Detection of DNA Gyrase Mutation among Clinical and Environmental Isolates of *Salmonella enterica* Serovar Typhi from Some Parts of Adamawa State, Nigeria

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Abstract: The emergence of quinolone resistance among *Salmonella enterica* serovar Typhi is a growing concern in the treatment and management of typhoid fever especially in developing countries. The quinolone resistance is attributed to point mutation in *GyrA* gene of the organism. This study was aimed at identifying the occurrence of point mutation in the *GyrA* gene of *Salmonella* Typhi from some parts of Adamawa state. Results from the study showed that only 30% of the isolates from the study area were susceptibly to ciprofloxacin while 45% demonstrated reduced susceptibility to it. Two isolates *S*. Typhi MUB34 and *S*. Typhi GMB 1 bored plasmids coding for resistance to nalidixic acid. BLAST sequence analysis of the *GyrA* gene with reference *S.* Typhi isolate from the NCBI website revealed that 14 (56%) of the isolates had point mutations at position 83-serine while 6 (24%) had mutation at position 87. Further observation of the mutation pattern indicated that 12 (48%) of isolates had single point mutation at position ser 83 while 4(16%) had double mutation at points 83 and 87 of QRDR of *gyrA* gene. The remaining isolates had no point mutations in the *gyrA* gene at either position 83 or 87. The mutation observed in the QRDR of GyrA gene of *Salmonella enterica* Typhi will mean that the selection of fluoroquinolones for treatment of *S*. Typhi in the study area must be done with caution to avoid treatment failure. This is because the presence of single mutation in the *Gyr* A gene is associated with reduced susceptibility to quinolone antibiotic while double mutation confers resistance to quinolones.

Keywords: Quinolone, *GyrA gene*, Resistant, Mutation *Salmonella* serovar Typhi

INTRODUCTION

Typhoid fever remains an important public health concern because it is a severe life-threatening systemic disease caused by *Salmonella enterica* serovar Typhi with case fatality rates which could be as high as 30 % in the absence of prompt and appropriate antibiotic treatment. Following the introduction chloramphenicol in 1948, the case fatality rates was reduced to as low as 1 % (Parry *et al.*, 2002). Unfortunately, this breakthrough was short lived as resistance to chloramphenicol emerged among *Salmonella enterica* serovar Typhi in 1950 (Colquhoun and Weetch, 1950) and by 1972, wide spread resistance to this drug was reported which posed a serious threat to the health of communities (Ackers *et al.*, 2000). However, by the 1980s, the emergence of multi drug resistant (MDR) strains of *S.* Typhi presented a major challenge to chemotherapy. The widespread dissemination of these multi-drug *Salmonella enterica* Typhi across the globe necessitated the use of ciprofloxacin as the drug of choice in the treatment of typhoid fever (Arora *et al.,* 2010).

The fluoroquinolones at inception have the advantage of easily penetrating tissues and killing *S.* Typhi in its intracellular stationary phase thereby facilitating rapid clearance and prompt clinical response with lower rates of chronic carriage. Unfortunately, shortly after the adoption of quinolones as treatment choice for typhoid fever, strains with reduced susceptibility to quinolones as well as resistance to first line drugs begun to emerge from these same areas thereby threatening the efficacy of the use of quinolone antibiotics for treatment of typhoid fever (Threlfall and Ward, 2011). Today, on the basis of antimicrobial susceptibility testing results, isolates are ranked as susceptible to ciprofloxacin (when susceptible to ciprofloxacin and nalidixic acid), reduced ciprofloxacin susceptibility (resistant to nalidixic acid and intermediate to ciprofloxacin), and resistant to ciprofloxacin (resistance to nalidixic acid and ciprofloxacin) (Gopal *et al.* 2016; Hakanen *et al*., 1999).

