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Recent Prevalence of Multi-Drug Resistant (MDR) Salmonella typhimurium from Tertiary Care Hospital of Lahore, Pakistan; Integrating 16S rRNA Profiling and *In Silico Analysis*

Hassan Raza Heral
University of Lahore
Dua Imran
University of Central Punjab
Ayesha Masood
University of Lahore
Mohsin Gulzar Barq
University of Central Punjab
Syed Zeeshan Haider Naqvi
University of Lahore
Asim : Shahzad
agron12@yahoo.com

Qingdao Hengxing University of Science and Technology

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Abstract

Multidrug-resistant (MDR) strains of Salmonella enterica serovar Typhimurium with biofilm-forming capability are important foodborne pathogens that pose a significant challenge to control and treatment. The present study was designed to explore the involvement of the second-messenger signaling gene adrA in biofilm formation and antimicrobial resistance in multidrug-resistant *S. Typhimurium* isolates. Approximately 2300 isolates were sampled from tertiary care hospitals. The isolates were subjected to antimicrobial susceptibility testing by the Kirby-Bauer disk diffusion method, and biofilm formation was assessed by the Congo red agar assay. The isolates were identified by conventional biochemical methods along with molecular identification by 16S rRNA analysis. Amplification and subsequent sequencing of the 16S rRNA gene and of the target gene *adrA* were done by polymerase chain reaction (PCR). Additionally, bioinformatics tools such as phylogenetic analysis, protein structure prediction, and docking were applied. All the isolates showed multidrug-resistant phenotypes with a high level of resistance to ampicillin, cefotaxime, and ciprofloxacin, but were sensitive to azithromycin and meropenem. All were able to form biofilm and were positive for the gene *adrA*. The phylogenetic analysis of Salmonella strains confirmed the conservation of these genes. Molecular docking analysis was carried out to show the interactions of the encoded proteins with the tested antibiotics. Finally, there was evidence that multidrug-resistant *S. Typhimurium* strains had high biofilm-forming abilities and contained important second messenger signaling genes that are linked with bacterial persistence. The results showed that the genes *adrA* could be associated with biofilm formation and antimicrobial resistance and could be used as a starting point for future research into how to control multidrug-resistant Salmonella infections.

1. Introduction

S. Typhimurium is a Gram-negative, facultative intracellular, rod-shaped bacterium that belongs to the family Enterobacteriaceae (RG et al., 2019). The organism is typically 2–5 μm long and has peritrichous flagella that are important to enable the organism to move and colonize the host (SM, 2019). *S. Typhimurium* is a facultative anaerobe that can survive and adapt in different environments, including contaminated foods, environmental reservoirs, and the human gastrointestinal tract (Oludairo et al., 2023). The pathogen is known worldwide as a major cause of food-borne gastroenteritis and is a big contributor to enteric infections (Eng et al., 2015). Infection is most commonly acquired through contaminated poultry products, water, and food-contact surfaces, and it can result in mild diarrhea and gastroenteritis, or in systemic infection, such as septicemia and typhoid fever-like syndrome.

The emergence of multidrug-resistant (MDR) strains of *S. Typhimurium* has been a problem in recent decades and is a global public health issue. Generally, MDR strains are considered to be resistant to at least one antimicrobial agent in three or more antibiotic classes (Antunes et al., 2016). The use of antibiotics in clinical medicine and animal agriculture is excessive and inappropriate, which has helped to drive the selection and spread of resistant strains all over the world (Ahmad et al., 2017; Khan & Shamim, 2022; Quinn et al., 2006). In *S. Typhimurium*, resistance is adding to the morbidity and mortality of infection and complicating therapeutic management (Jing et al., 2021). The emergence of resistance in fluoroquinolones and third-generation cephalosporins is a major threat to public health system especially (Li et al., 2017).

Multidrug resistant (MDR) strains of *S. Typhimurium* with possible increase in biofilm formation are important food borne pathogens that pose a great difficulty in control and treatment. The present study was designed to explore the involvement of second messenger signaling genes i.e. *adrA*, in biofilm formation and antimicrobial resistance of multi-drug resistant *S. Typhimurium* isolates.

The spread of MDR strains of *S. Typhimurium* that are capable of persistent biofilm formation is a huge public health problem throughout the world. Although antimicrobial resistance and biofilm production have been studied individually in many studies, little data are available on the role of second messenger signaling gene *adrA*, in these phenotypes. Furthermore, little to nothing is known about the association of changes in these signaling pathways and the resistance observed. Therefore, a wide range of investigation approaches, including phenotypic assays, molecular characterization and computational analysis is necessary to gain a better understanding of the molecular basis of multidrug resistance of *S. Typhimurum* biofilms.

This research study was performed to determine the prevalence of MDR and XDR *S. Typhimurium* from a tertiary care hospital in Lahore in the province of Punjab, Pakistan. The patterns of antibiotic sensitivity were also observed to study the resistance behavior of XDR and MDR *S. Typhimurium* isolates, respectively.

This study provides a comprehensive investigation of multidrug-resistant *S. Typhimurium* isolates from Lahore, Pakistan, by integrating antimicrobial susceptibility testing, biofilm characterization, *adrA* gene detection, phylogenetic analysis, protein structure prediction, and molecular docking. The combined use of phenotypic, molecular, and computational approaches offers new insights into the role of AdrA-mediated biofilm regulation in bacterial persistence and antimicrobial resistance.

2. Methodology

2.1. Sample type and Processing:

In this study, a total of 2300 samples were obtained over a period of twelve months (September 2024–August 2025) from University of Lahore Teaching Hospital, Lahore, Pakistan for the isolation and identification of *Salmonella*-associated bacterial strains. The samples were collected and appropriately labeled and were transported in sterile conditions and processed immediately for microbiological analysis in the laboratory. The samples were processed by the VersaTREK™ Automated Microbial Detection System (ThermoFisher Scientific, Waltham, MA, USA) for evidence of microbial growth, gas formation, and hemolysis. Each of the blood culture vials was monitored for seven consecutive days after its incubation at 37^{o}C. After seven days, a blood culture vial with no evidence of visible microbial growth was sub-cultured before ruling it to be negative for the patient. For the positive blood culture vials, the samples were cultured onto basic, enriched, and selective media for the growth of the suspected pathogen. Patients of positive samples were also asked about their medical history and background in a brief proforma.

2.2. Antimicrobial Susceptibility Testing (AST)

To determine the resistance and sensitivity profile to commonly prescribed antibiotics (chloramphenicol, azithromycin, ampicillin, cefotaxime, meropenem, and amoxicillin–clavulanic acid), antimicrobial susceptibility testing (AST) was carried out for *S. Typhimurium* isolates. Kirby–Bauer disk diffusion method was used based on the recommendations of the Clinical and Laboratory Standards Institute (CLSI, 2024). Susceptibility results were interpreted based on Clinical and Laboratory Standards Institute (CLSI) guidelines. The AST was performed in sterile laboratory conditions with fresh bacterial cultures. This test was created to evaluate the susceptibility of the isolated Salmonella strains to selected antibiotics and to find multidrug-resistant isolates related to biofilm-producing and virulence-associated genes.

