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Original Article

Analysis of chromosomal genes and proteins of gyrA and gyrB from Indonesian Local-Strain *Salmonella enterica* serovar Typhi

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Abstract

Introduction: Typhoid fever is an infection of the gastrointestinal tract caused by *Salmonella* Typhi. Ciprofloxacin is the most widely used second-line therapy; it provides good results in therapy. Indonesia has a unique resistance pattern of fluoroquinolones. Earlier studies of the *gyrA* and *gyrB* from local *S.* Typhi strains showed no mutation, and amino acid replacement was identified in all reported codons. In this study, we explored the whole sequence of the *gyrA* and *gyrB* further to understand the association of the unique characteristics of our local strains with fluoroquinolone resistance.

Methodology: This was an analytical study with a cross-sectional approach. Isolates collection, identification, antibiotic sensitivity test, DNA extraction, gene amplification, purification, and sequencing were carried out. Bioedit was used for the analysis of sequencing data.

Results: Four isolates were identified as *S.* Typhi. Three isolates were sensitive to nalidixic acid, ciprofloxacin, levofloxacin, and moxifloxacin. One isolate was intermediate to nalidixic acid, ciprofloxacin, levofloxacin, and resistant to moxifloxacin. The *gyrA* and *gyrB* genes were aligned with *S.* Typhi Ty2 reference sequence (NCBI GenBank AE014613.1). Three amino acid changes (Gly133Glu, Asn538Asp, and Thr856Ala) and one amino acid change (Ala416Ser) were found in *gyrA* and *gyrB*, respectively. Protein secondary structures of these isolates showed some changes in alpha helices, beta sheets, random coils, and beta turns, which could result in alterations of the properties of proteins.

Conclusions: Some variations and protein secondary structure alterations were found in the *gyrA* and *gyrB* among local strains of *S.* Typhi, which might be associated with fluoroquinolone susceptibility.

Key words: *Salmonella* Typhi; fluoroquinolone resistance; variation; *gyrA*; *gyrB*; quinolone resistance determining regions (QRDRs).

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Introduction

Typhoid fever is a gastrointestinal infection caused by *Salmonella enterica* serovar Typhi. This type of infection can lead to some serious complications and even death. The World Health Organization (WHO) in 2018 stated that typhoid fever was one of the global health problems, with the number of 11-20 million cases per annum and a mortality rate of 128,000-161,000 cases each year if patients were left without adequate treatment [1].

Treatment with antibiotics is required to cure typhoid fever. The first-line antibiotics for treating typhoid fever were ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole. However, the therapy triggered the emergence of multidrug-resistant *S.* Typhi. Later, fluoroquinolones, cephalosporins, and azithromycin, as the second-line antibiotics, were then used in most cases due to their promising effect in treating typhoid fever [2]. In 2019, the CDC reported that the number of ciprofloxacin-resistant *S.*Typhi around 74% [3]. During the implementation of antibiotic therapy, some *S.* Typhi have evolved into Multi-Drug-Resistant (MDR) strains and Extensively Drug-Resistant (XDR) strains, which have been resistant to all recommended antibiotics except macrolide and carbapenem. The XDR *S.* Typhi strain has been spreading in Pakistan since 2016 and has infected more than 5,000 people [3]. In 2017, the WHO published the global pathogens priority list in which XDR *S.* Typhi has been categorized as a high-priority group [4].

The resistance of *S.* Typhi strain to fluoroquinolones has been reported in several countries, such as China [5], Kuwait [6], Vietnam [7], Bangladesh

[8], and Ghana [9]. Meanwhile, there has been a particular resistance pattern being reported in Indonesia. Most of the *S.* Typhi strains in Jakarta and the Tangerang area were still sensitive to first-line antibiotics and fluoroquinolone. The decline in sensitivity of *S.* Typhi to the first-line antibiotics and fluoroquinolone has been reported in Surabaya [10]. According to Marchello *et al.* 2020, the data of fluoroquinolone resistance *S.* Typhi in Indonesia remains low compared to the *S.* Typhi antimicrobial-resistant data in Asia, in Africa, and worldwide [11]. The data from some countries in the Pacific regions also conferred a small number of fluoroquinolone resistance *S.* Typhi [12], which is closely similar to the data collected from Indonesia. These distinct resistance patterns were likely induced by strains of *S.* Typhi in different regions with certain resistance mechanism genes [10].

Moehario *et al.* 2001 [10], 2009 [13], and 2019 [14] explored the antibiotic susceptibility of *S.* Typhi in Indonesia against several first-line antibiotics and fluoroquinolones. The results showed that all the isolates were sensitive to all tested antibiotics, including ciprofloxacin, ofloxacin, levofloxacin, and nalidixic acid. These susceptibility patterns remained unchanged in the last 10 years. Lugito dan Cucunawangsih in 2017 showed nearly identical results, which were a low resistance number of *S.* Typhi to the first-line antibiotics and fluoroquinolones in Rumah Sakit Umum Karawaci, Tangerang [15].

There are two mechanisms of fluoroquinolone resistance in *S.* Typhi. Firstly, it occurs through the mutation of Quinolone Resistance Determining Regions (QRDRs), which consist of *gyrA, gyrB, parC,* and *parE* genes; secondly, via the Plasmid Mediated Quinolone Resistance (PMQR) genes mutation [16].

Yanagi *et al.* (2009) reported that 8 out of 17 *S.* Typhi isolates obtained from Surabaya and its surroundings showed resistance to nalidixic acid. Moreover, some codon mutations were detected in all isolates with lower sensitivity. The mutations in *gyrA* were found in codon 87 (GAC → TAC), but no mutation was identified in codon 83 [17]. Nathania *et al.* (2022) have conducted research utilizing 28 local *S.* Typhi isolates, with a particular focus on hotspot regions *gyr*A, *gyr*B, *par*C, and *par*E. The hotspot region is a region of the protein sequence that is known to be particularly susceptible to mutation. This study revealed a novel result that none of the 28 *S.* Typhi isolates obtained from Jakarta were resistant to fluoroquinolones. Based on the result of AST to nalidixic acid, levofloxacin, and ciprofloxacin, only 1 isolate was intermediate to ciprofloxacin. A point mutation in *gyrA, gyrB, parC,* and *parE* was not identified in all isolates, as well as the isolate that is an intermediate to ciprofloxacin. All the nucleotide and amino acid sequences were identical to the reference sequence *S.* Typhi Ty2 (NCBI GenBank AE014613.1) [18].