The evolving resistance to quinolone antibiotics among *S.* Typhi is attributed to a spontaneous point mutation occurring in the chromosomal gene in the quinolone

resistance determining regions (QRDR) that codes for DNA gyrase (*gyr* A, *gyr* B) and topoisomerase IV (*par* C, and *par* E) (Gopal *et al*. 2016). It has been reported that a single mutation of *gyr*A gene leads to resistance to nalidixic acid and reduced susceptibility to ciprofloxacin (minimum inhibitory concentration (MICs) of 0.125–0.25µg/mL) in *Salmonella Typhi* (Onyenwe *et al.* 2012). Complete resistance to ciprofloxacin (MIC > 4µg/mL) is attributed to double mutation in the Quinolone resistance determining region. This point mutation is known to alter these enzymes that are targets for quinolone drugs and therefore it is not transferable. Beside the chromosomal based quinolone resistance, it has been reported that a naturally occurring plasmid-encoded *qnr* gene (*qnrB* and *qnrS*) among *S*. Typhi can mediate reduced susceptibility to quinolones (Guan *et al*., 2013; Geetha *et a*l., 2014).

Although there are reports on *S*. Typhi occurrence as well as their antimicrobial susceptibility profile in Nigeria and in some parts of Adamawa State, there are no available data on the occurrence of point mutation in the QRDR of *Salmonella* Typhi in the study area. In the current study, we studied mutation pattern in *GyrA* gene of *Salmonella* Typhi isolates from clinical and environmental samples from some parts of Adamawa State.

MATERIALS AND METHODS:

Test Organism

The *Salmonella enterica* Typhi (214) isolates used in this study were organisms we previously isolated from clinical specimen (blood and stool), ready to eat foods and water in the three senatorial zones of Adamawa State and stored at 4^0 C in the laboratory (Sale *et al*., 2019, Sale *et al*., 2017). The isolates were reactivated on nutrient agar and sub cultured on bismuth sulphite agar and reconfirmed using *S.* Typhi polyvalent O antiserum (WHO, 2003). The isolates labelled according to the location from which they were isolated.

Antibiotic Susceptibility Testing

The antimicrobial susceptibility profile of the *S. enterica* serovar Typhi isolates for the study against the minimum inhibitory concentrations of nalidixic acid, (30 µg), ofloxacin (5 µg) and ciprofloxacin (5 µg) was determined using the disk diffusion method described by CLSI (2014).

Plasmid Curing

The Salmonella Typhi isolates were screened for the presence of plasmids using the plasmid curing method described by Mirmomeni *et. al.,* (2007). About 0.2 ml of overnight culture of S. Typhi was inoculated into 5 ml nutrient broth containing 10% Sodium dodecyl sulphate and incubated at 37 ^{0} C for 24 hours. After the period of incubation, the broth culture was agitated to homogenize the content and then sub cultured onto freshly prepared Mueller Hinton agar plates. The plates were then incubated at 37 ^{0}C for 24 hours. The resultant colonies from the Mueller Hinton agar plates were then subjected to antibiotic susceptibility screening as described previously. The antibiogram of the isolates were noted and compared with those of the isolates before the plasmid curing.

DNA Extraction and Quantitation

S. Typhi genomic DNA was extracted following the Qiagen DNA extraction protocol (Qiagen DNeasy, 2006). The spectrophotometer lens (nanodrop ND 1000) was used to measure the amount and purity level of the DNA. A DNA sample with an optical density (OD) of 1 at 260 nm corresponded to a DNA concentration of 50 µg/ml of double-stranded DNA. The purified chromosomal DNA pellets were then transferred into a new tube and stirred on ice. Purity levels were between 1.5-1.8 of 2 µl.

PCR Amplification of GyrA gene

PCR Amplification of the *GyrA* gene was done using the forward primer F-5'CGT TGG TGA CGT AAT CGG 3' and reverse primer R -5' CCG TAC CGT CAT AGT TAT-3' reported by Onyenwe *et al.,* (2012) in a final volume of 25 µl. The primers were synthesized at the Inqaba Biotech Genomic

laboratory, South Africa and obtained through the Inqaba Biotech West Africa (IBWA) office, Ibadan.