2.3. Biofilm Formation Assays

To determine the resistance and sensitivity profile to commonly prescribed antibiotics, antimicrobial susceptibility testing (AST) was carried out for *S. Typhimurium* isolates. Kirby–Bauer disk diffusion method was used based on the recommendations of the Clinical and Laboratory Standards Institute (Clinical and Laboratory Standards Institute, 2024). This method is commonly employed for the characterization of the antimicrobial susceptibility profile of clinically important bacterial pathogens. The AST was performed in sterile laboratory conditions with fresh bacterial cultures. This test was created to evaluate the susceptibility of the isolated Salmonella strains to selected antibiotics and to find multidrug-resistant isolates related to biofilm producers and virulence-associated genes.

2.4. Amplification of 16S rRNA and adrA gene

The DNA of the isolates was extracted using the Genome Jet DNA Extraction Kit (Thermo). Primers for the 16S rRNA and adrA gene were first confirmed with Virtual PCR amplification (Kalendar et al., 2024). To identify the presence of genes (16S rRNA and adrA), primers specific for conserved regions were used and listed in Table 1. The PCR was set at 35 cycles with initial denaturation at 95 ^{o}C for 5 mins, denaturation at 95 ^{o}C for 30 secs, annealing at 61 ^{o}C for 30 secs, extension at 72 ^{o}C for 90 secs and final extension at 72 ^{o}C for 10 minutes. The amplicons were sequenced the phylogenetic analysis was

performed using Mega XI (Tamura et al., 2021). The reliability of the relationships inferred was tested by bootstrap analysis, the results expressed as bootstrap values (%).

Table 1
adrA gene primers and 16S rRNA primers used for PCR amplification

Primers	Primer sequence	Amplicon size (bp)
Forward primer --- *adrA*	ACCGAAAAGCGGTTGAACAG	1000
Reverse primer --- *adrA*	GGTTACGTCCGGCATTCTTT	1000
Forward primer 515	AGAGTTTGATCMTGGCTCAG	300
Reverse primer 806	TACGGYTACCTTGTTACGACTT	300

2.5. Protein Structure Prediction

Protein structure prediction was used in order to find the three-dimensional (3D) structural conformations of the *adrA* protein of *S. Typhimurium*. The sequences of the gene *adrA* were first translated into the corresponding amino acid sequences using the ExPASy Translate tool.

Three-dimensional structure prediction of a protein was done by the I-TASSER server (Yang et al., 2014). The server aligns the sequences multiple times with different types of threading and selects structural templates from the Protein Data Bank (PDB) and iteratively assembles the structure to predict protein tertiary structure.

The amino acid sequences of the *adrA* protein were each submitted to I-TASSER server for structural modeling. The server has created several structural templates, using homologous proteins in the PDB database, during the prediction process. Using the threading algorithms the proteins with structure that are structurally similar to the known proteins were identified and likely tertiary structure was built by iterative simulations of refinements.

A list of protein structure models provided by the server was returned for the protein sequences submitted: the models are provided with the confidence scores (C-Score, TM-Score, and estimated RMSD in Å). C-score values gave an estimated measure of the reliability of the model while TM-scores and RMSD values gave a measure of the similarity in the structure between the predicted models and the native proteins.

The best 3D-structural properties generated predicted proteins were selected for additional validation and docking. The resulting structures were downloaded in the Protein Data Bank (PDB) file format and analyzed further in BIOVIA Discovery Studio Visualizer (BIOVIA, 2005).

The structure validation was done by using UCLA-DOE LAB — SAVES v6.1 in which we run PROCHECK (Laskowski et al., 2012) and ERRAT which give quality factor and tells about how much atom are in most allowed regions.

2.6. Molecular Docking Analysis

Standard structure-based computation methods (Meng et al., 2011) were used to evaluate the interaction between proteins and ligands by molecular docking. Molecular docking analysis was done to understand the interaction of the predicted bacterial proteins with selected antibiotics.

Docking analysis was done using PyRx software. Interaction analysis of AdrA protein with selected antibiotics such as ampicillin, amoxicillin, azithromycin, cefotaxime, chloramphenicol, ciprofloxacin, and meropenem in the docking study. The 3D structures of the selected antibiotics were downloaded from the PubChem database in Protein Data Bank (PDB) format.

The I-TASSER predicted protein structures were prepared for docking by removing the water molecules from the structures, correcting the structural errors, adding missing hydrogen atoms, and optimizing the protein geometry. The pre-prepared

protein models were then displayed in the docking platform PyRx. Protein was selected as a macromolecule and converted into pdbqt format.

In docking simulations, the software produced several different conformations and orientations of the ligand in the binding sites of the target protein. The binding affinities of each protein–ligand complex were determined and reported in kilocalories per mole (kcal/mol). The docking scores were compared, and the antibiotics with better binding affinity towards the *AdrA* protein were identified. Those protein–ligand complexes with the best binding energies were chosen for in-depth analysis of the interactions between the proteins and their ligands.

Visualization and structure interpretation of docking interactions were done using BIOVIA Discovery Studio (BIOVIA, 2005). Hydrogen bond interactions and hydrophobic contacts between the antibiotics and the target proteins were carefully examined since they can play an important role in the stability of the interaction and in the inhibitory effect of the antibiotic.

The docking study provided the information regarding antibiotic interactions with the proteins involved in biofilm formation by Salmonella isolates, as well as information on the molecular-level antibiotic resistance behavior.

3. Results

3.1. Sample Collection, Isolation, and Identification

Bacterial growth was observed for all samples on selective culture media, and all samples had typical colony morphology indicative of *S. Typhimurium*. The isolates were pale, non-lactose fermenting on MacConkey agar. The presumptive isolates that were obtained by cultural characterization were subsequently biochemically tested for confirmation of *S. Typhimurium*.

3.2. Antimicrobial Susceptibility Testing (AST)

The isolates were found to show different resistance profiles, as shown in Table 2. The result presents the Clinical and Laboratory Standards Institute (Clinical and Laboratory Standards Institute, 2024) breakpoint zone diameter standards used for interpretation of antimicrobial susceptibility patterns of *S. Typhimurium* isolates. The inhibition zone diameter of the isolates HZA-1 to HZA-10 is shown in Table 3, and the same results were represented by the majority of the samples. The isolates were resistant to ampicillin, cefotaxime, and ciprofloxacin and sensitive to azithromycin and meropenem. The intermediate susceptibility was mostly seen to chloramphenicol and amoxicillin–clavulanic acid.

Table 2

The table presents the Clinical and Laboratory Standards Institute (CLSI) breakpoint zone diameter standards used for interpretation of antimicrobial susceptibility patterns of S. Typhimurium isolates. Antibiotic susceptibility was categorized as resistant (R), intermediate (I), or sensitive (S) based on the measured inhibition zone diameters around antibiotic discs on Mueller–Hinton agar. The tested antibiotics included chloramphenicol, azithromycin, ampicillin, cefotaxime, meropenem, ciprofloxacin, and amoxicillin–clavulanic acid. µg = Microgram, mm = Millimeter.

