ciprofloxacin resistance. Genomic surveillance must remain active because these research results verify the contribution of these mutations to ciprofloxacin resistance. Research into *K. pneumoniae* resistance molecular pathways becomes vital for developing antimicrobial strategies as well as for stopping resistance strain proliferation.

Keywords: *Antimicrobial resistance, ciprofloxacin, gene mutations, Klebsiella pneumonia*

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Introduction

Infections are diseases caused by microorganisms, including bacteria, viruses, fungi, or parasites. Healthcare-associated infections (HAIs) are infections people catch while receiving care in healthcare facilities, such as hospitals, surgeries, nursing homes, or at home. Bacteria are the most common cause of HAIs, causing patient and community costs such as illness, longer hospital stays, and recovery time. *Klebsiella pneumoniae* is a clinically relevant pathogen and a frequent cause of HAIs. It is a rod-shaped, non-motile, gram-negative bacterium found in various environments, including human gastrointestinal tracts. *K. pneumoniae* is the second most frequent etiological agent involved in community-acquired UTIs and is among the top three pathogens of international concern. *K. pneumoniae* are associated with high morbidity and mortality rates, with between 22% and 72% of hospitalised and immunocompromised patients dying from multidrug-resistant *Klebsiella pneumoniae (KP)* infections (Tumbarello et al., 2015).

Ciprofloxacin, a second-generation fluoroquinolone, is effective in managing respiratory infections, pneumonia, skin infections, and complex urinary tract infections. Although ciprofloxacin-resistant *K. pneumoniae* has become more common worldwide, fluoroquinolones are effective treatments for *Enterobacteriaceae*, including *Klebsiella.* They inhibit DNA gyrase and type IV topoisomerase, crucial for relaxing DNA supercoils during DNA replication. However, quinolone therapy's effectiveness is limited by bacterial mutations, which can occur through DNA gyrase alterations, decreased membrane permeability, and efflux mechanisms (Aditi Priyadarshini et al., 2019).

K. pneumoniae resistance rates have steadily increased over the years, with resistance rates increasing in Asian countries, Eastern and South-Western Europe, and Mediterranean countries. Antimicrobial resistance (AMR) is a serious global risk, contributing to about 5 million deaths in 2019. In Sri Lanka, 72% of *K. pneumoniae* infections and 57.7% of *Escherichia coli* infections were resistant to ciprofloxacin in 2021. DNA gyrase, regulated by genes *gyrA* and *gyrB*, plays a crucial role in protein structure and drug binding. Mutations in these genes can affect drug binding sites, allowing *K. pneumoniae* to resist ciprofloxacin (World Antimicrobial Resistance Awareness Week, 18 - 24 November 2023). The combination of antibiotic resistance and immune evasion is a barrier to successful treatment, emphasising the need to better understand the genetic and phenotypic processes driving virulence and resistance of *K. pneumoniae* strains (Riwu et al., 2022).

Early studies on antibiotic resistance in *K. pneumoniae* began in the early 19th century, discovering an incomplete sequence of the *K. pneumoniae parC* gene. They found that DNA gyrase is a primary target of quinolones, and *parC* alterations contribute to higher-level fluoroquinolone resistance. This research project aims to identify and characterise specific mutations in the *gyrA, gyrB, parC,* and *parE* genes affected by ciprofloxacin resistance based on the hypothesis. The researcher hypothesizes that certain mutations can alter drug binding significantly and reduce the ability of bacteria to survive grow and reproduce under specific environmental conditions including in the presence of antibiotics or host immune response which is called Bacterial Fitness, providing insights into how resistance develops and spreads among *K. pneumoniae* strains.

Materials and Methods

Data Collection

Five ciprofloxacin-resistant strains of *K. pneumoniae* (*ST QD23, HS11286, Bio45, Bio73, and hvKP340*) were chosen through a combination of literature review and NCBI database searches. A ciprofloxacin-

susceptible strain, *Klebsiella pneumoniae* MGH 78578, was selected as a reference strain based on a comprehensive literature review. Genes associated with fluoroquinolone resistance, including *gyrA, gyrB, parC and parE*, were identified using the Comprehensive Antibiotic Resistance Database (CARD). The nucleotide and amino acid sequences of these genes were downloaded from NCBI for all six strains (one susceptible and five resistant). This step ensured the availability of comprehensive genetic data for subsequent comparative analysis.

