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Research article

Genetic and in-silico approaches for investigating the mechanisms of ciprofloxacin resistance in *Salmonella typhi*: Mutations, extrusion, and antimicrobial resistance

Noman Khan [a,b,*], Syed Maaz Gillani [c], Mashooq Ahmad Bhat [d], Ihsan ullah [e,f], Muhammad Yaseen [f,**]

[a] Department of Biosciences, Mohammad Ali Jinnah University Karachi Pakistan, Pakistan
[b] The Gene-omics Bioinformatics Laboratory, Karachi, Pakistan
[c] Department of Microbiology, University of Karachi, Karachi, 75270, Pakistan
[d] Department of Pharmaceutical Chemistry, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia
[e] Institut für Chemie, Universität Rostock, Albert-Einstein-Str. 3a, 18059, Rostock, Germany
[f] Institute of Chemical Sciences, University of Swat, Charbagh, 19130, Swat, Pakistan

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ABSTRACT

Salmonella enterica serovar Typhi spreads typhoid infection in humans through the consumption of contaminated food and water. Poor sanitation plays a pivotal role in its dissemination. Over time, the bacterium has acquired resistance to many promising antibiotics, posing a growing global health concern and hindering the achievement of sustainable development goals. This study aims to elucidate the molecular complexity of fluoroquinolone resistance, a first-line treatment for typhoid infection. To achieve this aim, 80 clinical isolates were collected from various diagnostic laboratories. These isolates were confirmed based on morphological characteristics and biochemical tests. Multidrug-resistant (MDR) and extensively drug-resistant (XDR) isolates were identified using the Kirby-Bauer disc diffusion method. The mechanism of ciprofloxacin resistance was investigated by sequencing the quinolone resistance-determining region (QRDR) genes and identifying the presence of the *qnrS1* gene. As a result of this study, 60 % of isolates showed resistance to ciprofloxacin. At the same time, *the qnrS1* gene was present in all the selected strains while mutation analysis identified significant mutation in QRDR of DNA gyrase subunit A (*gyrA*) and Topoisomerase IV (*parC*) gene. The combinatorial effect was further investigated by downloading 286 draft genomes. The Mutation analysis reveals significant mutations at *gyrA* S83F, *gyrA* D87N, *gyrA* S83Y, *gyrB* S464F, *parC* S80I, *and parE* L416F. Additionally, docking analysis indicates reduced binding affinity and altered solvent accessibility, which show the structural changes at mutation sites. This study provides crucial insights that mutation reduces the binding affinity while *qnrS1* acts as a transport channel to extrude the ciprofloxacin. In the future, further validation through experimental mutagenesis is recommended, for targeted therapeutic interventions against the mounting threat of antibiotic-resistant *S. Typhi*.

* Corresponding author. Department of Biosciences, Mohammad Ali Jinnah University Karachi Pakistan, Pakistan.
** Corresponding author.
E-mail addresses: knoman915@gmail.com (N. Khan), syedmaaz088@gmail.com (S.M. Gillani), mabhat@ksu.edu.sa (M.A. Bhat), ihsanmtk@uswat.edu.pk (I. ullah), muhammadyaseen.my907@gmail.com (M. Yaseen).

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1. Introduction

Typhoid fever affects approximately 21 million individuals each year, leading to 128,000 to 161,000 deaths around the world. It is a serious disease with a mortality rate of 10 %–30 % if not treated [1]. Despite its low prevalence in developed countries, this disease remains a significant health concern and presents a danger of emerging in underdeveloped Asia and Africa due to inadequate hygiene and sanitation conditions. Typhoid fever is mainly caused by *Salmonella enterica serovar Typhi*, which is a gram-negative, rod-shaped, facultative anaerobe that belongs to the Enterobacteriaceae family. *S. Typhi* is transmitted to humans via the oral-fecal route and contaminated food or water [2]. It exhibits human specificity, causing systemic infection characterized by prolonged fever, headache, nausea, loss of appetite, abdominal discomfort, and potentially life-threatening complications [3,4]. Effective treatment for typhoid disease requires better hygiene, immunization, and acceptable antibiotic usage. Irrational antibiotic usage causes resistance to first- and second-line antibiotics, leading to the formation of multidrug-resistant microorganisms. Typhoid fever is primarily treated with antibiotics to inhibit the replication machinery of bacteria. Frequently prescribed antibiotics include fluoroquinolones, third-generation cephalosporin, and aminoglycosides. In severe cases, when antimicrobial resistance (AMR) is a concern, treatment becomes more challenging, and healthcare professionals may need to adjust the treatment plan based on individual circumstances and resistance patterns by evaluating the susceptibility [5,6].

AMR has become a significant global health concern, particularly with the growing resistance in *S. Typhi*, which has limited the effectiveness of traditional antibiotics such as chloramphenicol, ampicillin, and trimethoprim-sulfamethoxazole. Fluoroquinolones and third-generation cephalosporin have been widely prescribed to treat typhoid fever, but resistance to these antibiotics is also well-documented [7,8]. The increasing resistance has led to a greater reliance on azithromycin and carbapenem for treating MDR and XDR infections [9]. Effective surveillance and tracking of AMR patterns are crucial for determining appropriate treatment options. The spread of resistance in *S. Typhi* is driven by numerous factors, such as the overuse and misuse of antibiotics, limited healthcare access, poor sanitation, and global travel [10–12]. These factors contribute to the development and dissemination of resistance, especially in developing countries where typhoid fever is endemic. Inadequate hygiene practices exacerbate the transmission of resistant bacteria through contaminated water and food sources. Global travel and migration further facilitate the introduction of resistance to new areas. The exchange of mobile genetic elements among strains also enhances the rapid spread of resistance [13].