Antibiotic	Disk Potency (µg)	Resistant (R) (mm)	Intermediate (I) (mm)	Sensitive (S) (mm)
Chloramphenicol (C30)	30	≤ 12	13–17	≥ 18
Azithromycin (AZM15)	15	≤ 12	13–14	≥ 15
Ampicillin (AM10)	10	≤ 13	14–16	≥ 17
Cefotaxime (CTX30)	30	≤ 22	23–25	≥ 26
Meropenem (MEM10)	10	≤ 19	20–22	≥ 23
Ciprofloxacin (CIP5)	5	≤ 20	21–30	≥ 31
Amoxicillin–Clavulanic Acid (AMC30)	30	≤ 13	14–17	≥ 18

Table 3

The table presents the inhibition zone diameters and susceptibility interpretations of the ten prominent samples HZA-1 to HZA-10 against different antibiotics following CLSI guidelines. Antibiotic responses were categorized as resistant (R), intermediate (I), or sensitive (S) based on measured zone diameters (mm). The tested antibiotics included chloramphenicol, azithromycin, ampicillin, cefotaxime, meropenem, ciprofloxacin, and amoxicillin–clavulanic acid. NZ represents no zone.

Sample No.	Chloramphenicol (C30)	Azithromycin (AZM15)	Ampicillin (AM10)	Cefotaxime (CTX30)	Meropenem (MEM10)	Ciprofloxacin (CIP5)	Amoxicillin–Clavulanic Acid (AMC30)
HZA-1	14 (I)	16 (S)	09 (R)	NZ (R)	21 (S)	8 (R)	15 (I)
HZA-2	23 (S)	14 (S)	NZ (R)	21 (R)	26 (S)	11 (R)	NZ (R)
HZA-3	16 (I)	22 (S)	NZ (R)	NZ (R)	21 (S)	22 (I)	20 (S)
HZA-4	17 (I)	13 (S)	08 (R)	NZ (R)	23 (S)	NZ (R)	18 (I)
HZA-5	20 (S)	18 (S)	10 (R)	20 (R)	28 (S)	10 (R)	17 (I)
HZA-6	15 (I)	17 (S)	10 (R)	20 (R)	22 (S)	10 (R)	15 (I)
HZA-7	15 (I)	13 (S)	NZ (R)	NZ (R)	32 (S)	8 (R)	15 (I)
HZA-8	38 (S)	14 (S)	NZ (R)	18 (R)	30 (S)	30 (I)	16 (I)
HZA-9	14 (I)	13 (S)	10 (R)	NZ (R)	26 (S)	NZ (R)	18 (I)
HZA-10	16 (I)	17 (S)	10 (R)	NZ (R)	28 (S)	12 (R)	16 (I)

3.3. Congo Red Assay and Tube Biofilm Assay

Strong biofilm-forming ability is demonstrated by all the isolates. All the isolates that formed black crystalline colonies on Congo red agar. The dark black pigmentation observed in the colonies resulted from the interaction of Congo red dye with extracellular polysaccharides and curli fimbriae produced by the bacterial cells. Strong biofilm-producing isolates demonstrated intense black coloration with a dry crystalline appearance, which is considered a characteristic feature of exopolysaccharide synthesis and enhanced biofilm formation, as shown in Fig. 1. All tubes showed the formation of a visible violet film along the inner walls and the bottom of the tubes, which showed positive biofilm formation. Following incubation and crystal violet staining, visible violet-colored films were observed along the inner walls and bottom surfaces of the test tubes in isolates HZA-1 to HZA-4, indicating positive biofilm formation as shown in Fig. 2. The intensity of crystal violet retention reflected the degree of bacterial adherence and extracellular polymeric substance production. Strongly adherent isolates exhibited thick violet staining with dense surface attachment, demonstrating significant biofilm-producing ability. Similar biofilm formation patterns were observed among the majority of isolates analyzed in this study.

Biofilm-forming ability was observed, indicating that the mechanism of persistence and antimicrobial resistance could be associated with the formation of biofilm by *S. Typhimurium* isolates.

3.4. In Silico, In Vitro PCR and Phylogenetic Analysis

The computational PCR (*in silico* PCR) amplification results are obtained for the *adrA* gene for the specific forward and reverse primers and are shown in Fig. 3. Primers were found to have high specificity for the target gene sequences as they successfully amplified a 1059 bp DNA fragment from the genome of *Salmonella enterica subsp. Enterica* serovar Typhimurium DT104. No mismatches were observed with the primers, which indicated that the primers bind correctly and the selected genomic region was amplified efficiently.

The sequence amplified has a GC content of 52.1%, which is indicative of moderate stability of the nucleotides and good primer annealing characteristics for PCR amplification. The amplified segment was found to be at position 476273 of the

bacterial genome of about 4,933,631 bp. The successful in silico amplification confirmed the primer set to be used for molecular detection of the *adrA* gene and recommended its use in experimental PCR analysis of *S. Typhimurium* isolates.

For *in vitro* PCR, visible bands were obtained near the wells in the figure, showing successful extraction of DNA. Amplification of 16S rRNA of extracted DNA by universal primers 515F and 806R produced the band of about 300 bp of two isolates (HZA-1 to HZA-6). Approximate sizes of amplicons of amplified DNA fragments of HZA-1 to HZA-6 were observed on the gel shown in Fig. 3. As clear bands on the gel were seen, which indicated the specific binding of the primer to elongate DNA along with no dimer formation. The presence of clear and specific PCR bands indicated successful molecular confirmation of the isolates as *S. Typhimurium*. The amplified fragments appeared within the expected base pair range when compared with the molecular marker. The successful amplification of the 16S rRNA gene further validated the biochemical and cultural identification results of the bacterial isolates.

The phylogenetic analysis based on the 16S rRNA gene showed that the isolates HZA-1 to HZA-6 were members of *Salmonella enterica*. The isolate belonged to the *Salmonella enterica* clade with close evolutionary relatedness to the *Salmonella enterica subsp. Enterica* serovar Typhimurium reference strains, and different strains of the other subspecies: arizonae, indica, houtenae and salamae. The parsimony of the phylogenetic relationships was supported by bootstrap values of at least 90% and up to 99%. The sequenced datasets generated during the current study are available in the NCBI repository with the accession numbers PZ688175 to PZ688180.

Polymerase chain reaction (PCR) was used to detect genes associated with the presence of biofilm and multidrug resistance in the samples. Genomic DNA from confirmed *S. Typhimurium* isolates was amplified with specific primers for the genes *adrA*.

For adrA gene, after PCR amplification, the gel electrophoresis showed band of approximately ~ 1000 bp corresponding to the expected amplicon sizes when gel is visualized under UV illumination as shown in Fig. 4. Amplified products indicated that the target genes were there in the tested isolates.

The amplified products were seen as clear sharp bands without much of the nonspecific amplification reflecting efficiency and specificity of the PCR reaction. The presence of the *adrA* gene suggests its involvement in cyclic di-GMP signaling and regulation of biofilm formation in *S. Typhimurium*. .