Due to the different resistance patterns of *S.* Typhi in Indonesia, further research could explore any variations or mutations in the *gyrA* and *gyrB* genes, which have the potential to be associated with unique patterns of susceptibility.

Methodology

Sample collection and bacterial identification

This study analyzed a total of four *S.* Typhi isolates from 2019 to 2022. One isolate was obtained from the Laboratory of Microbiology, School of Medicine and Health Sciences, Atma Jaya Catholic University of Indonesia. Prior research has indicated that this isolate exhibits intermediate resistance to ciprofloxacin. A comprehensive gene analysis was conducted to ascertain the presence of any additional variations in the gyrA and gyrB genes. Three additional isolates were obtained from other medical facilities in Jakarta. All isolates were subjected to reidentification through the implementation of standard microbiological procedures. The isolates were inoculated on Nutrient Agar (NA) and Blood Agar (BA). The subsequent step was to identify the isolates using *Salmonella Shigella* agar (SS), Xylose Lysine Deoxycholate agar (XLD), and Triple Sugar Iron agar (TSIA). The positive results of the *S.*Typhi isolates on XLD and SS agar were characterized by the presence of a black-centered colony. The presence of a red slant, a yellow butt, and black precipitate on TSIA were indicative of the *S.* Typhi isolates. All inoculated agars plates were incubated at 35°C for 24 hours. Further characterization was performed using Gram staining, which revealed red or pink color bacteria. Serology tests were carried out using the slide agglutination method with commercial antiserum O9 and O12 (Difco®). The presence of *S.* Typhi was confirmed with positive agglutination of both antiserums.

Antibiotic susceptibility test

The Kirby–Bauer or disk diffusion method was utilized to perform the antibiotic susceptibility test on Mueller-Hinton agar (MHA). This was conducted in accordance with the standard set out by the Clinical and Laboratory Standards Institute (CLSI) M100. In this study, a range of antibiotics were employed, including

ciprofloxacin (5 µg), nalidixic acid (30 µg), levofloxacin (5 µg), and moxifloxacin (5 µg). The positive and negative controls were Escherichia coli ATCC 25922 and a blank disc, respectively. The results of the zones of inhibition were compared with the guidelines from the Clinical and Laboratory Standards Institute (CLSI) [19] and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [20].

DNA extraction

The genomic DNA was extracted using QIAamp DNA mini Kit (Qiagen®) from the NA slant culture as instructed in the kit. The extracted DNA was then confirmed by electrophoresis in 1% agarose gel with 2 µL of FloroSafe DNA Stain (1st Base®) at 100 V for 60 minutes. Five µL of extracted DNA with 1 µL of loading buffer was loaded onto the gel. The marker used in this electrophoresis was PhiX174 HaeIII. The expected size of the *S.*Typhi genome was 4,768,352 bp. The entire extracted DNA was stored at -20 ºC.

Amplification of gyrA and gyrB

The genes of *gyrA* and *gyrB* were amplified with the polymerase chain reaction (PCR) conditions in Table 1 using the primers as shown in Table 2. PCR amplification was performed using Taq PCR Master Mix Kit (Qiagen®). The primers were designed based on the reference sequence *Salmonella enterica subsp. enterica* Typhi Ty2 from GenBank AE014613.1.

DNA sequencing

The DNA sequences were obtained by using the Sanger sequencing method [17]. The designs of the internal primers are shown in Table 2. The sequencing results were analyzed using Bioedit and aligned with the reference sequence *S.* Typhi Ty2 from GenBank AE014613.1.

Table 1. PCR condition for amplifying *gyr*A and *gyr*B.

Step	Temperature (ºC)	Time
Initial Denaturation	94	3 minutes
Denaturation	94	1 minute
Annealing	60[a], 56[b]	30 seconds
Extension	72	2.5 minutes
Cycle	35x	
Final Extension	72	10 minutes
Hold	4	∞

[a] Annealing temperature for amplifying *gyr*A. [b] Annealing temperature for amplifying *gyr*B.

Homology modeling and protein analysis

The secondary and tertiary protein structures were visualized using the Network Protein Sequence @nalysis/NPS@ and SWISS-MODEL, respectively.

Ethics approval of research

This study was conducted in the Laboratory of Microbiology and Biomolecular, School of Medicine and Health Sciences, Atma Jaya Catholic University of Indonesia, and approved by the Ethical Committee of the School of Medicine and Health Science, Atma Jaya Catholic University of Indonesia, with ethical approval numbers: 08/03/ KEP-FKIKUAJ/2021 and 9/08/KEP-FKIKUAJ/2021.

Results

Isolate identification

A total of 4 isolates were collected and reidentified, which were AW1, AW2, AW3, and AW4. AW1 was obtained from the study conducted by Nathania *et al.* 2022 [18] and was employed as an internal control, with the relevant accession numbers being ON220762 (*gyrA*) and ON220790 (*gyrB*).

Antibiotic susceptibility profile

AW1 exhibited sensitivity to nalidixic acid, levofloxacin, moxifloxacin, and ciprofloxacin. AW2 isolate demonstrated sensitivity to all four antibiotics.

Table 2. PCR Primer and internal primer sequences of *gyr*A and *gyr*B.