The present study aims to elucidate the molecular complexity of fluoroquinolone resistance, which is a first-line treatment for typhoid infection. Fluoroquinolones, particularly ciprofloxacin, are widely used antibiotics for treating typhoid. However, frequent use of fluoroquinolones leads to resistance, triggered by various genetic factors. These factors include mutations in the target genes responsible for DNA replication and repair, namely gyrase (*gyrA* and *gyrB*) and topoisomerase IV (*parC* and *parE*) [14–16]. These mutations alter the structure of these enzymes, particularly in the quinolone resistance-determining region (QRDR), ultimately reducing the binding affinity of the antibiotics. QRDR acts as a binding site for fluoroquinolones. Additionally, the acquisition of plasmids or mobile genetic elements carrying quinolone resistance genes acts as transport channels that extrude the antibiotic. Plasmid transfer across species by horizontal gene transfer contributes to the dissemination of resistance within bacterial populations [17,18]. Understanding the genetic mechanisms underlying quinolone resistance is crucial for clinicians to adopt effective strategies to combat the spread of infection [19]. Clinicians must be aware of these mechanisms to make informed decisions regarding treatment options. Continued research and surveillance are essential to stay ahead of the evolving landscape of AMR in *S. Typhi*.

2. Material and methodology

2.1. Isolation and screening of S. Typhi

This prospective study was conducted at the Department of Biosciences, Mohammad Ali Jinnah University. A total of 80 *S. Typhi* isolates were collected from various hospitals and clinical laboratories across Karachi, including Dr. Ruth K. M. Pfau Civil Hospital (n = 45), Ziauddin Hospital (n = 20), and Chughtai Laboratory (n = 15). The strains were isolated from both blood and stool samples obtained from outpatients and inpatients, with 48 samples from males and 32 from females, ranging from infants to adults of up to 40 years. The collected strains were stored in a 70 % glycerol solution at −20 °C for further analysis. Serologically confirmed *S. Typhi* strains were initially identified at the hospitals and clinical labs. For identification *S. typhi* ATCC 6539 were used as control strain. Further biochemical testing was performed to confirm these identifications. The colony characteristics of the strains were observed on MacConkey agar. Biochemical tests included the IMViC series (Indole, Methyl Red, Voges-Proskauer, and Citrate tests), Triple Sugar Iron (TSI) agar test, and Sulfur Indole Motility (SIM) test, following the protocols described in Bergey's Manual of Systematic Bacteriology [20].

2.2. Antimicrobial susceptibility test (AST)

The Kirby-Bauer disc diffusion method was used to determine the antibiotic susceptibility profile of *S. Typhi*, following the guidelines set by the Clinical and Laboratory Standards Institute (CLSI) [21]. *S. Typhi* ATCC 6539 was used as the quality control strain. The antibiotics tested included ampicillin (10 μg), amoxicillin (25 μg), ceftriaxone (30 μg), cefotaxime (30 μg), cefixime (30 μg), azithromycin (15 μg), ciprofloxacin (5 μg), meropenem (30 μg), chloramphenicol (30 μg), and cotrimoxazole (25 μg). The diameter of the inhibition zones around the discs was measured and compared with CLSI interpretive criteria to categorize the isolates as susceptible, intermediate, or resistant to each antibiotic.

2.3. DNA extraction

The preserved isolates were inoculated into nutrient broth and grown overnight at 37 °C. Genomic DNA was then extracted using the cetyltrimethylammonium bromide (CTAB) method [22]. After the overnight culture, the bacterial cells were harvested by centrifugation, and the resulting cell pellet was treated with CTAB buffer to lyse the cells. This lysis step releases the cellular contents, including the DNA. The lysate was then mixed with chloroform: isoamyl alcohol solution (24:1) to separate the DNA from proteins and other cellular debris. Following centrifugation, the upper aqueous DNA phase was carefully collected into a separate microcentrifuge tube. Chilled isopropanol was added to the solution to precipitate the DNA, and the mixture was centrifuged to form a DNA pellet. The DNA pellet was subsequently washed twice with 70 % ethanol to remove any remaining impurities. To eliminate any RNA contamination, the DNA was treated with 10 μL of RNase and incubated for 30 min at 37 °C. Finally, the purified genomic DNA was resuspended in Tris-EDTA (TE) buffer and stored at −20 °C for subsequent experimentation. This method ensured the extraction of high-quality genomic DNA suitable for further molecular analyses.