3.5. Protein Structure Prediction

The results show the 6 open reading frames (ORF). The amino acid sequences of the genes detected were used to predict the three-dimensional (3D) proteins structure of *AdrA* proteins using the I-TASSER server. The models of proteins were selected based on highest confidence score and stability. The predicted protein model showed a very clearly defined tertiary structure, with the majority of it consisting of α-helices (red), and there were also several β-sheets (cyan), along with connecting loop regions (green and gray) as shown in Fig. 5. The high proportion of α-helical domains implies that the protein structure is stable, thus typical of signaling and regulatory proteins associated with the cellular processes of bacteria.

3.6. PROCHECK results

The Ramachandran plot obtained from PROCHECK is used to assess the stereochemical quality of the *AdrA* protein model, based on the backbone dihedral angles (φ and ψ) of the amino acid residues. The plot reveals that 205 residues (out of 208) (98.6%) are in the most favored regions and 3 residues (1.4%) are in other allowed regions, which are not glycine and proline residues, as shown in Fig. 6. There are no residues in the allowed or disallowed, regions. The results show that the *AdrA* model has a backbone geometry of excellent quality and a stereochemical quality that is reliable, as high-quality protein structures usually have over 90% of the residues in the most favored regions.

3.7 ERRAT results

The non-bonded atomic interactions in the *AdrA* protein model were evaluated using ERRAT analysis as shown in Fig. 7. The overall quality factor obtained at the structure was 96.476, much higher than the generally accepted value for good quality

protein models. The error values are low, with a few regions towards the C-terminal end with slightly higher error values but still acceptable. A high ERRAT score indicates good atomic interactions and a high level of structural accuracy of the protein model.

3.8. Molecular Docking Analysis of the adrA Protein with Selected Antibiotics

Molecular docking was carried out with seven widely used antibiotics and compared with their phenotypic antibiotic susceptibility profiles to assess the possible interaction of *AdrA* with antibiotic molecules, as shown in Table 4. Docking analysis showed that the binding affinity of all tested antibiotics towards the AdrA protein was moderate, with binding energies ranging from − 4.8 to − 5.5 kcal/mol. Ampicillin had the highest binding affinity of − 5.5 kcal/mol, followed by ciprofloxacin (− 5.4 kcal/mol), cefotaxime (− 5.3 kcal/mol), amoxicillin/clavulanic acid (− 5.2 kcal/mol), azithromycin (− 5.1 kcal/mol), meropenem (− 5.0 kcal/mol), and chloramphenicol (− 4.8 kcal/mol).

Table 4

Molecular docking analysis of the AdrA protein with seven antibiotics. Binding affinities (kcal/mol) obtained from AutoDock Vina indicate moderate interactions between AdrA and the tested antibiotics, with ampicillin showing the highest binding.

Macromolecule	Ligand	Binding Affinity	rmsd/ub	rmsd/lb
adrA Protein	Ciprofloxacin	-5.4	0	0
adrA Protein	Amoxicillin	-5.2	0	0
adrA Protein	Meropenem	-5	0	0
adrA Protein	Cefotaxime	-5.3	0	0
adrA Protein	Chloramphenicol	-4.8	0	0
adrA Protein	Azythromycin	-5.1	0	0
adrA Protein	Ampicillin	-5.5	0	0

A different trend was observed by phenotypic susceptibility testing. The isolates were uniformly susceptible to meropenem and azithromycin, but exhibited high levels of resistance to ampicillin, cefotaxime, and ciprofloxacin. Frequent intermediate susceptibility was seen to the drugs chloramphenicol and amoxicillin–clavulanic acid.

Data comparisons between docking and susceptibility revealed that the antibiotic effectiveness was not directly related to binding affinity. For instance, ampicillin and ciprofloxacin had some of the highest binding energies to *AdrA* and were also highly phenotypically resistant for the isolates. In contrast, meropenem showed only moderate binding affinity (− 5.0 kcal/mol) but 100% phenotypic susceptibility. These results indicate that *AdrA* has the potential to interact with a variety of antibiotics; however, there are other mechanisms of resistance that affect susceptibility of the bacteria including efflux pumps, membrane permeability, target-site alterations and biofilm-associated resistance. Thus, docking outcomes should be considered as an indicator of the possibility of molecular interaction instead of direct prediction of antimicrobial susceptibility.

4. Discussion

The findings of the current investigation are highly comparable with the recent reports in central Pakistan, where MDR and XDR have shown increased prevalence. The isolates exhibited multidrug resistance against commonly used antibiotics, i.e., ampicillin, cefotaxime, and ciprofloxacin, while retaining susceptibility to azithromycin and meropenem. These β-lactams and fluoroquinolone antibiotics have long served as the cornerstone for treating salmonellosis, and the resistance against them is becoming more and more prevalent (Eng et al., 2015; Li et al., 2017). Previously, such resistance profiles of MDR and XDR *S.*

Typhimurium have been observed and strongly support our findings (Zakir et al., 2021). Similarly, established widespread dissemination of resistance factors among clinical Salmonella isolates, while (Fasih et al., 2023) reported high levels of antimicrobial resistance among Salmonella strains recovered from retail meat. Results of antimicrobial susceptibility testing (AST) showed that a high proportion of the Salmonella isolates were resistant to ampicillin, cefotaxime, and ciprofloxacin. Amoxicillin-clavulanic acid (Augmentin) and chloramphenicol were frequently intermediate, while there was no resistance to azithromycin and meropenem. The results of these studies suggest the occurrence of multidrug-resistant phenotypes among the isolates tested, especially for commonly prescribed β-lactam antibiotics and the fluoroquinolones. Collectively, these studies signify that antimicrobial-resistant Salmonella is no longer constrained to hospital environments and may prevail between human, food, and environmental reservoirs.

An important finding of the current investigation was the comprehensive susceptibility of isolates to meropenem and azithromycin. Comparable interpretations were suggested by (Jabeen et al., 2023), where carbapenems remained vastly effective against XDR Salmonella isolates. The conserved activity of meropenem imitates its restricted clinical use associated with previous antimicrobial classes. However, increasing dependence on azithromycin and carbapenems raises concerns about the progression of resistance, stressing the importance of antimicrobial stewardship programs (Jabeen et al., 2023; Zakir et al., 2021).

The antimicrobial resistance patterns observed in the present study are consistent with reports from neighboring Asian countries. In India, multidrug-resistant *Salmonella enterica* serovar Typhimurium isolates exhibiting resistance to ampicillin, cefotaxime, and ciprofloxacin have been increasingly reported from both clinical and food sources (Dutta et al., 2014; Dahiya et al., 2023). Similarly, studies conducted in China demonstrated widespread dissemination of MDR *Salmonella* isolates carrying resistance determinants against β-lactams and fluoroquinolones, with resistance profiles closely resembling those observed in the current investigation (Li et al., 2017; Jing et al., 2021). These findings suggest that antimicrobial resistance among *Salmonella* has become a major concern throughout Asia.