Gene		Size (bp)	Sequences	Base Position
PCR primer				
*gyr*A	F	2.637	5'- TTG CGA CCT TTG AAT CCG GG -3'	
	R		5'- GGC CCT CGC ACA GCA ATA A -3'	
*gyr*B	F	2.415	5'- GAT GTT TAC CGT GGA AAA GGG -3'	
	R		5'- GGC CGG GGA TTA AGG CA -3'	
Internal primer				
*gyr*A	AF		5'- TTG CGA CCT TTG AAT CCG GG -3'	1 – 1,100
	Ai1F		5'- GCA GTA GGT ATG GCG ACG AA -3'	600 – 1,600
	Ai2F		5'- TCC GTG GGA TCT GGG CAA C -3'	1,300 – 2,400
	Ai3F		5'- TCG CGG TAA ACC TCA ACG AC -3'	2,100 – 2,637
*gyr*B	BF		5'- GAT GTT TAC CGT GGA AAA GGG -3'	1 – 500
	Bi1F		5'- AGG TCT GGG TGA AAT GAA CC -3'	2,250 – 2,415
	Bi2F		5'- GAA TAA AAC GCC GAT CCA CCC -3'	750 – 1,300
	Bi3F		5'- GAC GAC GAA GCG ATG GAT CA -3'	1,600 – 2,300
	Bi4F		5'- GTC GCA AAA ACT GGA GCT GG -3'	400 – 1,200
	Bi5F		5'- GCA AAC TGG CGG ATT GTC AG -3'	1,250 – 2,200

Table 3. Susceptibility profile of isolates to fluoroquinolone antibiotics.

Code	Isolate	LVX (mm)	S/I/R*	NAL (mm)	S/I/R	CIP (mm)	S/I/R	MFX (mm)	S/I/R	*Blank Disk*
AW1	STY19	34	S	26	S	36	S	31	S	-
AW2	STY2	35	S	24	S	35	S	27	S	-
AW3	STY070622RYT	22	I	15	I	25	I	16	R	-
AW4	STY050922RYT	36	S	37	S	37	S	31	S	-

*S: sensitive; I: intermediate; R: resistant.

AW3 isolates showed an intermediate response to nalidixic acid, ciprofloxacin, and levofloxacin while exhibiting resistance to moxifloxacin. AW4 was sensitive to all four antibiotics. A summary of the antibiotic susceptibility test is provided in Table 3.

PCR amplification of gyrA and gyrB

The length of the PCR products for each gene is consistent with the anticipated outcomes, with the *gyrA* product measuring 2,637 bp and the *gyrB* product measuring 2,415 bp.

Genetic sequence analysis of the gyr gene of S. Typhi

An earlier study conducted by Nathania *et al.* (2022) explained the hotspot regions of *gyr*A, *gyr*B, *par*C, and *par*E from local S. Typhi [18]. The present study used these hotspot references, i.e., Accession number ON220744 *gyr*A and ON220772 *gyr*B, to assess the presence or absence of mutations, both within and outside these hotspot regions.

Genetic Sequence Analysis of the gyrA Gene

The sequencing results for isolates AW1, AW2, AW3, and AW4 of the *gyr*A gene were aligned with the reference sequence of *S.* Typhi Ty2 (NCBI GenBank accession number AE014613.1). The *gyrA* gene sequences of isolates AW1, AW2, AW3, and AW4 yielded a length of 2,637 base pairs, which aligned with the reference sequence. The sequence was obtained using four internal primers, specifically AF, Ai1F, Ai2F, and Ai3F (Figure 1). The results of the alignment of the *gyrA* gene are as follows:

- The alignment of the isolate AW1 *gyr*A gene with the reference sequence *S.* Typhi Ty2 (NCBI GenBank AE014613.1) revealed a variation at position 1612 (A to G), accompanied by an amino acid alteration at codon Asn538Asp. The amino acid alteration occurs outside the hot spot region. Isolate AW1 of the *gyr*A gene was found to be identical to the Accession number ON220744.
- The alignment of the isolate AW2 *gyrA* gene with the reference sequence *S.* Typhi Ty2 (NCBI GenBank AE014613.1) revealed 51 variations with an alteration in the amino acid at three codons: the amino acid substitutions at positions Gly133Glu, Asn538Asp, and Thr856Ala. The amino acid substitution at position 133, from Gly to Glu, occurred within a hot spot region. In contrast, the

Figure 1. The internal primers used in the sequencing of the *gyr*A gene. Primer AF has been designed to bind to a sequence of 1,100 base pairs, while primer Ai1F has been optimized for binding to a sequence of 600 to 1,600 base pairs. Similarly, primer Ai2F is designed to bind to a sequence of 1,300 to 2,400 base pairs, while primer Ai3F is optimized for binding to a sequence of 2,100 to 2,637 base pairs.

Figure 2. Amino acid alterations in the *gyr*A gene. Amino acid substitutions at the Gly133Glu position were identified in isolates AW2 and AW3. An alteration in the amino acid Asn538Asp was noted in isolates AW1, AW2, and AW3. Amino acid alterations occurring at the Thr856Ala position were observed in isolates AW2 and AW3.

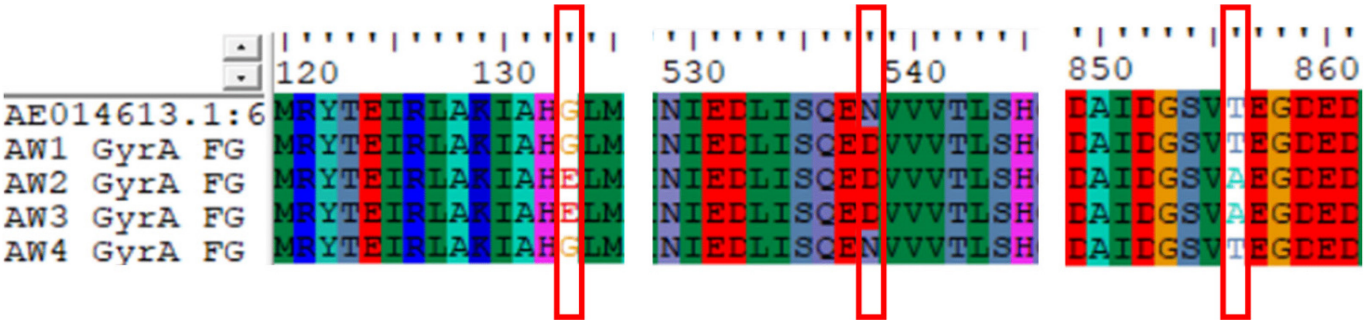
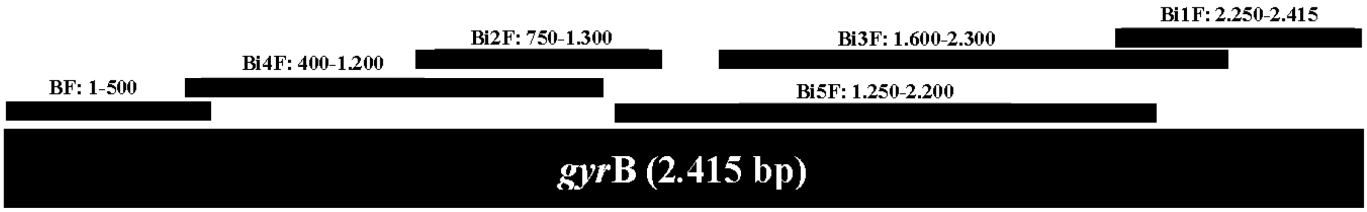