2.4. PCR amplification and sequencing of gyrA, parC, and qnrS1 gene

To elucidate the mechanism of resistance, the *gyrA*, *parC*, and *qnrS1* genes were amplified from 10 randomly selected ciprofloxacin-resistant isolates. A conventional thermal cycler was used to perform polymerase chain reaction (PCR) for this analysis. Primers for the amplification were designed using Primer3 Web (v4.1.0), based on reference sequences: *gyrA* (Accession No. MG596303.1), *parC* (Accession No. MK112508.1), and *qnrS1* (Accession No. HQ214119.1). While the PCR reaction was optimized according to the parameters detailed in Supplementary Table 2 [23–25]. The PCR reaction mixture, totaling 33 μL, was prepared as follows: 16.5 μL of Thermo Scientific™ PCR Master Mix (2X), 3 μL of each primer (forward and reverse), 0.5 μL of nuclease-free water, and 10 μL of extracted DNA. *S. typhi* ATCC 6539 was used as a positive control for the PCR reaction. The thermal cycling conditions included an initial denaturation step, followed by cycles of denaturation, annealing, and extension, with a final extension step to ensure complete amplification of the target genes. The amplified PCR products were then visualized using gel electrophoresis on a 1 % agarose gel run at 60 V for 1 h. This process allowed for the confirmation of successful amplification by comparing the bands to a DNA ladder. The resulting amplicons were sent to BGI Genomics for Sanger sequencing. Once sequenced, the partial sequences of *gyrA* and *parC* were aligned using MEGA 11 software with ClustalW for multiple sequence alignment [26]. These sequences were then uploaded to GenBank, where they were assigned the accession numbers PP747319-PP747338.

2.5. Extraction of draft genomes from databases and antimicrobial resistance genes profiling

A total of 283 MDR *S. Typhi* draft genomes were extracted from the Pathogenwatch database (https://pathogen.watch/). To ensure relevance and accuracy, the inclusion criteria were set and only genomes reported between 2019 and 2023 were selected, and each had to have a documented phenotypic resistance profile. Antimicrobial resistance genes (ARG) and point mutations mediating resistance were annotated using Resfinder 4.1 (http://genepi.food.dtu.dk/resfinder) [27].

2.6. In silico validation of quinolone resistance by docking analysis

Protein models, particularly for DNA gyrase subunit A (*gyrA*) and topoisomerase IV (*ParC*), were prepared using Swiss-Model for subsequent docking analysis. The protein sequences were used to develop protein models, which were downloaded in PDB format to facilitate their utilization in docking studies. Ciprofloxacin was selected as the ligand molecule and downloaded in PDB format from DrugBank. This ligand molecule is essential for the docking analysis to explore its interaction with the prepared protein models.

Molecular docking simulations were conducted using AutoDock Vina (v. 1.2), and the interactions between the ligand (ciprofloxacin) and receptor molecules (*gyrA* and *ParC*) were visualized using Discovery Studio [28]. Before docking, the ligand and receptor structures were prepared by removing alternate locations of residues, adding hydrogen atoms, and assigning charges to protein atoms [29]. The grid was generated around the QRDR. For *gyrA*, the grid was adjusted with a spacing of 0.325 Å, comprising 106 points along the x-coordinate, 96 points along the y-coordinate, and 126 points along the z-coordinate. The center of the grid was located at

Table 1
Antimicrobial resistance test of 80 isolated strains against different antibiotics.

Antibiotic class	Antibiotic	No. of Resistant	Percentage
Penicillin	Ampicillin	77	96.2
	Amoxicillin	78	97.5
3rd Generation Cephalosporin	Ceftriaxone	73	91.2
	Cefotaxime	72	90
	Cefixime	74	92.5
Macrolides	Azithromycin	10	12.5
Fluoroquinolone	Ciprofloxacin	48	60
Carbapenem	Meropenem	0	0
Phenicols	Chloramphenicol	80	100
Sulfonamides	Co-trimoxazole	74	92.5

coordinates (−8.715, −1.601, 19.044) in three-dimensional space. For *ParC*, the spacing was 0.439 Å, comprising 86 points along the x-coordinate, 56 points along the y-coordinate, and 98 points along the z-coordinate. The center of the grid was located at coordinates (−7.929, 8.189, 1.065) in three-dimensional space.

AutoDock Vina was used for docking simulations, exploring various ligand conformations and orientations to predict binding affinities [30,31]. Post-docking analysis in BIOVIA Discovery Studio and PyMol facilitated the visualization of binding poses, identification of key binding residues, and assessment of potential interactions [32].

3. Results and discussions

In this study, 80 isolates of *S. Typhi* were tested to evaluate their resistance profiles, as detailed in Table 1. All 80 isolates (100 %) were found to be resistant to chloramphenicol. There was a significant range of resistance observed against different classes of antibiotics, including ampicillin (96.2 %), amoxicillin (97.5 %), ceftriaxone (91.2 %), cefotaxime (90 %), cefixime, and co-trimoxazole (92.5 %). Notably, 60 % of the isolates were resistant to ciprofloxacin, 12.5 % to azithromycin, while meropenem remained effective against all isolates (100 % susceptibility). The results indicate a high prevalence of MDR among the clinical isolates, with some showing XDR. The mechanisms of resistance can be intrinsic or acquired resistance. Intrinsic resistance often refers to resistance that is present across species. The mutations in the antibiotic binding sites or the presence of antimicrobial resistance genes in the chromosome are referred to as acquired resistance. The acquired resistance is mainly acquired through plasmids, horizontal gene transfer, mobile genetic elements, and mutations at the antibiotic binding sites [33–35].