Comparable findings have also been reported in Middle Eastern countries. In Saudi Arabia, *Salmonella* isolates from clinical and food sources exhibited high resistance rates to ampicillin and fluoroquinolones while retaining susceptibility to carbapenems, highlighting the continued effectiveness of last-resort antibiotics (Alhumaidan, 2024; El-Kholy et al., 2020). Likewise, Egyptian studies have reported a high prevalence of MDR *Salmonella* isolates recovered from poultry and food products, with resistance predominantly directed against β-lactam antibiotics and quinolones (Abd-Elghany et al., 2022; Moawad et al., 2017). The similarities between these reports and our findings suggest a regional dissemination of MDR *Salmonella* strains across the Middle East and North Africa.

Similar resistance trends have been observed in Europe despite the implementation of stringent antimicrobial stewardship policies. Surveillance reports from the European Food Safety Authority (EFSA) have documented increasing resistance among non-typhoidal *Salmonella* isolates to ampicillin and fluoroquinolones, particularly those originating from poultry and food-animal reservoirs (EFSA, 2023). Studies from Portugal, Spain, and other European countries have also demonstrated the persistence of MDR *Salmonella* strains associated with food production systems (Antunes et al., 2016). However, carbapenem resistance remains uncommon in Europe, which is consistent with the complete susceptibility to meropenem observed in the present study.

Biofilm formation characterizes one of the most significant adaptive mechanisms employed by Salmonella species. In the current study, the majority of the isolates displayed strong biofilm-forming capability in Congo red agar as well as tube biofilm assays. Previous studies have verified the facilitation of biofilm persistence in increased tolerance to environmental stress and reduced susceptibility to antimicrobial agents (Costerton J.W et al., 1999; Flemming & Wingender, 2010; Hall & Mah, 2017).

Phenotypic and genotypic data showed that the isolates that showed resistance to multiple antibiotics also had some of the key genes to regulate the formation of biofilms. The presence of *adrA* does not necessarily lead to antibiotic resistance, but it may be involved in lowering antimicrobial susceptibility by favoring the formation of biofilms that allow less penetration of

antimicrobial agents and enhance bacterial persistence. The presence of multidrug-resistant genes and genes related to biofilms implies that the ability to form biofilms could be significant in the survival and adaptation of the isolates to antimicrobial pressure.

The PCR-based detection of the *AdrA* gene amongst all under consideration isolates was important, since *AdrA* functions as a diguanylate cyclase involved in cyclic-di-GMP signaling pathways that regulate extracellular matrix production, cellulose biosynthesis, and biofilm maturation (Ahmad et al., 2017). PCR analysis revealed that 100% of the isolates contained the biofilm gene *adrA.* Previous studies have confirmed that *AdrA*-mediated cyclic-di-GMP production shows a crucial role in the development of the rdar morphotype and long-term bacterial persistence (Ahmad et al., 2017). Therefore, the detection of *AdrA* in every isolate suggests a strong genetic basis for biofilm formation among the studied Salmonella population. The synchronicity of multidrug resistance and biofilm-associated genetic determinants detected in this study supports the hypothesis that biofilm formation contributes indirectly to antimicrobial resistance (Alhumaidan, 2024).

The universal detection of the *adrA* gene in the present study is also consistent with international findings. Studies from China, Sweden, and Germany have demonstrated that *adrA* is highly conserved among biofilm-forming *Salmonella* isolates and plays a central role in cyclic-di-GMP-mediated regulation of cellulose production and rdar biofilm development (Ahmad et al., 2017; Römling et al., 1998). Furthermore, European investigations have reported significant associations between biofilm-associated genes and enhanced bacterial persistence under antimicrobial stress conditions (Vestby et al., 2020). These observations support the notion that biofilm-mediated persistence represents a globally conserved adaptive mechanism in *Salmonella*.

The bioinformatics module of the investigation provided supplementary insights into the predicted biological role of *AdrA*. The predicted protein model suggested structural stability, as demonstrated by favorable PROCHECK and ERRAT scores. Current advances in computational structural biology have significantly developed the accuracy of protein structure prediction, enabling the identification of functional domains, ligand-binding sites, and molecular interaction networks (Jumper et al., 2021). The predominance of α-helical regions observed in the predicted *AdrA* structure is consistent with the architecture of signaling and regulatory proteins involved in bacterial adaptation processes.

From a public health perspective, the findings of this study are highly relevant. Pakistan continues to face a substantial burden of antimicrobial-resistant Salmonella infections, particularly in urban centers such as Lahore (Jabeen et al., 2023; Zakir et al., 2021). The identification of MDR, biofilm-forming *S. Typhimurium* strains carrying *adrA* suggests the existence of highly persistent bacterial populations capable of surviving antimicrobial treatment and environmental stresses. The continued emergence and dissemination of such strains may compromise therapeutic options and increase healthcare costs and disease burden.

The present study possesses several strengths, including the integration of phenotypic antimicrobial susceptibility testing, molecular characterization, phylogenetic analysis, protein modeling, and molecular docking approaches. This multidisciplinary strategy enabled a more comprehensive understanding of the relationship between antimicrobial resistance, biofilm formation, and bacterial persistence. Nevertheless, future studies should include larger sample sizes, quantitative biofilm assays, transcriptomic analyses, and investigation of additional biofilm-associated genes such as *csgD*, *bcsA*, and *bapA* to further elucidate the molecular mechanisms underlying MDR phenotypes.

Collectively, the present findings are in agreement with reports from Pakistan, India, China, Saudi Arabia, Egypt, and Europe, demonstrating that multidrug resistance and biofilm-associated persistence in *S. Typhimurium* represent a global public health challenge rather than a geographically restricted phenomenon. The widespread occurrence of MDR biofilm-forming strains across diverse regions underscores the urgent need for coordinated international surveillance programs, antimicrobial stewardship initiatives, and the development of novel anti-biofilm therapeutic strategies.

Overall, the present findings demonstrate that MDR *S. Typhimurium* isolates circulating in Lahore possess strong biofilm-forming capabilities and universally harbor the *adrA* gene. The combined phenotypic, molecular, and computational evidence

supports the hypothesis that cyclic-di-GMP-mediated biofilm regulation contributes to bacterial persistence and may indirectly facilitate antimicrobial resistance. These findings highlight the urgent need for continuous surveillance, rational antibiotic use, and the development of novel anti-biofilm therapeutic strategies to combat multidrug-resistant Salmonella infections.

5. Conclusion

The current study encompasses multidrug-resistant (MDR) *S. Typhimurium* isolates, the role of second messenger signaling gene adrA in biofilm formation, and the development of antimicrobial resistance in *S. Typhimurium*. Phenotypically, all strains were found to be biofilm producers as well as MDR, exhibiting high-level resistance to ampicillin, cefotaxime, and ciprofloxacin, and low-level resistance to azithromycin and meropenem. The isolates were molecularly characterized and were found to contain the gene adrA, which is associated with biofilm development and bacterial persistence. The results demonstrated that *adrA* gene is highly conserved among all Salmonella strains examined, indicating its importance in bacterial adaptation and survival. Furthermore, molecular docking studies confirmed the binding interactions of selected antimicrobial agents with the signaling proteins. Comparison of docking results with phenotypic resistance profiles revealed that antimicrobial resistance is a complex phenomenon involving factors beyond protein–ligand interactions. Overall, the findings suggest that second-messenger signaling pathways are involved in biofilm formation and in the survival of MDR *S. Typhimurium*, with particular importance attributed to cyclic di-GMP regulation. The integration of microbiological, molecular, and computational approaches in this study provided a comprehensive understanding of bacterial persistence and antimicrobial resistance mechanisms.