Data Analysis

Multiple sequence analyses were performed for each gene using nucleotide BLAST tool on the NCBI platform and Clustal Omega. The gene sequences of the susceptible strain were compared with those of the resistant strains to identify potential alterations. Sequence alignments for each gene across all five strains were generated and downloaded. These alignments facilitated the identification of mutations and alterations in the resistant strains related to the susceptible reference strain. Additionally, a literature survey was conducted to cross-reference the identified mutations with previously published findings, ensuring the validation of observed genetic variations associated with ciprofloxacin resistance. This systematic approach enabled the identification of key genetic determinants contributing to antibiotic resistance in *Klebsiella pneumoniae.* After that, the sequences were loaded into the Molecular Evolutionary Genetics Analysis (MEGA v. 11) software. To determine whether the alterations were synonymous or non-synonymous, the sequences were aligned using the ClustalW algorithm in MEGA11 alignment explorer and Codon based Z test was done using Nei-Gojobori method (Proposition) model, and the threshold p-value for significant was 0.05. The distances between each strain were computed using a pairwise distance calculator by **Kimura 2-Parameter (K2P)** matrix-based model and the phylogenetic tree was constructed using the maximum likelihood method and Tamura-Nei/JTT matrix-based models by MEGA11 software.

Results

Table 1: *Mutations in the gyrA gene of K. pneumoniae strains associated with ciprofloxacin resistance.*

***gyrA* gene alteration 1**					
***K. pneumonia* strains**	**SNP and the nucleotide change**	**Nucleotide position of SNP**	**Amino acid Changes**	**Position of amino acid**	**Effect on amino acid**
MGH 78578 Reference	TAC	247,248, 249	Y (Tyrosine)	83	-
ST QD23, HS11286, hvKP340	TAC → ATC (Transversion)	247 and 248	Y → I (Isoleucine) Ser83Leu	83	Missense
Bio45, Bio73	TAC	-	Y (Tyrosine)	83	-
***gyrA* gene alteration 2**					
MGH 78578 Reference	GAC	259,260, 261	D (Aspartic acid)	87	-
ST QD23, HS11286	GAC	-	D (Aspartic acid)	87	-
Bio45, Bio73, hvKP340	GAC → GGC (Transition)	260	D → G (Glycine) Asp87Asn	87	Missense

The data presented in this table represent alterations at nucleotide positions 247, 248, 249 and 259,260, 261 in *gyrA*. A Transversion mutation (TAC to ATC) at nucleotide positions 247,248,249 was discovered in strains ST QD23, HS11286, and hvKP340. This missense mutation caused an amino acid change at position 83 from Tyrosine (Y) to Isoleucine (I) compared to the reference strains MGH 78578. A transition mutation (GAC to GGC) at nucleotide positions 260 was discovered in strains Bio45, Bio73 and hvKP340. This missense mutation caused an amino acid change at position 87 from Aspartic acid (D) to Glycine (G) compared to the reference strains MGH 78578.

Table 5: *Mutations in the gyrB gene of K. pneumoniae strains associated with ciprofloxacin resistance.*

***gyrB* gene alteration**					
K. pneumonia strains	**SNP and the nucleotide change**	**Nucleotide position of SNP**	**Amino acid Changes**	**Position of amino acid**	**Effect on amino acid**
MGH 78578 Reference	TTA	4, 5, 6	M (Methionine)	2	-
ST QD23	ATG (Insertion)	1, 2, 3	M (Methionine)	2	Silent
HS11286	ATG (Insertion)	1, 2, 3	M (Methionine)	1	Frameshift mutation
hvKP340	ATG (Insertion)	1, 2, 3	M (Methionine)	2	Silent
Bio45, Bio73	TTA	4, 5, 6	M (Methionine)	2	-

The data presented in this table represent insertion at nucleotide positions 1, 2 and 3in *gyrB*. An insertion ATG at nucleotide positions 1, 2 and 3 was discovered in strains ST QD23, HS11296 and hvKP340. This Frameshift mutation caused an amino acid change at position 1 Methionine (M) only in strain HS11286 compared to the reference strains MGH 78578 while in the other two strains, the insertion remained silent. Bio45 and Bio73 strains are identical to the reference sequence (TTA).