S. typhi infections are commonly treated with fluoroquinolones and third-generation cephalosporins [36]. Among fluoroquinolones, ciprofloxacin is considered a first-line drug due to its ability to inhibit DNA replication in pathogenic microorganisms. However, resistance to fluoroquinolones, including ciprofloxacin, is gradually increasing [37]. This resistance can be both mutations induced and presence of extrinsic factors. Bacterial DNA gyrase and topoisomerase IV is essential for DNA replication and repair and targeted by ciprofloxacin leading to disrupted DNA synthesis and bacterial cell death. The Mutations in the *gyrA* and *parC* genes, which encode these enzymes, can induce resistance by causing conformational changes that reduce ciprofloxacin's binding affinity [38–40]. Additionally, the *qnrS1* gene can act synergistically with these mutations, further diminishing ciprofloxacin's efficacy. In this study, 48 (60 %) of the isolates were resistant to ciprofloxacin. Among them, 10 isolates were randomly selected for detailed analysis. As previously noted, fluoroquinolone resistance is induced by mutations or the presence of plasmid-mediated factors. Plasmid-mediated quinolone resistance (PMQR) involves the *qnrS1* gene, which aids in ciprofloxacin extrusion. The *qnrS1*, *gyrA*, and *parC* genes were amplified as illustrated in Supplementary Table 2. Molecular characterization revealed the presence of *qnrS1* in seven selected isolates [Fig. 1(A)]. Mutation-induced resistance is often associated with the QRDR of *gyrA* and *parC* genes. The partial sequence of *gyrA* and *parC* was amplified and sequenced [Fig. 1(B)]. Sequence analysis using MEGA 11 (v. 11.0.13) indicated mutations at *gyrA* S83F (60 %), *gyrA* S83Y (30 %), *gyrA* D87N (30 %), and *gyrA* D87G (40 %), with 60 % of the isolates harboring mutations at both sites. Additionally, mutations in topoisomerase IV (*parC*) were identified at *parC* S80I (10 %), *parC* S80R (10 %), *parC* E84K (10 %), and *parC* E84G (20 %), as illustrated in [Fig. 2] [41–44].

To build a comprehensive understanding of fluoroquinolone resistance, we further analyzed 283 draft genomes of MDR *S. Typhi*.

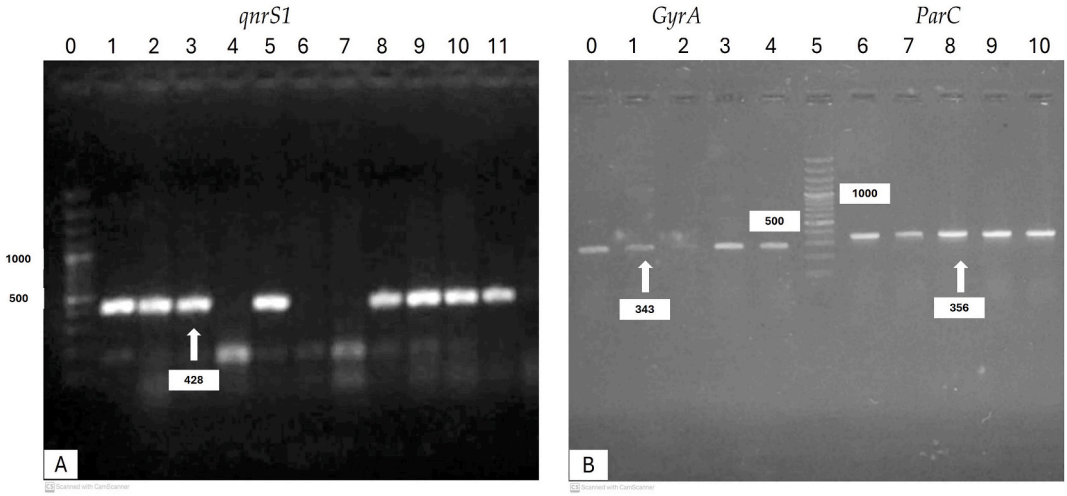

Fig. 1. (A): Gel electrophoresis for the *qnrS1* gene. Lane 0: 1400 bp DNA ladder, Lane 1: positive control, Lanes 2–11: clinical isolates (ST-07, ST-23, ST-24, ST-33, ST-34, ST-58, ST-66, ST-72, ST-77, and ST-80). The product size was around 428 bp. The *qnrS1* gene was not amplified in three isolates. Full, non-adjusted images are available Supplementary Fig. 3 **(B):** Gel electrophoresis for *gyrA* and *parC* genes. Lane 0: positive control for *gyrA*, Lanes 1–4: clinical isolates (ST-07, ST-24, ST-58, and ST-77), Lane 5: 1400 bp DNA ladder, Lane 6: positive control for *parC*, Lanes 7–10: clinical isolates (ST-07, ST-24, ST-58, and ST-77). The product size was around 350 bp for both genes. Full, non-adjusted images are available Supplementary Fig. 4.

The isolates were collected from various sample types, including blood (23.07 %), stool (4.9 %), and unknown sample types (72.02 %). The phenotypic resistance data indicate that the isolates were resistant to ampicillin (97.17 %), third-generation cephalosporins (89.4 %), chloramphenicol (98.6 %), ciprofloxacin (42.65 %), sulfonamides (67.9 %), trimethoprim (66.08 %), tetracycline (1.4 %), azithromycin (5.7 %), and meropenem (0.0 %). Additionally, 56.3 % of the isolates showed intermediate resistance to ciprofloxacin. To validate these phenotypic resistance profiles, we identified genetic markers responsible for AMR in the isolates. The genotypic analysis revealed the presence of several key resistance genes, including $bla_{CTX\text{-}M\text{-}15}$ (55.30 %), $bla_{TEM\text{-}1D}$ (91.79 %), *catA1* (97.43 %), *dfrA7* (96.82 %), *qnrS1* (54.35 %), *sul1* (98.46 %), *sul2* (65.64 %), and *tetA(B)* (1.02 %). These genes confer resistance to a variety of antibiotics across different classes, including beta-lactams, chloramphenicol, sulfonamides, trimethoprim, quinolones, and tetracycline. Importantly, the analysis confirmed that *Salmonella* spp. remains susceptible to carbapenems both phenotypically and genotypically, with no detected genetic markers conferring resistance to these antibiotics. Similarly, no resistance markers for macrolides were found in the genotypic analysis.