The expected band size of the amplicon produced by the primer was 251 bp. Each reaction mixture contained 5.5 µl molecular grade water, 12.5 µl PCR master mix 2 X (0.05 u/µl *Taq* DNA polymerase, reaction buffer, 4 mM $MgCl_2$, 0.4 mM of each dNTP), 2.5 µl of each primer and 2 µl of template bacterial DNA. Amplification reaction was carried on a GeneAmp System 2700 PCR thermocycler (Applied Biosystems, Foster City, CA, USA) with an initial denaturation at 94° C for 5 minutes, followed by 35 cycles of denaturation (94° C, 30 seconds), annealing (55 °C, 30 seconds) and extension (72° C, 2 min 30 seconds) and a final extension for 10 minutes at 72° C. The amplified products were separated by gel electrophoresis on 1.5% agarose at -100 volts and stained with ethidium bromide and visualised using Enduro gel documentation system. Molecular weight markers (50 base pairs) was used to determine the sizes of the amplicons.

DNA Gyrase Screening and Sequencing

The sequencing of the *gyrA* gene at the quinolone resistant determining region was done at the Bioscience Centre of the International Institute for Tropical Agriculture (IITA) Ibadan. The Basic Local Alignment Search Tool (BLAST) was used to compared the resulting sequences with those of reference *Salmonella* Typhi deposited in the National Centre for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/BLAST) for the presence or absence of mutation in the quinolone determining region of the *GyrA* gene of the isolates.

RESULTS

Results from the antimicrobial susceptibility profile of the *S*. Typhi isolates against quinolone antibiotics (Table 1) showed that 76.4% from the study were susceptible to ofloxacin while only 37% demonstrated in vitro susceptibility against ciprofloxacin. Furthermore, 55% of the isolates were resistant to nalidixic acid while 38% demonstrated reduced susceptibility to ciprofloxacin. The result also showed that isolates from all the all zones demonstrate greater resistance to nalidixic acid, although it is highest in the Adamawa northern zone (67%) followed by the Southern zone (56%). It was also clear from the results that the isolates from the southern zone had greater ciprofloxacin susceptibility (52%) (Table 1).

On the basis of susceptibility to nalidixic acid and ciprofloxacin, the result (Table 2) revealed that only 30% of the isolates from the study area can be said to be susceptible to ciprofloxacin (NAL^S CIP^S) while 25% were resistant to ciprofloxacin ($NAL^R CIP^R$). The majority of the isolates (45%) from the study demonstrated reduced susceptibility to ciprofloxacin (NAL^R CIP^I). The highest resistance to ciprofloxacin was observed in the central zone followed by the northern zone (Table 2)

Result of plasmid carriage among the isolates in shown in Figure 1. The results showed that 49% of the isolates from the study area bore plasmid coding for resistance to antibiotic. From this number of isolates bearing plasmids, only isolates: *Salmonella* Typhi GMB 1 and *Salmonella* Typhi MUB 34 bore plasmids coding for resistance to nalidixic acid. While majority of the isolates from the southern zone (53.2%) and central zone (54.8%) bore plasmids, the majority of the isolates from the northern zone (67.3%) had no plasmid (Figure 1).

PCR analysis of amplicons revealed the presence of the quinolone resistance determining region (QRDR) of *gyrA* gene (Figure 2). Further analysis of the nucleotide sequences obtained after the sequencing of the QRDR of *GyrA* using Basic Local Alignment Search Tool (BLAST) with that of *Salmonella* Typhi CT 18 deposited in the NCBI website revealed the presence of varying type of mutation in *gyrA* gene as shown in Table 3 at position Serine 83 or asparagine 87 codon.