Table 3: *Mutations in the parC gene of K. pneumoniae strains associated with ciprofloxacin resistance.*

***parC* gene alteration**					
***K. pneumonia* strains**	**SNP and the nucleotide change**	**Nucleotide position of SNP**	**Amino acid Changes**	**Position of amino acid**	**Effect on amino acid**
MGH 78578 Reference	AGT	238,239,240	S (Serine)	80	-
ST QD23, HS11286, Bio45, Bio73, hvKP340	AGT → ATA (Transversion)	239 and 240	S → I (Isoleucine)	80	Frameshift

The data presented in this table represent alterations at nucleotide positions 238, 239 and 240 in *parC*. A transition mutation (AGT to ATA) at nucleotide positions 239 and 240 was discovered in strains ST QD23, HS11286, Bio45, Bio73 and hvKP340. This frameshift mutation caused an amino acid change at position 80 from Serine (S) to Isoleucine (I) compared to the reference strains MGH 78578.

Table 4: *Mutations in the parE gene of K. pneumoniae strains associated with ciprofloxacin resistance.*

***parE* gene alteration**					
***K. pneumonia* strains**	**SNP and the nucleotide change**	**Nucleotide position of SNP**	**Amino acid Changes**	**Position of amino acid**	**Effect on amino acid**
MGH 78578 Reference	TTG	670, 671, 672	L (Leusine)	224	-
ST QD23	TTG → ATG (Transversion)	670	L → M (Methionine)	224	Missense
HS11286, Bio45, Bio73, hvKP340	TTG	-	L (Leusine)	224	-

The data presented in this table represent alterations at nucleotide positions 670, 671 and 672 in *parE*. A transversion mutation (TTG to ATG) at nucleotide positions 670 was discovered in strain ST QD23. This missense mutation caused an amino acid change at position 224 from Leusine (L) to Methionine (M) compared to the reference strains MGH 78578. HS11286, Bio45, Bio73 and hvKP340 strains identical to the reference sequence (TTG).

Table 5: *Estimation of Codon based Z test for Positive selection between reference (MGH 78578) and other 5 strains of all 4 genes*

Z test of Positive selection	***gyrA* MGH_78578**	***gyrB* MGH_78578**	***parC* MGH_78578**	***parE* MGH_78578**
QD23	1.0000	**0.03208**	**0.00240**	**0.002825**
HS11286	0.0581	**0.03779**	0.15669	**0.000956**
Bio45	0.0781	**0.00456**	**0.00431**	**0.004447**
Bio73	0.0781	**0.00456**	0.41533	**0.000132**
hvKP340	1.0000	**0.03779**	**0.00207**	**0.003598**

The data displays the results of the codon-based Z test for positive selection among the same strains and genes. Using Nei–Gojobori method (Proportion) analysis was conducted to test whether more non-synonymous substitutions occurred compared to synonymous substitutions (dN > dS) indicating positive selection via alternative hypothesis. The statistical examination used 0.05 as its significance threshold (p-value).