Declarations

Ethics approval and consent to participate:

We authors declare that we give our consent to publish this article and give ethical approval for this submission. The manuscript constitutes original research work and has never been submitted elsewhere, either completely, in part, or in another form for publication. The current study included human clinical samples and ethical approval was obtained from the Ethical Review Committee of the University of Lahore, Pakistan (Ref-IMBB/BBBC/24/648-B). All study procedures were performed in accordance with the ethical principles of the Declaration of Helsinki and relevant institutional guidelines and regulations. Written informed consent was obtained from all adult participants before their inclusion in the study. For participants under 18 years of age, written informed consent was obtained from a parent or legal guardian, and all required ethical approvals were secured where applicable. Participant confidentiality was maintained throughout the study. Clinical, microbiological, and genomic data were anonymized before analysis and reporting, and no personally identifiable information is included in this manuscript. All data were collected and used solely for research purposes in compliance with applicable ethical and data protection requirements.

Consent for publication

Not Applicable

Conflict of Interests

We authors also declare that we have no conflict of interest.

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Author Contribution

H.R.H conceived the study, performed the experiments, collected and analyzed the data, and drafted the manuscript. D.I contributed to the molecular and bioinformatics analyses, including 16S rRNA sequencing, phylogenetic analysis, and molecular docking. A.M assisted with laboratory experiments, data interpretation, and manuscript revision. M.G.B contributed to the experimental design, statistical analysis, and critical review of the manuscript. S.Z.H.N supervised the study, secured resources, validated the findings, and critically revised the manuscript. A.S contributed to the study design, interpretation of the results, manuscript editing, and overall scientific review. All authors read and approved the final version of the manuscript.

Data Availability

The datasets used and/or analyzed during the current study are included. The sequenced datasets generated during the current study are available in the NCBI repository with the accession numbers PZ688175 to PZ688180.

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Figures

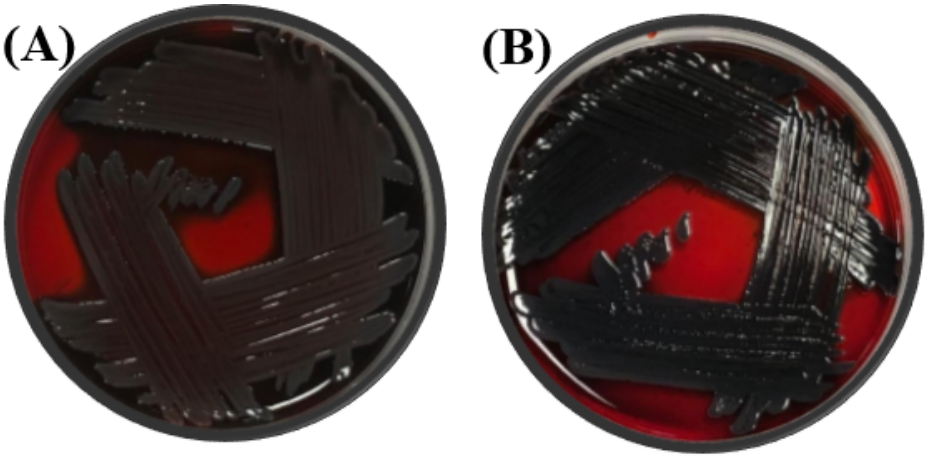


Figure 1

The figure illustrates the results of the Congo red agar assay performed to evaluate the biofilm-forming ability of S. Typhimurium isolates. Representative isolates HZA-1 (A), and HZA-2 (B) exhibited black crystalline morphology on Congo red agar plates, indicating strong biofilm production. Similar biofilm-producing patterns were observed among the majority of isolates examined in this study.

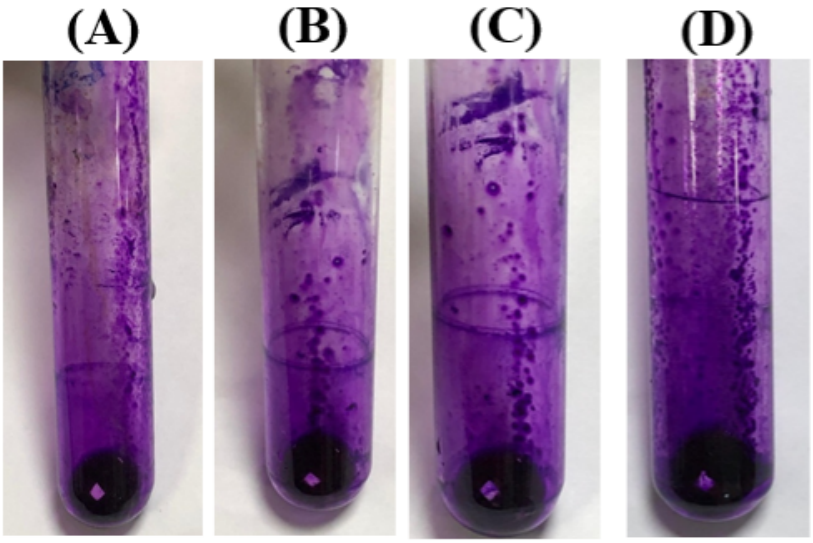


Figure 2

The figure presents the results of the tube biofilm assay performed to evaluate the adherence and biofilm-forming capability of representative S. Typhimurium isolates.

The results confirmed that the tested (HZA-1 to HZA-4) isolates possessed strong biofilm-forming potential, which may contribute to enhanced environmental persistence, bacterial survival, and increased resistance to antimicrobial agents.