The results revealed that 14 (56%) of the isolates had point mutations at position 83-serine while 6 (24%) had mutation at position 87. Further observation of the mutation pattern indicated that 12 (48%) of isolates had single point mutation at position ser 83 while 4(16%) had double mutation at points 83 and 87 of QRDR of *gyrA* gene. The remaining isolates had no point mutations in the *gyrA* gene at either position 83 or 87. The single point mutation showed that 10 isolates had mutation in the 83-position serine which was substituted by glycine, alanine or valine. Also, two single point mutation were observed in the 87 position Aspartic acid which was substituted with Leucine and alanine.

Table 1Susceptibility Profile of S. Typhi Isolates from the study area to quinolone antibiotics

	Nalidixic Acid (30 µg)			Ciprofloxacin (5 µg)			Ofloxacin (5 µg)		
	S	**I**	**R**	**S**	**I**	**R**	**S**	**I**	**R**
ANZ	17(31)	1(2)	37(67)	21(38)	19(35)	15(27)	50(91)	1(2)	4(7)
ACZ	28(33)	17(20)	39(46)	19(23)	37(44)	29(35)	60(71)	15(18)	9(11)
ASZ	29(38)	5(6)	43(56)	40(52)	26(35)	10(13)	55(71)	6(8)	16(21)
Total	74(34.3)	23(10.6)	119(55.1)	80(37.0)	82(38.0)	54 (25)	165(76.4)	22(10.2)	29(13.4)

KEY
Values in parenthesis are percentages
ANZ= Adamawa Northern Senatorial zone
ACZ = Adamawa Central Senatorial zone
ASZ = Adamawa Southern Senatorial zone

Table 2 Susceptibility to Ciprofloxacin on the Basis of Nalidixic acid and Ciprofloxacin Susceptibility

	$NAL^S CIP^S$	$NAL^R CIP^I$	$NAL^R CIP^R$
ANZ	17(31)	23 (41.8)	15 (27.2)
ACZ	19(22.6)	36 (42.9)	29 (34.5)
ASZ	29(37.7)	38(49.4)	10(13)
TOTAL	**65 (30)**	**97 (45)**	**54 (25)**

Values in parenthesis are percentages
ANZ= Adamawa Northern Senatorial zone
ACZ = Adamawa Central Senatorial zone
ASZ = Adamawa Southern Senatorial zone
NAL = Nalidixic Acid
CIP = Ciprofloxacin
S = Susceptible
R = Resistant