Figure 3. The internal primers were utilized in the sequencing of the *gyr*B gene. The primer BF reads base sequences 1-500; the Bi4F primer reads base sequences 400-1,200; the primer Bi2F reads base sequences 750-1,300; the primer Bi5F reads base sequences 1,250-2,200; the primer Bi3F reads base sequences 1,600-2,300; and finally, the primer Bi1F reads base sequences 2,250-2,415.



substitutions Asn538Asp and Thr856Ala occurred outside the hot spot region.

- The alignment of the isolate AW3 *gyrA* gene with reference sequence *S.* Typhi Ty2 (NCBI GenBank AE014613.1) revealed 51 variations in the base sequence, accompanied by alterations in the amino acids at three codons: the amino acid substitutions at positions Gly133Glu, Asn538Asp, and Thr856Ala. The amino acid substitution at position 133, from Gly to Glu, occurs within the hot spot region. The alterations in the amino acid sequence at Asn538Asp and Thr856Ala are not located within the hot spot region.
- The alignment of isolate AW4 *gyrA* with the reference sequence *S.* Typhi Ty2 (NCBI GenBank AE014613.1) yielded identical results to those of the reference sequence.
- The alignment of isolates AW1, AW2, AW3, and AW4 *gyr*A gene with reference sequences (NCBI GenBank accession number AE014613.1) revealed a multitude of variations that did not result in amino acid changes. Amino acid alterations were observed in only three codons: Gly133Glu, Asn538Asp, and Thr856Ala (Figure 2).

All the sequence data retrieved from this study have been submitted and are available at NCBI GenBank with the accession numbers listed in Supplementary Table 1.

Genetic Sequence Analysis of the gyrB Gene

The sequencing results of isolates AW1, AW2, AW3, and AW4 of the *gyrB* gene were aligned with the reference sequence of *S.* Typhi Ty2 (NCBI GenBank AE014613.1). The *gyrB* gene sequences of isolates AW1, AW2, AW3, and AW4 exhibited a length of 2, 415 base pairs, which aligned with the reference sequence. The sequence was obtained using six internal primers, namely BF, Bi1F, Bi2F, Bi3F, Bi4F, and Bi5F (Figure 3). The results of the *gyrB* gene alignment are as follows:

- The alignment of the AW1 isolate *gyrB* gene with the reference sequence of *S.* Typhi Ty2 (NCBI GenBank AE014613.1) yielded identical results with the reference sequence. Furthermore, the AW1 isolate of the *gyr*B gene was found to be identical to Accession number ON220772.
- The alignment of the AW2 isolate *gyrB* gene with the reference sequence of *S.* Typhi Ty2 (NCBI GenBank AE014613.1) revealed 33 base variations, with no observed changes in amino acids. This base variation occurred outside the identified hot spot area.
- The alignment of the AW3 isolate *gyrB* gene with the *S.* Typhi Ty2 reference sequence (NCBI GenBank AE014613.1) revealed a single 27-base variation resulting in the substitution of an amino acid at codon 416, from alanine (Ala) to serine (Ser). This alteration took place within the hotspot

Figure 4. Amino acid alterations in the *gyr*B gene. Amino acid substitutions at the Ala416Ser position were identified in isolates AW2 and AW3.

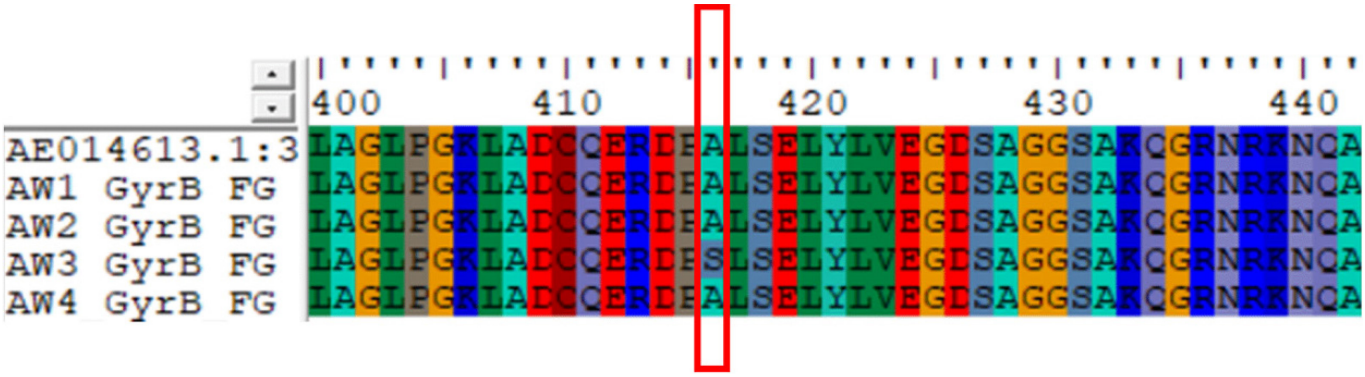


Table 4. The antibiotic susceptibility test profiles of isolates and the amino acid changes in the *gyr*A and *gyr*B genes of AW1, AW2, AW3 and AW4 isolates.

Code	Phenotype				Alterations of amino acids	
	CIP	LVX	NAL	MOX	*gyr*A	*gyr*B
AW1	S	S	S	S	Asn538Asp	-
AW2	S	S	S	S	Gly133Glu^{hs,} Asn538Asp, Thr856Ala	-
AW3	I	I	I	R	Gly133Glu^{hs,} Asn538Asp, Thr856Ala	Ala416Ser^{hs}
AW4	S	S	S	S	-	-

hs: hot spot.

region.