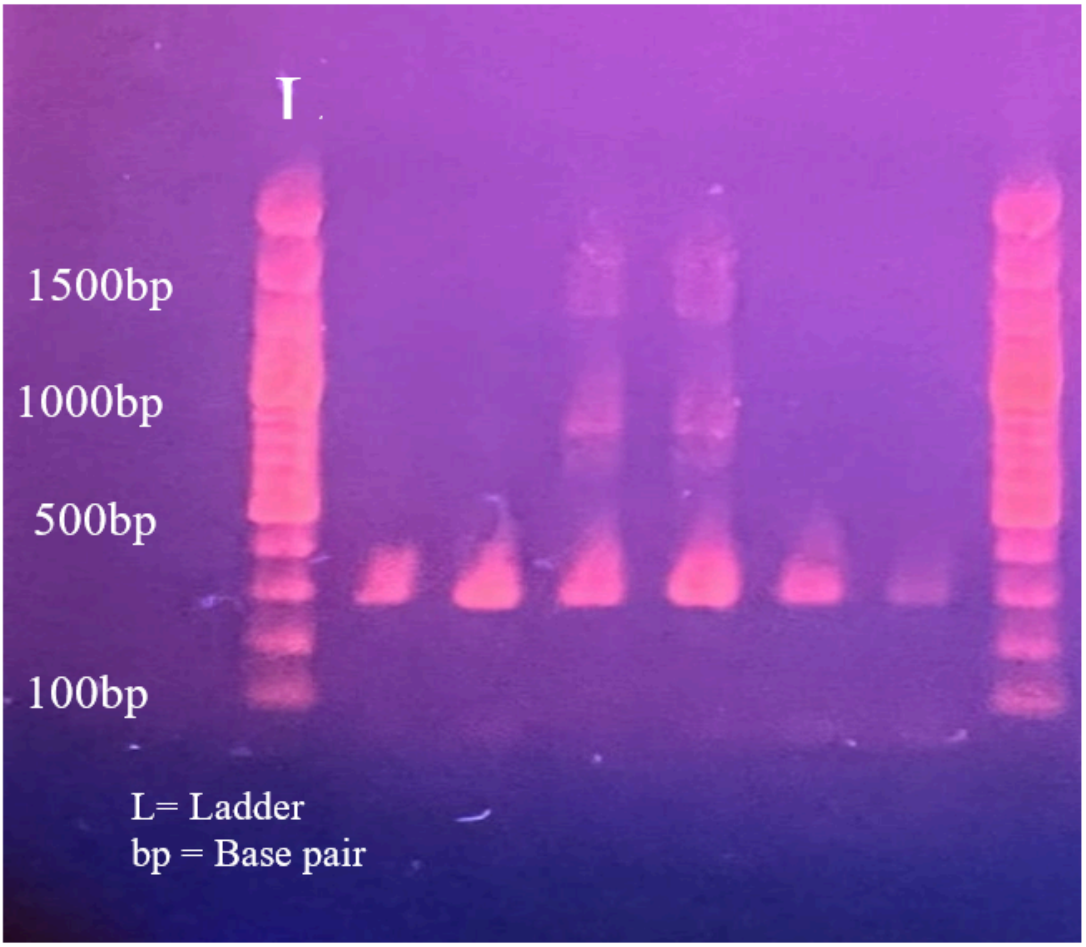


Figure 3

The figure shows agarose gel electrophoresis analysis of PCR-amplified 16S rRNA gene fragments obtained from representative HZA-1 to HZA-6 isolates.

The lanes labeled “L” contain DNA ladder (100bp) used as molecular size markers for estimation of PCR product size. Distinct amplified DNA bands of ~300 bp were observed in the gel.

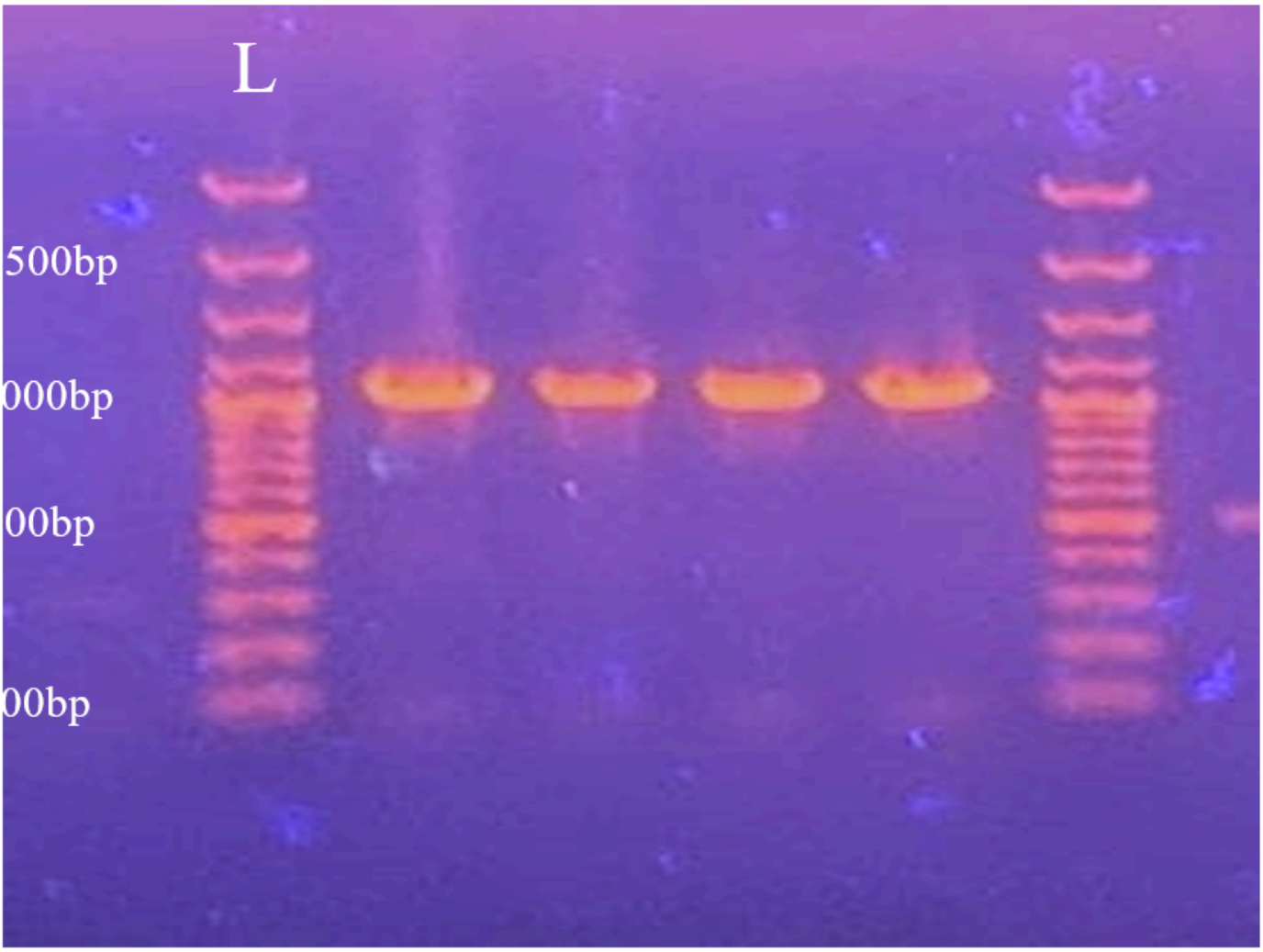


Figure 4

The figure presents the PCR amplification results of the adrA gene of ~1000bp amplicon obtained from representative S. Typhimurium isolates.

The lanes labeled “L” contain DNA ladder (100bp). Distinct amplicons were observed HZA-1 to HZA-4 at the expected amplicon size region.



Figure 5

Three-dimensional predicted structure of AdrA protein showing α-helices (red), β-sheets (cyan), and loop regions (green/gray) involved in maintaining structural stability and regulatory function in c-di-GMP-mediated biofilm formation.