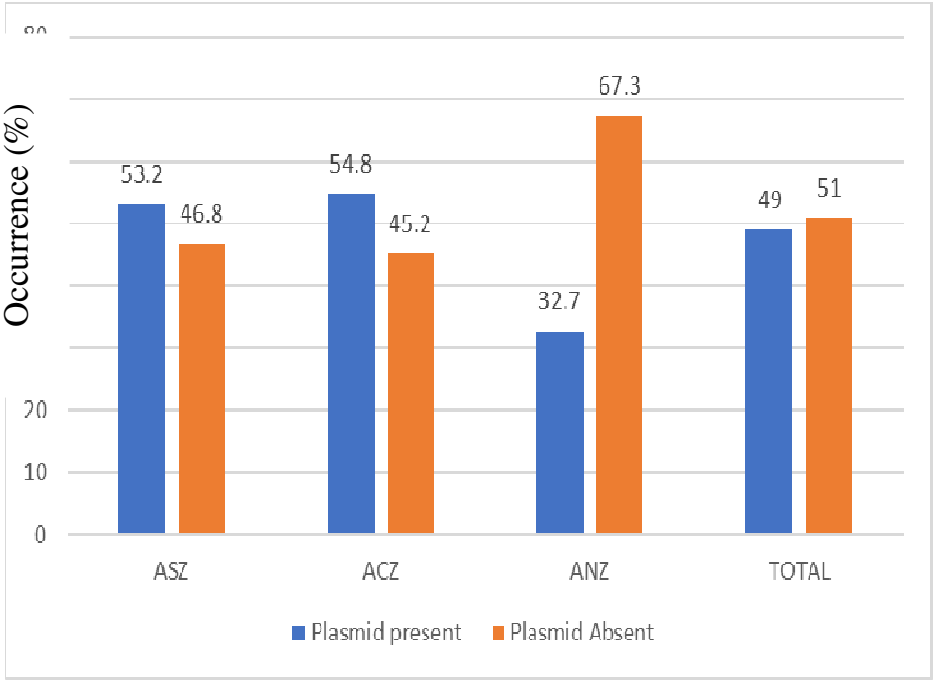

Figure 1 Plasmid Carriage among *S.* Typhi isolates from the study area

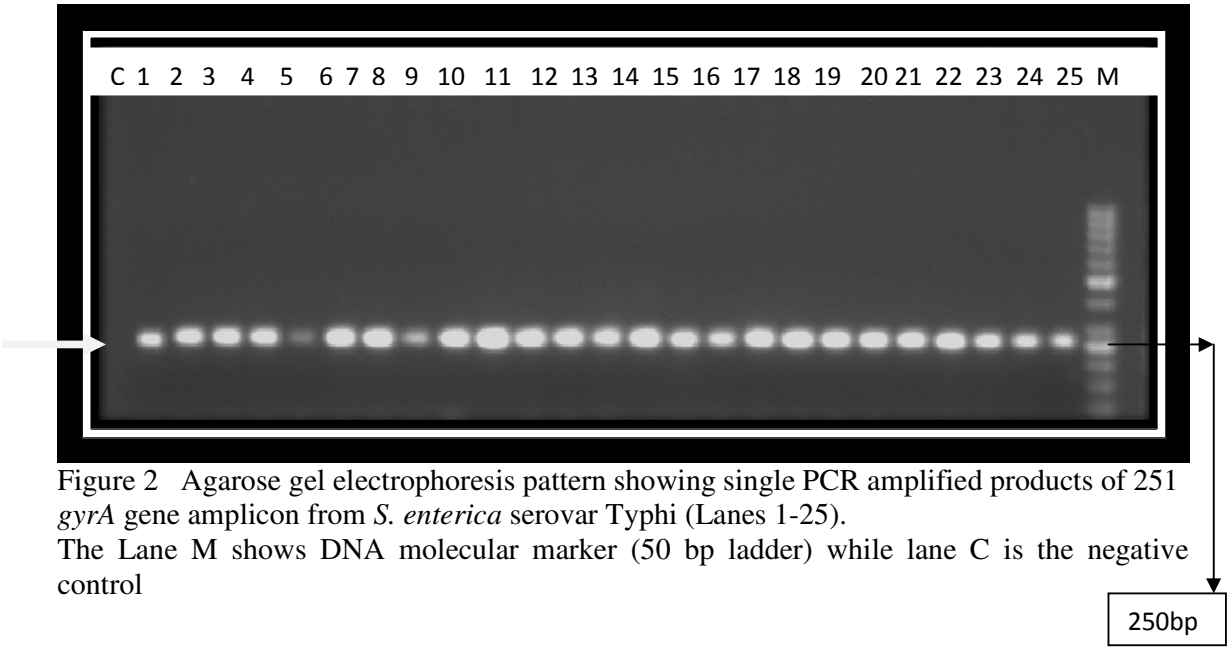

Figure 2 Agarose gel electrophoresis pattern showing single PCR amplified products of 251 *gyrA* gene amplicon from *S. enterica* serovar Typhi (Lanes 1-25).
The Lane M shows DNA molecular marker (50 bp ladder) while lane C is the negative control

Table *3: Salmonella enterica subsp. enterica* serovar Typhi *GyrA* Gene Mutation