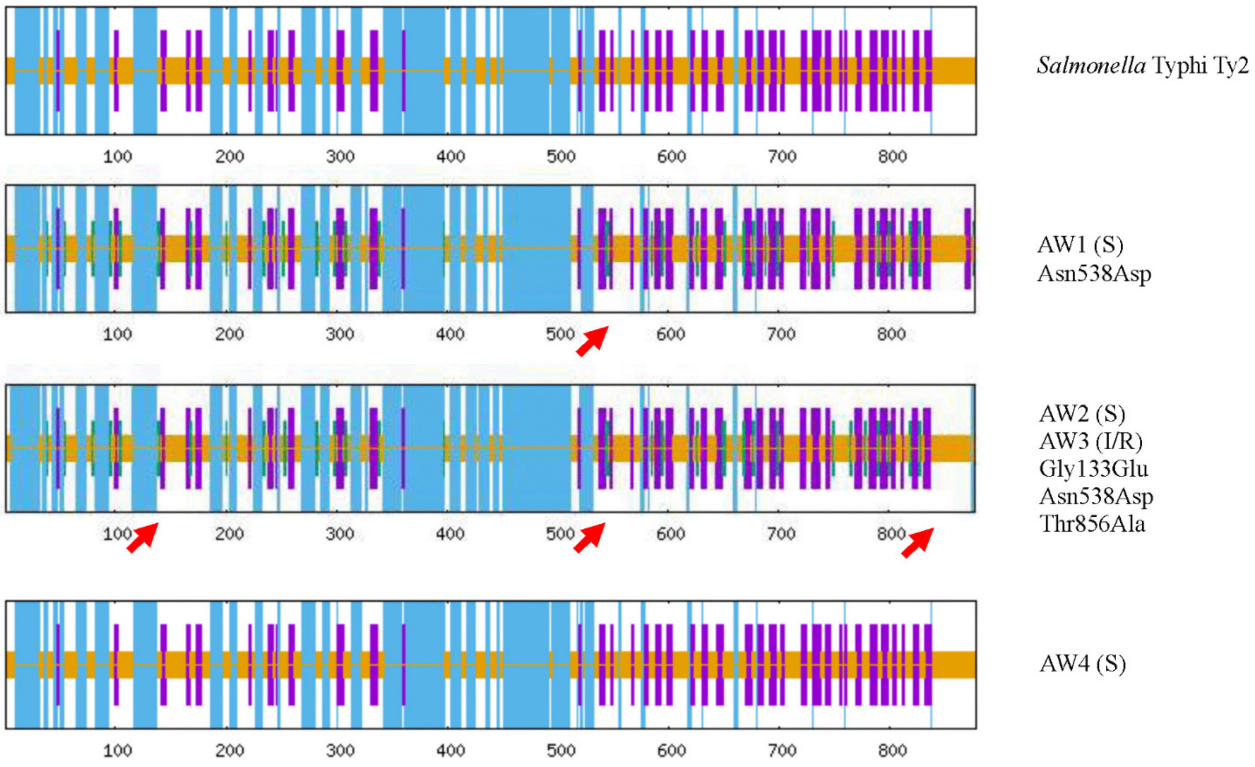
- The alignment of the AW4 isolate *gyrB* gene with the reference sequence of *S.* Typhi Ty2 (NCBI GenBank AE014613.1) revealed that it was identical to the reference sequence.
- The alignment of the isolate AW1, AW2, AW3, and AW4 *gyrB* gene with reference sequences (NCBI GenBank accession number AE014613.1) revealed a multitude of variations that did not result in amino acid changes. Amino acid alterations were observed in only one codon: Ala416Ser (Figure 4).

The results of the sequencing analysis, conducted on the *gyrA* and *gyrB* genes of isolates AW1, AW2, AW3, and AW4, are summarized in Table 4. Isolate AW1 exhibited amino acid alterations in a single gene, specifically the *gyrA* gene (Asn538Asp). Isolate AW2 exhibited amino acid alterations in a single gene, specifically the *gyrA* gene (Gly133Glu, Asn538Asp, Thr856Ala). Isolate AW3 demonstrated alterations in the amino acid sequences of two genes. These include *gyrA* (Gly133Glu, Asn538Asp, Thr856Ala) and *gyr*B (Ala416Ser). Isolate AW4 showed no variation from the Ty2 reference in either of the genes examined.

Analysis of the gyrA protein

The amino acid sequences of the gyrA protein were analyzed using Network Protein Sequence @nalysis/NPS@ in order to generate a profile overview of the gyrA protein secondary structure, specifically alpha helix and beta sheet. The results of the gyrA protein analysis were then compared with those of the reference protein, *S.* Typhi Ty2. The results of the NPS@ analysis are presented in Figure 5. The alteration of a single amino acid at codon Asn538Asp in isolate

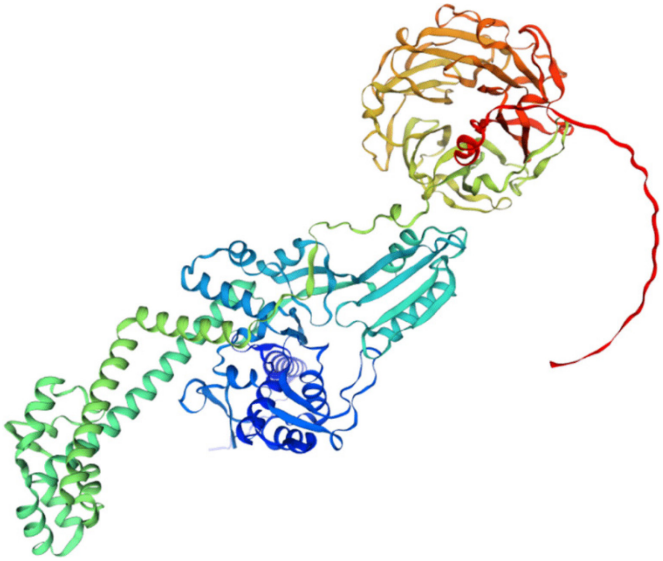
Figure 5. Structure of *Salmonella* Typhi gyrA secondary protein. Secondary protein structure of *Salmonella* Typhi Ty2 reference sequence gyrA compared with isolates AW1, AW2, AW3, and AW4. Arrows indicate regions of secondary structure changes caused by amino acid changes. S: sensitive. I: intermediet. R: resistant. This analysis was made using NPS@: Network Protein Sequence@nalysis Combet C., Blancet C., Geourjon C., and Deleage G. TIBS 2000 March Vol. 25, No. 3 [291]:147-150.