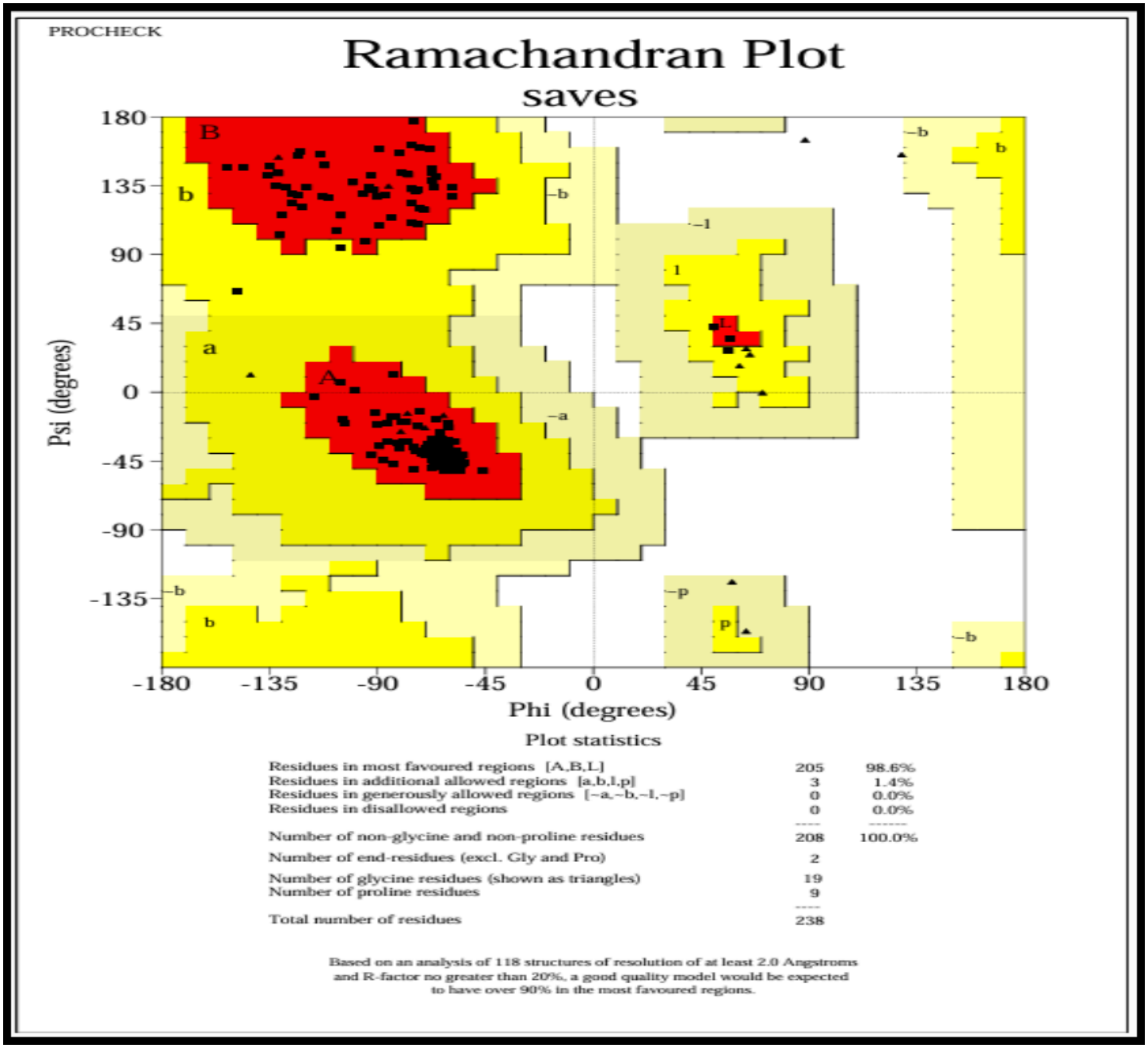


Figure 6

Ramachandran plot analysis of the AdrA protein structure generated using PROCHECK. A total of 98.6% of residues are located in the most favored regions, with the remaining 1.4% in additionally allowed regions and no residues in disallowed regions, indicating excellent stereochemical quality and structural reliability.

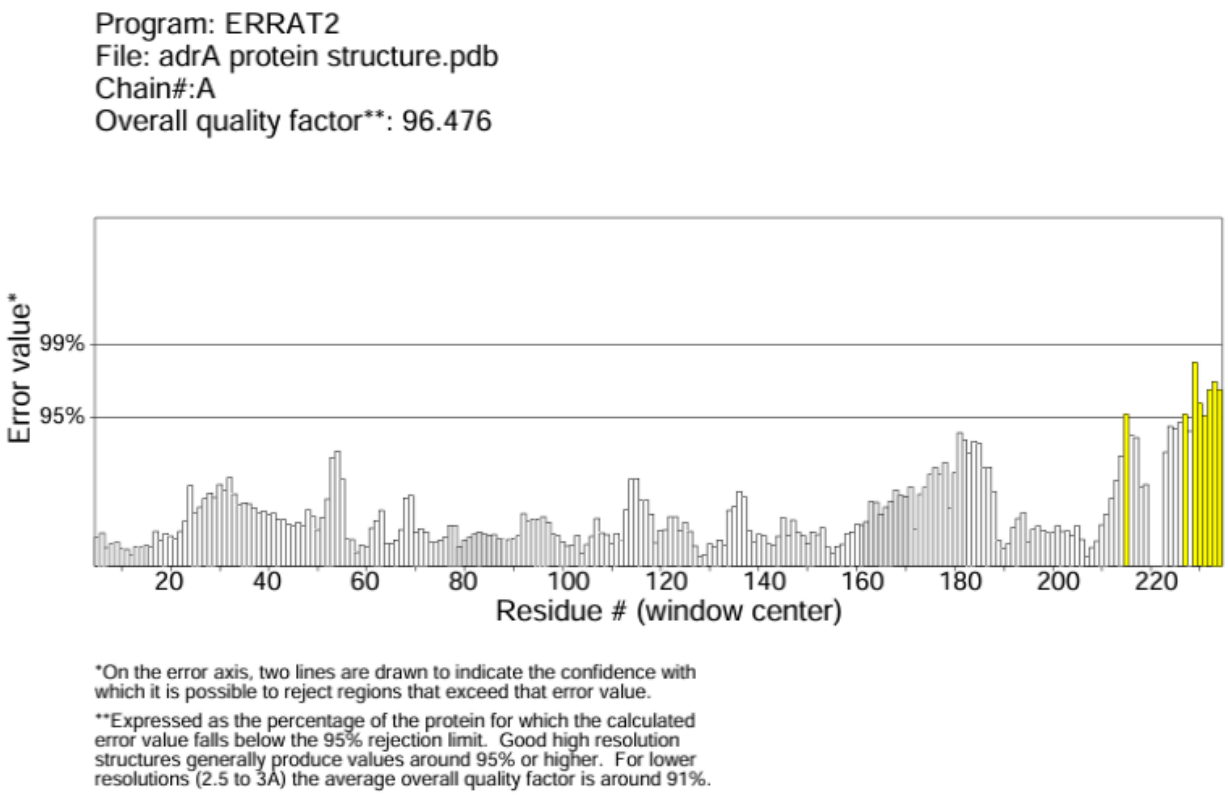


Figure 7

ERRAT validation plot of the AdrA protein structure showing an overall quality factor of 96.476.

The high-quality score and generally low error values across the sequence indicate good non-bonded atomic interactions and confirm the reliability