The results of the antimicrobial susceptibility test (AST) demonstrated a strong correlation between ciprofloxacin resistance and specific genetic markers, including mutations and the presence of the *qnrS1* gene. Mutation analysis revealed significant mutations at several key sites as Illustrated in [Fig. 3]: *gyrA* S83F (found in 258 isolates), *gyrA* D87N (20 isolates), *gyrA* S83Y (3 isolates), *gyrB* S464F (18 isolates), *parC* S80I (16 isolates), and *parE* L416F (4 isolates). Additionally, the mutations *gyrA* S83F, *gyrA* S83Y, *gyrA* D87N, and *gyrA* S83F, D87N were detected in 10 partially sequenced isolates. The predominant mutation in *parC* was *parC* S80I. Notably, among the 283 genomes analyzed, three isolates lacked mutations at these key sites and did not possess the qnrS1 gene, indicating susceptibility to ciprofloxacin. This finding suggests that while the presence of specific mutations and the qnrS1 gene are significant factors in quinolone resistance, their absence can predict susceptibility to ciprofloxacin.

The data indicate that mutations at sites such as *gyrA* S83F, *gyrA* D87N, *gyrA* S83Y, and gyrB S464F contribute to intermediate resistance [Fig. 4]. However, a combination of the *gyrA* S83F mutation and the presence of the *qnrS1* gene results in full resistance. This suggests that these mutations reduce the binding ability of ciprofloxacin to its target, while the qnrS1 transport protein aids in extruding ciprofloxacin from the cell. Furthermore, the data show that multiple mutations, such as *gyrA* S83F, D87N, and *parC* S80I within the same isolate, can confer resistance even in the absence of the plasmid-mediated quinolone resistance factor. The alterations in the cellular replication machinery account for the overall resistance observed in these isolates. The effects of the mutations and extrusion factors are discussed in detail in Supplementary Table 1.

Understanding the combined effects of mutations in the QRDR is key to understanding the ciprofloxacin resistance in *S. Typhi*. This study found that specific mutations, such as *gyrA* S83F and *gyrA* D87N, significantly enhance resistance by further decreasing ciprofloxacin binding affinity and altering solvent accessibility. The synergistic interaction where the combined impact of these mutations exceeds the sum of their individual effects, leading to a higher level of resistance. Similarly, the combination of *gyrA* S83F and *parC* S80I mutations within the same isolate contributes to strong resistance, even without the presence of plasmid-mediated resistance factors like *qnrS1*. The frequency of these co-occurring mutations highlights their selective advantage, with mutations such as *gyrA* S83F being prevalent in many isolates, often in combination with other mutations. This widespread occurrence suggests these combinations are particularly advantageous in the presence of ciprofloxacin. In contrast, some mutations, like *gyrA* S83Y, are rare, indicating they may not offer the same resistance benefits or might even be antagonistic when combined with other mutations,

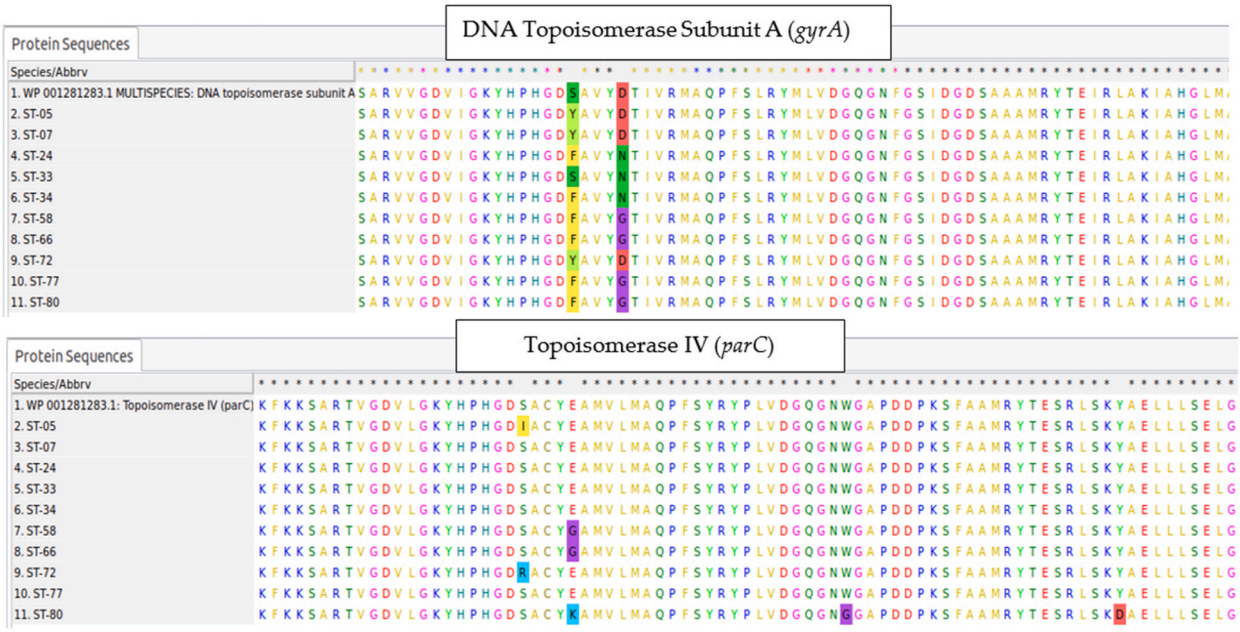

Fig. 2. Multiple sequence alignment of quinolone resistance determining region (*gyrA*, and *parC*) with MEGA CLUSTAL W.