AW1 resulted in a change in the percentage of alpha helix from 34.51% to 35.19%, beta sheet from 20.05% to 20.27%, random coil from 45.44% to 37.36%, and the emergence of a beta turn structure by 7.18%. The secondary protein structure of isolate AW2 and isolate AW3, which exhibited alterations in three amino acids at codon Gly133Glu, Asn538Asp, and Thr856Ala, demonstrated a shift in alpha helix percentage from 34.51% to 36.67% beta sheet from 20.05% to 19.70%, random coil from 45.44% to 36.45%, and the emergence of a beta turn structure by 7.18%. The secondary protein structure of isolate AW4 was found to be identical with the reference amino acid sequence of *S.* Typhi Ty2, with a percentage of 36.67% for alpha helix, 19.70% for beta sheet, 36.45% for random coil, and 7.18% for beta turn structure.

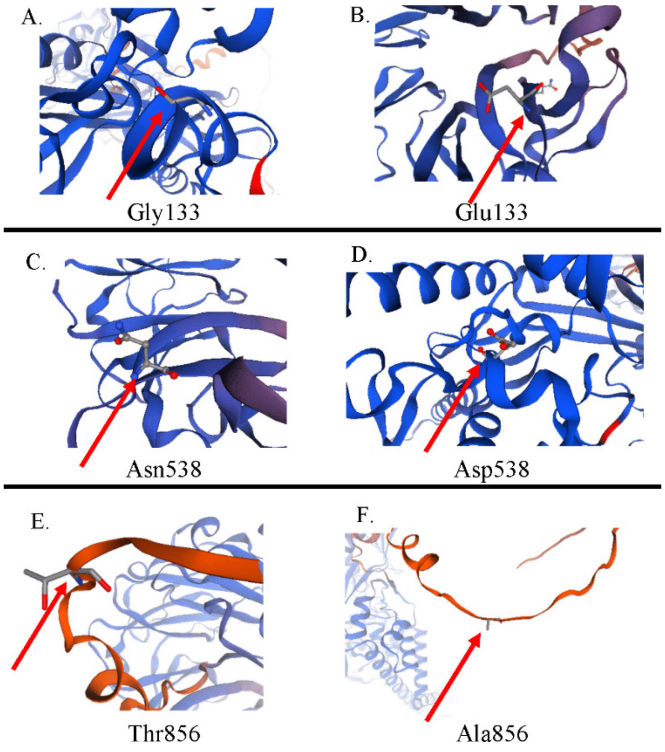
The three-dimensional structure of the *S.* Typhi Ty2 reference sequence gyrA protein is illustrated in Figure 6. The three-dimensional structure of the gyrA protein

Figure 6. The three-dimensional structure of the *Salmonella* Typhi Ty2 reference sequence gyrA protein is presented herewith. The model was constructed using the SWISS-MODEL.



variation at the codons Gly133Glu, Asn538Asp, and Thr856Ala can be observed in Figure 7. The 3D structures of the gyrA protein variation and NPS@ analysis of the gyrA protein demonstrate that alterations in amino acids can influence the proportion of the secondary structure of the gyrA protein.

Figure 7. The three-dimensional structure of the gyrA protein from *Salmonella* Typhi. A. Three-dimensional structure of the Gly codon at position 133, with the reference amino acid as the basis for comparison. B. Three-dimensional structure of Glu codon 133 of AW2 and AW3 isolates. C. Three-dimensional structure of the amino acid corresponding to the Asn codon 538. D. Three-dimensional structure of Asp codon 538 in isolates AW1, AW2, and AW3. E. Three-dimensional structure of Thr codon 856 of the reference amino acid. F. Three-dimensional structure of Ala codon 856 of AW2 and AW3 isolates. The model was constructed using the SWISS-MODEL.



Analysis of the gyrB protein

The amino acid sequences of the gyrB protein were analyzed using NPS@ to generate an overview of the percentage of the gyrB protein secondary structure,

Figure 8. The three-dimensional structure of the *Salmonella* Typhi Ty2 reference sequence gyrA protein is presented herewith. The model was constructed using the SWISS-MODEL.