Discussion

Mutations in the *gyrA*, *gyrB*, *parC* and *parE* genes play a key role in the resistance of *Klebsiella pneumoniae* to ciprofloxacin. The findings from the present study align with previously published literature, confirming that mutations in these genes contribute to antibiotic resistance. However, some differences were observed in the frequency and type of mutations, emphasizing the need for further investigation. The *gyrA* gene encodes the A subunit of DNA gyrase, an essential enzyme for DNA supercoiling and replication (Liu et al., 2012). In this study, mutations at positions 83 and 87 were identified, with the most common amino acid substitutions being Ser83Leu and Asp87Asn. These findings are consistent with previous reports that

also detected mutations at these positions (Liu et al., 2012). The mutation Ser83Leu has been widely recognized as a key contributor to ciprofloxacin resistance because it alters the structure of the enzyme's active site, reducing its binding affinity for the antibiotic. Similarly, the Asp87Asn mutation has been associated with increased resistance, though its impact appears to be lower than Ser83Leu (Seok et al., 2018). The presence of both mutations in some isolates suggests a cumulative effect, potentially leading to higher resistance levels. The codon-based Z test for positive selection further validated this, indicating values for both strains, demonstrating that non-synonymous mutations were favored over synonymous ones—clear evidence of adaptive evolution in response to ciprofloxacin pressure.

Mutations in the *gyrB* gene, which encodes the B subunit of DNA gyrase also detected in this study. The most notable alterarions occurred at positions 4, 5, and 6. These mutations have not been previously reported in *Klebsiella pneumonia.* While *gyrB* mutations are generally considered to have a lesser impact on resistance compared to *gyrA*, the combination of mutations in both genes has been shown to significantly enhance bacterial survival against fluoroquinolones (Azargun et al., 2019). The codon-based Z test revealed a value of 0.03779, suggesting moderate but meaningful positive selection in these strains. These statistical analyses confirm that changes in gyrB are evolutionarily advantageous under antibiotic pressure.

The *parC* gene encodes a subunit of topoisomerase IV, another enzyme involved in DNA deactivation. Mutations in this gene have been linked to increased fluoroquinolone resistance, often in combination with *gyrA* mutations (Liu et al., 2012). In the present study, nucleotide changes at positions 238,239,240 were identified, resulting in the amino acid substitution Ser80Ile. These findings support previous reports indicating that Ser80Ile mutations impair ciprofloxacin binding, further contributing to resistance (Liu et al., 2012). The detection of these mutations in a significant number of resistant isolates highlights their role in reducing the effectiveness of ciprofloxacin treatment. Statistical validation through Codon-based Z test results of 0.00240 (QD23), 0.000431 (Bio45), and 0.00207 (hvKP340) indicates strong selective pressure on this gene. These findings support the concept that mutations in *parC* are not random but are actively selected for in the presence of ciprofloxacin, especially when co-occurring with *gyrA* mutations.

The *parE* gene, encoding the B subunit of topoisomerase IV, was also analyzed in this study. The most frequently detected mutations occurred at positions 458 and 529, leading to the amino acid substitutions Ser458Ala and Ile529Leu. The present study found an alteration in nucleotide position 670, amino acid position 224. The *parE* mutations alone may not significantly impact ciprofloxacin resistance, but in combination with mutations in *gyrA*, *gyrB*, and *parC*, they enhance the overall resistance profile of the bacterial strain. Codon-based Z test values revealed mild but important positive selection.

Overall, the results of this study confirm that mutations in the *gyrA*, *gyrB*, *parC*, and *parE* genes contribute to ciprofloxacin resistance in *Klebsiella pneumoniae*. The findings align with previously published research. However, variations in mutation frequencies and types suggest that different bacterial populations may exhibit distinct resistance mechanisms.

Conclusion

The study investigates the mechanisms of ciprofloxacin resistance in *Klebsiella pneumoniae* by examining genetic alterations in *gyrA, gyrB, parC*, and *parE* genes. The findings suggest that gene alterations decrease the effectiveness of ciprofloxacin by modifying DNA gyrase and topoisomerase IV structures, impairing drug receptor-binding sites. Genomic surveillance system data confirms the identification of main mutations responsible for antibiotic resistance progression. The research underscores the importance of

proper antibiotic administration to prevent antibiotic resistance formation. The increasing number of resistant *K. pneumoniae* strains necessitates the development of combination treatments and next-generation fluoroquinolone medications. Further research is needed to explore alternative resistance mechanisms, such as efflux pumps, porin loss, and horizontal gene transfer. The future investigations require further examination into alternative strains.

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