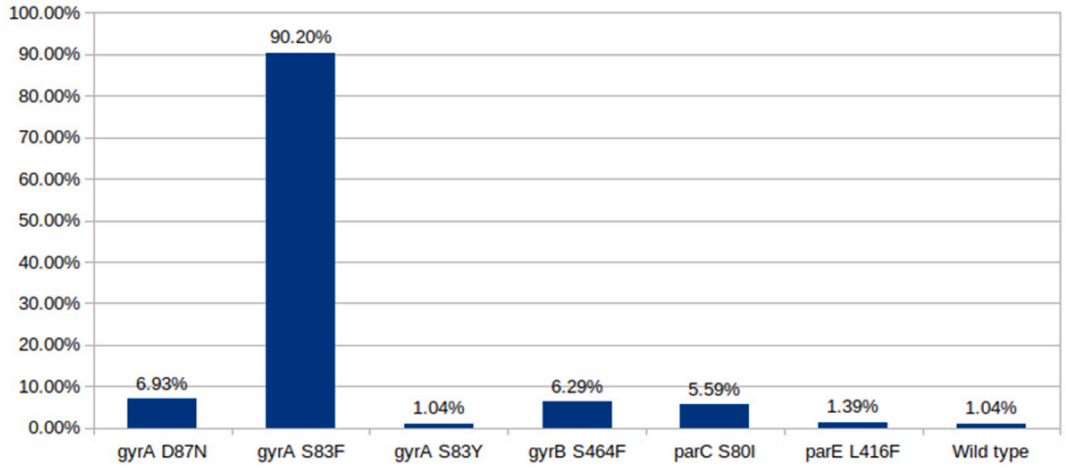

Fig. 3. Frequency of mutations in quinolone resistance determining region.

Frequency	*gyrA* D87N	*gyrA* S83F	*gyrA* S83Y	*gyrB* S464F	*parC* S80I	*parE* L416F	*qnrS1*	Ciprofloxacin
N=106								Resistant
N=16								Resistant
N=4								Intermediate
N=18								Intermediate
N=136								Intermediate
N=3								Intermediate
N=4								Intermediate

Fig. 4. Distribution of QRDR mutations and *qnrS1* gene in ciprofloxacin-resistant and intermediate-resistant *S. Typhi* isolates.
Heat map legend.
Red = Presence of Mutation/gene.
Green = Absence of Mutation/Gene.

potentially reducing bacterial fitness. Understanding these synergistic and antagonistic effects is crucial for predicting resistance patterns and developing effective treatment strategies.

The effect of the mutation at the QRDR and its role in resistance was further elucidated by docking analysis. The docking results offer valuable insights into the potential impact of specific mutations in the *gyrA* and *parC* genes on the binding affinity of ciprofloxacin. The wild type *gyrA* exhibited a relatively strong binding affinity with ciprofloxacin, reflected by a binding energy of −8.5 kcal/mol.

The molecular interactions of Ligand (Ciprofloxacin), and Protein (*gyrA* and *ParC*) active sites is illustrated in [Fig. 5 (A – G)]. This suggests a stable and effective interaction between ciprofloxacin and the un-mutated *gyrA*, supporting the drug's efficacy against the wild-type strain. However, when mutations were introduced into the *gyrA* gene, such as *gyrA* S83F, *gyrA* S83Y, and *gyrA* D87N, a consistent decrease in binding affinity was observed. The *gyrA* S83F mutation resulted in the lowest binding affinity among the single mutations, indicating that this specific change in the *gyrA* gene could significantly impact the drug's binding efficacy [45–47]. The double mutation *gyrA* S83F, D87N did not further decrease the binding affinity, suggesting a saturation effect or potential structural changes that hinder effective drug binding. The solvent accessibility values provide additional context. Solvent accessibility reflects the degree to which the binding site is accessible to the surrounding solvent molecules [48,49]. In the wild-type *gyrA*, a solvent accessibility of 2830.99 was observed, suggesting a reasonably accessible binding site. The mutations, however, showed a decrease in solvent accessibility, indicating potential alterations in the local environment that might hinder ciprofloxacin's accessibility to the binding site.