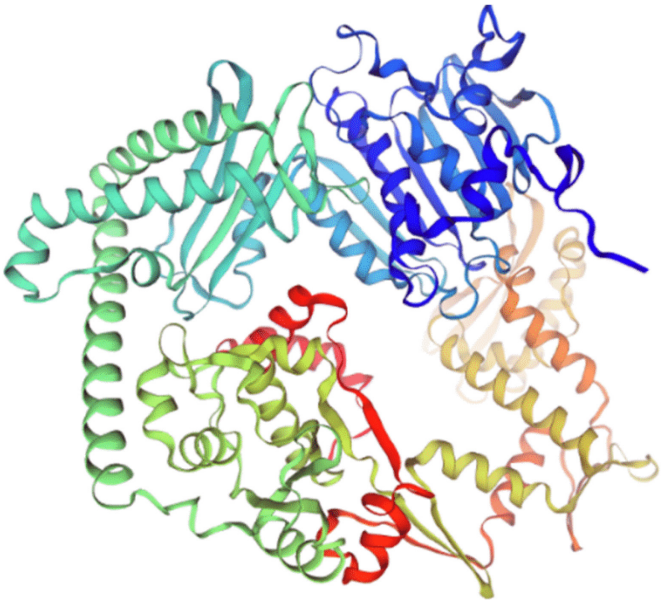
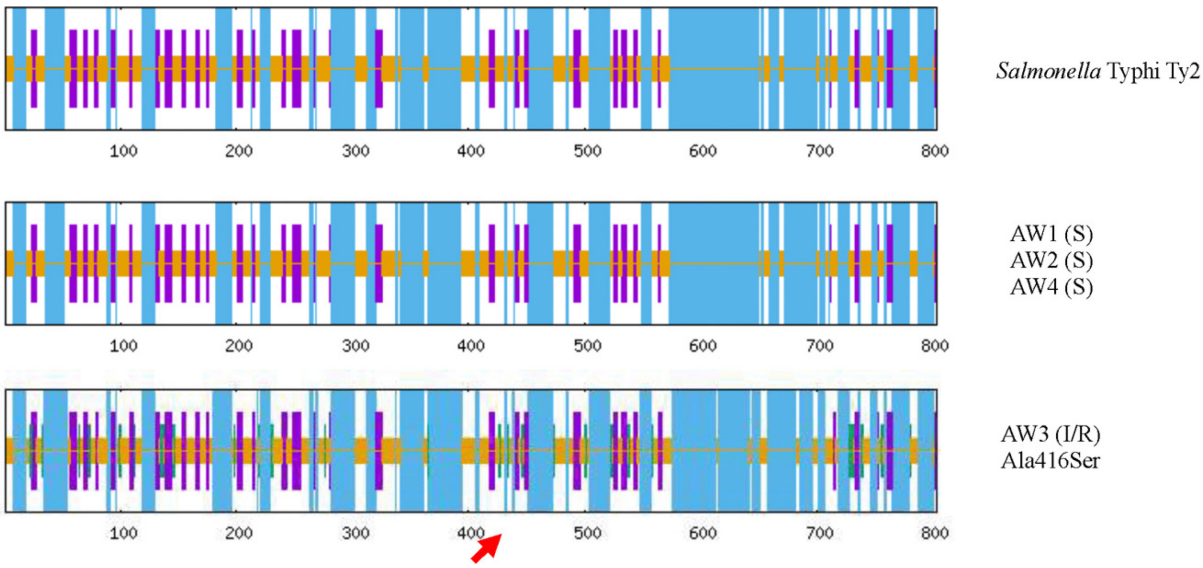
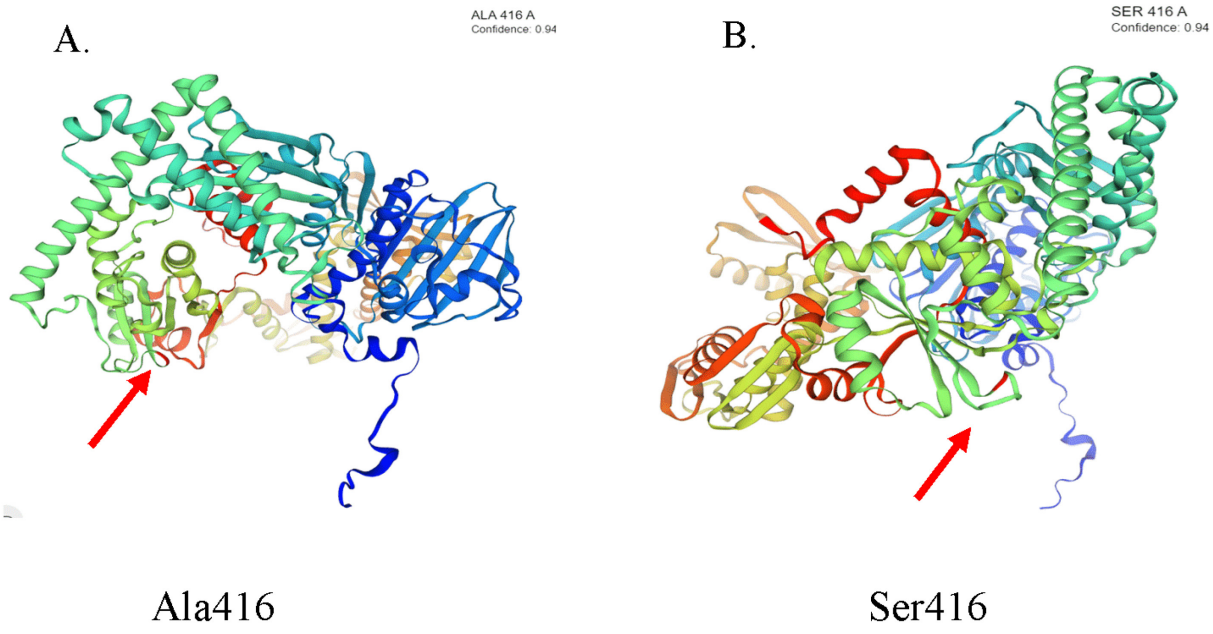


Figure 9. Structure of *Salmonella* Typhi gyrB secondary protein. Secondary protein structure of *Salmonella* Typhi Ty2 reference sequence gyrB compared with isolates AW1, AW2, AW3, and AW4. Arrows indicate regions of secondary structure changes caused by amino acid changes. S: sensitive. I: intermediate. R: resistant. This analysis was made using NPS@: Network Protein Sequence@nalysis Combet C., Blancet C., Geourjon C., and Deleage G. TIBS 2000 March Vol. 25, No. 3 [291]:147-150.



comprising alpha helices and beta sheets. The results of the gyrB protein analysis were then compared with those of the reference protein, *S.* Typhi Ty2. The results of the NPS@ analysis are presented in Figure 9. The secondary protein structure of the AW1 isolate is consistent with the amino acid sequence of the *S.* Typhi Ty2 reference. The secondary protein structure of isolate AW2 is consistent with the reference amino acid sequence of *S.* Typhi Ty2. The secondary protein structure of isolate AW3, which has undergone a single amino acid substitution at codon Ala416Ser, results in a slight alteration in the percentage composition of its secondary structure. Specifically, the alpha helix percentage increases from 48.26% to 49%, and the beta sheet percentage increases from 14.05% to 14.3%. Additionally, the random coil percentage declines from 37.69% to 30.85%, and a new beta turn structure emerges with a percentage of 5.85%. The secondary

Figure 10. The three-dimensional structure of the gyrB protein, which is a component of the *Salmonella* Typhi bacterium, is presented herewith. A. Three-dimensional structure of the Ala416, with reference to the amino acids in the surrounding region. B. Three-dimensional structure of Ser416 of AW3 isolate. The model was constructed using the SWISS-MODEL software.



protein structure of AW4 isolate is consistent with the reference amino acid sequence of *S.* Typhi Ty2.