Furthermore, the *parC* wild-type gene demonstrated a binding affinity of −7.8 kcal/mol, slightly lower than that of the unmutated *gyrA*. Despite this, it still indicates a relatively strong interaction. The introduction of the *parC* S80I mutation resulted in a further decrease in binding affinity, suggesting that this mutation could confer resistance to ciprofloxacin similarly to the mutations in the *gyrA* gene (Table 2). These mutations are likely compromising the competitive binding of ciprofloxacin to *gyrA* and *parC* genes. This alters the structure of the enzyme, changing the shape and chemical properties of the binding site. This structural alteration hinders

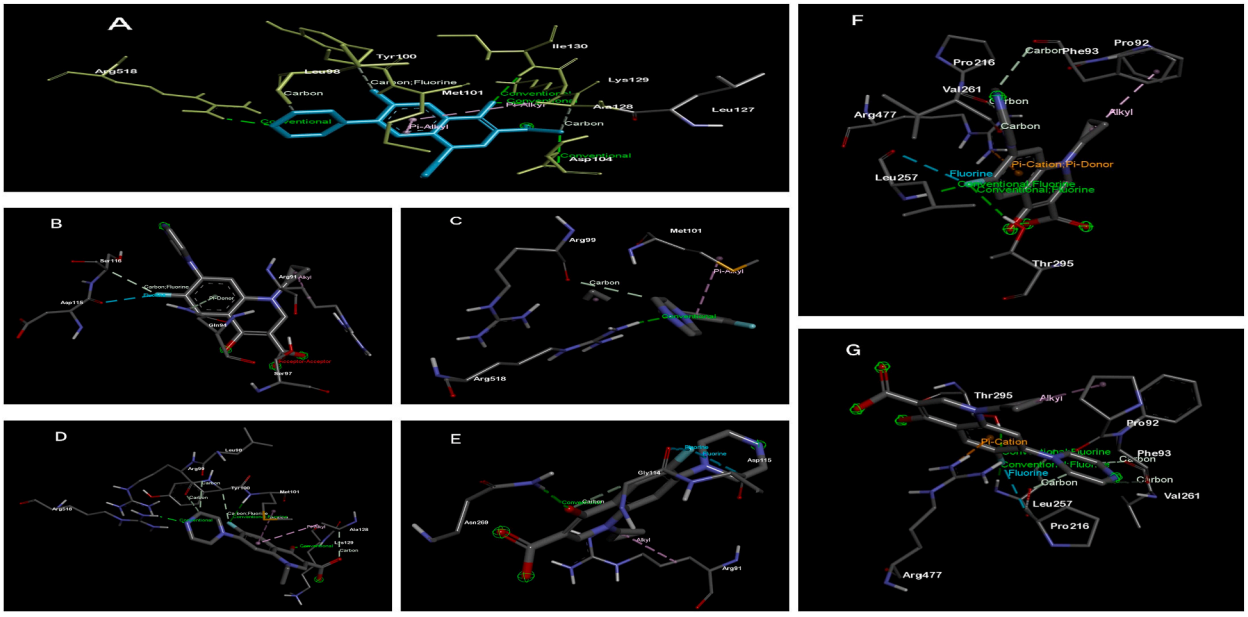

Fig. 5. Representing the molecular interactions of ciprofloxacin (ligands molecule) with a protein *gyrA* (A–E), and *ParC* (F–G) active sites. **(A):** Shows hydrogen bonds (Asp104, Lys129, Ala128, Ile130), carbon-hydrogen bonds (Ala128, Leu98, Tyr100), and alkyl interactions (Met101, Ala128). **(B):** Displays hydrogen bonds (Gln94, Ser116), a halogen bond (Asp115 with fluorine), and an unfavorable acceptor-acceptor bond (Ser97). **(C):** Highlights hydrogen bonds (Arg518), additional non-covalent interactions (Arg99), and alkyl interactions (Met101, Ile130). **(D):** Presents hydrogen bonds (Lys129, Met101, Arg518) and non-covalent interactions (Leu98, Tyr100, Arg99, Ala128). **(E):** Shows hydrogen bonds (Asn269), carbon-hydrogen bonds (Gly114), halogen bonds (Asp115), and alkyl interactions (Arg91). **(F):** Exhibits hydrogen bonds (Tyr24, His75), carbon-hydrogen bonds (Asp28), halogen bonds (Ala81), and alkyl interactions (Arg29, Ala30, Val41, Ala85). **(G):** Illustrates hydrogen bonds (Gln260), carbon-hydrogen bonds (Ala81), halogen bonds (Thr171), pi-sigma interactions (Leu88), and alkyl interactions (Lys113, Phe115).

ciprofloxacin from effectively binding to the target site, reducing its ability to inhibit the enzyme's function. Overall, the findings highlight the significant impact of specific mutations in the QRDR on the binding efficacy of ciprofloxacin. The mutations in *gyrA* and *parC* genes alter the binding site's structure, reducing the drug's efficacy and leading to resistance. The combination of decreased binding affinity and solvent accessibility changes provides a comprehensive understanding of how these mutations contribute to ciprofloxacin resistance in *S. Typhi* isolates.

Visualization of the docking results and protein structures provided an important observation that there were no direct interactions between ciprofloxacin and the molecules at the mutation sites [Supplementary Fig. 2 (A-G)]. This suggests that the decrease in binding affinity may be attributed to conformational changes induced by the mutations rather than direct interactions with the drug [50,51]. The reduction in binding affinity, along with changes in solvent accessibility and the absence of direct interactions at mutation sites, suggests that these mutations significantly contribute to the reduced effectiveness of ciprofloxacin, potentially leading to antibiotic resistance. The findings underscore the importance of understanding molecular interactions in the context of antibiotic resistance,

Table 2
Effect of mutations on binding affinity, solvent accessibility, and interacting amino acids.