The three-dimensional structure of the *S.* Typhi Ty2 reference sequence gyrB protein is illustrated in Figure 8. The three-dimensional structure of the gyrB protein variation at codon Ala416Ser is illustrated in Figure 10. The 3D structure of the gyrB protein variation and the NPS@ analysis of the gyrA protein demonstrate that alterations in amino acids can influence the proportion of the secondary structure of the gyrA protein.

Amino acid alterations in the gyrA and gyrB proteins result in modifications to the secondary protein structure. The alterations in secondary protein structure may be attributed to modifications in amino acid characteristics**.**

Discussion

Our findings on the *gyrA* sequence revealed amino acid alterations within the hot spot area (Gly133Glu) and outside the hot spot area (Asn538Asp and Thr856Ala). Substitution found on codon 133 agreed with earlier research by Nathania *et al.,* where codon 82, 83, & 133 substitutions also occurred [18]. However, the study by Acheampong *et al.* indicated that a single mutation in codon 133 of the *gyrA* gene may not be sufficient to cause fluoroquinolone resistance. The variation of codon 133 of the *gyrA* gene differs between countries. In Ghana, no association was found between this variation and fluoroquinolone resistance. However, in Kenya, it was concluded that codon 133 of the *gyrA* gene could be associated with fluoroquinolone resistance [21]. The study by Emran *et al.* showed 11 isolates with decreased fluoroquinolone sensitivity were found to have mutations in the codon Gly133Glu [22]. Variations in codons 538 and 856 of the *gyrA* gene have never been reported. Another study reported variations of codon Ile203Ser to occur outside the hot spot region [21]. The *gyrA* gene has been demonstrated to play an important role in fluoroquinolone resistance. Roumagnac *et al.* analyzed 199 gene fragments, including *gyrA*, and identified at least 15 independent mutations in the *gyrA* gene [23].

The sequencing result on the gyrB gene revealed an amino acid substitution Ala416Ser within the hot spot region. The hot spot areas of the gyrB gene that are frequently investigated in relation to fluoroquinolone resistance are codons 426, 435, 464, 465, 466, and 468 [18]. Our study found variations in codon 416 of the gyrB gene, which has not been reported elsewhere in *S.* Typhi. Acheampong et al. identified mutations outside the hot spot area Phe601Leu [21].

The study conducted by Emran *et al.* concluded that *S.* Typhi isolates that displayed decreased ciprofloxacin sensitivity exhibited double mutations in genes associated with fluoroquinolone resistance. In his study, all isolates showed mutations in codons 83 and 133 of the gyrA gene and codon 464 of the gyrB gene. Double mutations in both the gyrA gene and gyrB gene have been associated with ciprofloxacin resistance [22]. Our study seemed to be in agreement that fluoroquinolone resistance is associated with double mutations, i.e., intermediate and resistant to fluoroquinolone AW3 isolates exhibited variations in both genes: gyrA (Gly133Glu, Asn538Asp, and Thr856Ala) and gyrB (Ala416Ser). Isolate AW1, susceptible to 4 antibiotics, showed an amino acid change in one codon (Asn538Asp) of the *gyrA* gene. AW2, which is sensitive to 4 antibiotics, found changes in 3 codons in the *gyrA* gene (Gly133Glu, Asn538Asp, Thr856Ala) without changes in the *gyrB* gene. Fluoroquinolone resistance in *S.* Typhi was associated with a single QRDR mutation (D87G and S83F of the *gyrA*) [24]. Further research involving a larger number of isolates is required to substantiate the conclusions drawn from these results.

Changes in amino acids within the gyrA and gyrB proteins may impact the secondary structure of the protein, thereby influencing the characteristics of the amino acids, including polarity and hydrophobicity, that may affect protein function. The analysis of protein secondary structure is of great importance to gain insight into the role of secondary structure in protein function. This encompasses protein folding, functional conformation, and protein interaction with other molecules. The gyrA protein of isolates AW1, AW2, and AW3 and the gyrB protein of isolate AW3 exhibited amino acid alterations and modifications in the composition of the secondary protein structure. It is yet unclear whether changes in the percentage of secondary protein structures and the emergence of beta-turn structures are the cause of fluoroquinolone resistance. A beta turn is a distinctive hydrogen backbone configuration, comprising four residues arranged at an angle. This structural element might affect the nature of the protein, which may be associated with fluoroquinolone resistance.

The potential causal relationship between amino acid changes in the *gyrA* and *gyrB* genes and alterations in fluoroquinolone sensitivity needs to be elaborated. To gain a comprehensive understanding, it is essential to increase the number of samples exhibiting similar phenotypes.

Conclusions

In conclusion, the continuous investigation of

antibiotic susceptibility is paramount in the fight against the growing threat of antibiotic resistance, particularly in regions where resistant pathogens, such as Salmonella typhi, are becoming increasingly prevalent. As antibiotic resistance continues to rise globally, maintaining a robust system of research and surveillance is vital to protect public health, safeguard the effectiveness of existing antibiotics, and prevent the onset of severe, drug-resistant diseases that could otherwise be difficult to manage and treat. Further studies are required to elucidate the association of these changes with susceptibility to fluoroquinolones. It would be beneficial to implement adequate protective measures if antibiotic resistance is increasing in this region and to gain a deeper understanding of the mechanisms behind resistance, which could potentially be targeted to prevent severe disease.

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Author Contributions

Conceived and designed the study: LHM, IMN. Performed research, primer design, and data analysis: AW and IMN. Manuscript preparation: AW and IN. Review and revise the manuscript: LHM, AW, IN, and ADKT. Proofread: ADKT. All authors read and approved the final manuscript.

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Conflict of interest

No conflict of interest is declared.

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Annex – Supplementary Items

Supplementary Table 1. NCBI Genbank accession number of all isolates.

Code	Gene	Accession Number
AW1	gyrA	PQ053332
AW2	gyrA	PQ053333
AW3	gyrA	PQ053334
AW4	gyrA	PQ053335
AW1	gyrB	PQ053336
AW2	gyrB	PQ053337
AW3	gyrB	PQ053338
AW4	gyrB	PQ053339