Gene Variants	Binding Affinity in QRDR (Kcal/mol)	Solvent Accessbility in QRDR (A^o)	Changes in bonds and interacting residues
gyrA Wild type	−8.5	2830.99	Conventional hydrogen bond (Asp104, Lys129, Ala128, and Ile130), Carbon Hydrogen Bond (Ala128, Leu98, and Tyr100), and Alkyl Interactions (Met101 and Ala128)
gyrA S83F	−5.8	2731.51	Conventional hydrogen bond (Gln94 and Ser116), Halogen bond (Asp115 and the fluorine atom of Ciprofloxacin), and Unfavorable Acceptor-Acceptor bond (Ser97),
gyrA S83Y	−6.2	2741.95	Conventional hydrogen bond (Arg 518), Additional noncovalent Interactions (Arg 99), Alkyl interactions (Met 101, and Ile 130)
gyrA D87N	−6.1	2756.78	Conventional hydrogen bond (Lys 129, Met 101, and Arg 518), Additional noncovalent Interactions (Leu 98, Tyr 100, Arg 99, and Ala 128)
gyrA S83F, D87N	−5.9	2758.2	Conventional hydrogen bond (Asn 269), Carbon Hydrogen bond (Gly 114), Halogen bond (Asp 115), and Alkyl interaction (Arg 91)
parC Wild type	−7.8	3326.01	Conventional hydrogen bond (Tyr 24, and His 75), Carbon Hydrogen bond (Asp 28), Halogen bond (Ala 81), and Alkyl interaction (Arg 29, Ala 30, Val 41, Ala 85)
parC S80I	−6.0	3014.57	Conventional hydrogen bond (Gln 260), Carbon Hydrogen bond (Ala 81), Halogen bond (Thr 171), Pi Sigma (Leu 88), and Alkyl interactions (Lys 113, and Phe 115)

providing valuable information for the development of more effective therapeutic strategies against resistant bacterial strains.

Despite the significant insights gained from the docking analysis regarding the impact of specific mutations in the *gyrA* and *parC* genes on ciprofloxacin binding, this study has certain limitations. The observed differences in binding affinity between wild-type and mutant forms of *gyrA* and *parC*, while indicative of reduced drug efficacy, are insufficient to conclude that these interactions alone are the primary drivers of ciprofloxacin resistance. The energy differences observed in docking simulations suggest that factors other than direct interaction with active site residues might contribute to resistance.

One arguable explanation is that these mutations induce conformational changes in the protein structure, which in turn affect the binding site's overall shape and chemical properties. These alterations may reduce the ability of ciprofloxacin to effectively interact with its target enzymes, leading to diminished drug efficacy. However, to conclusively demonstrate this mechanism, further studies involving molecular dynamics (MD) simulations or experimental structural biology approaches, such as X-ray crystallography or cryo-electron microscopy, are necessary. These methods could provide a more detailed understanding of the conformational changes induced by the mutations and their impact on drug binding. Additionally, the study did not explore the potential compensatory mechanisms that bacteria might employ to overcome the fitness costs associated with these mutations. Future research should aim to integrate these factors to develop a more comprehensive understanding of ciprofloxacin resistance in *S. Typhi*.

4. Conclusion

The current study focuses on ciprofloxacin resistance in *S. Typhi*, a fluoroquinolone typically used as a primary treatment for this infection, which is now facing widespread resistance due to various factors. Our findings suggest that ciprofloxacin resistance in *S. Typhi* is fundamentally linked to mutations and the presence of extrinsic factors. Mutation-induced factors include mutations in the QRDR, while extrinsic factors involve the presence of transport channels that aid in the extrusion of antibiotics. Docking studies indicate a significant impact of these mutations on the binding affinity of ciprofloxacin with *gyrA* and *parC*. The lack of direct interactions at mutation sites, along with changes in solvent accessibility, indicates a structural basis for the reduced effectiveness of the drug. In addition to the mutation induced resistance, the presence of transport channels plays a crucial role in extruding the antibiotic from the cell, contributing to resistance. Importantly, the study highlights instances where the presence of resistance genes does not necessarily correlate with phenotypic resistance, underscoring the importance of careful interpretation of genotypic data. To further validate and comprehend these findings, molecular dynamic simulation, mutagenesis studies are recommended to confirm the impact of specific mutations on ciprofloxacin resistance. This comprehensive approach will enhance our understanding of the mechanisms driving antibiotic resistance in *S. Typhi*, informing the development of targeted therapeutic strategies to address the increasing threat of resistant bacterial strains.

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Institutional review board statement

The study was approved by the Institutional Ethical Review Board at Mohammad Ali Jinnah University.

Informed consent statement

Not applicable.

Data availability statement

The data associated with this study has been deposited into a publicly available repository GenBank with an accession number PP747319-PP747338.

Sample availability

All clinical Isolates are available and stored culture bank.

CRediT authorship contribution statement

Noman Khan: Writing – original draft, Validation, Supervision, Software, Methodology, Data curation, Conceptualization. **Syed Maaz Gillani:** Writing – original draft, Software, Investigation, Formal analysis, Data curation, Funding acquisition. **Mashooq Ahmad Bhat:** Writing – review & editing, Writing – original draft, Validation, Supervision, Investigation. **Ihsan ullah:** Visualization, Validation, Investigation, Data curation. **Muhammad Yaseen:** Writing – review & editing, Visualization, Methodology, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:Noman Khan reports financial support and article publishing charges were provided by King Saud University. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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