Isolate No	83 -Serine	87- Aspartic acid	Point Mutation
Salmonella Typhi YLA 1	Valine	Aspartic Acid	Single
Salmonella Typhi YLA 30	Alanine	Lysine	Double
Salmonella Typhi GMB 4	Alanine	Lysine	Double
Salmonella Typhi R03C	Glycine	Serine	Double
Salmonella Typhi W01S	Glycine	Aspartic acid	Single
Salmonella Typhi W02N	Glycine	Aspartic acid	Single
Salmonella Typhi W02C	Serine	Aspartic Acid	None
Salmonella Typhi YLA 41	Glycine	Aspartic acid	Single
Salmonella Typhi MUB 24	Glycine	Aspartic acid	Single
Salmonella Typhi YLA 7	Serine	Aspartic acid	None
Salmonella Typhi MUB 1	Glycine	Alanine	Double
Salmonella Typhi MCH17	Valine	Aspartic acid	Single
Salmonella Typhi MUB 18	Alanine	Aspartic acid	Single
Salmonella Typhi MCH 19	Serine	Aspartic acid	None
Salmonella Typhi R08N	Glycine	Aspartic acid	Single
Salmonella Typhi NUM 11	Serine	Aspartic acid	None
Salmonella Typhi NUM13	Valine	Aspartic acid	Single
Salmonella Typhi MBW 6	Serine	Aspartic acid	None
Salmonella Typhi MBW 20	Serine	Aspartic acid	None
Salmonella Typhi MCH2	Glycine	Aspartic acid	Single
Salmonella Typhi GMB 21	Serine	Aspartic acid	None
Salmonella Typhi GMB 1	Serine	Lysine	Single
Salmonella Typhi NUM 27	Serine	Serine	Single
Salmonella Typhi MBW 18	Serine	Aspartic acid	None
Salmonella Typhi R02S	Serine	Aspartic acid	None

DISCUSSION

A total of 37% of S. Typhi isolates demonstrated in vitro susceptibility against ciprofloxacin. while 55% of the isolates were resistant to nalidixic acid. This result showed that the use of ciprofloxacin in managing typhoid fever may in the long run not be effective because the probability of treatment failure is high and majority of the isolates are either resistant to ciprofloxacin or have reduced susceptibility to it. The resistance observed in this study is however lower than those reported by Islam *et al*., 2008 who reported 33% ciprofloxacin resistance in Dhaka, Bangladesh and the 15.4% reduced ciprofloxacin susceptibility reported in central Africa by Lunguya *et al.,* (2012). The high rate of reduced susceptibility to ciprofloxacin in vitro (38%) could imply a widespread and /or inappropriate use of fluoroquinolones in Adamawa State. It could also be attributed to socioeconomic constraints as reported by Muthu *et al.,* (2011). Fluoroquinolones are under strict prescription of clinicians in advanced countries (Fangtham and Wilde, 2008) but freely available in developing countries like Nigeria. The easy access of the public to prescription drug is a n illegal practice and must be controlled as this will help to prevent widespread drug resistant *Salmonella* in the future. The widespread use of ciprofloxacin especially in the study area in managing other bacterial infection or their prophylactic use could be responsible for this.

Resistance and treatment failures are increasingly being observed and reported directly or indirectly.

Based on the susceptibility to nalidixic acid and ciprofloxacin, the result revealed that only 30% of the isolates from the study area can be said to be susceptible to ciprofloxacin (NAL^S CIP^S) while 25% were outrightly resistant to ciprofloxacin (NAL^RCIP^R). This is very important because nalidixic acid is said to be an important predictor of ciprofloxacin susceptibility (Gopal *et al.,* 2016*)*. The use of 30 µg disk of nalidixic acid as a surrogate marker of low level ciprofloxacin resistance has been reported to have 100% sensitivity and 98.8% specificity by Hakanen *et al.,* (1999), and the ability of Nalidixic acid resistance to predict reduced susceptibility to ciprofloxacin was reported to have a sensitivity of 99% and specificity of 99.7% by Rahman *et al.,* (2014). This implies that the isolates in the study area predominantly are resistant to ciprofloxacin and treatment failure is bound the happen when the selection of the antibiotics is based on laboratory evidence of inhibitory action against pathogen, sadly this most times don't happen because the selection of antibiotic is based pure on empirical approach.

About 49% of the isolates from the study area bore plasmid coding for resistance to different antibiotics only isolates *Salmonella* Typhi GMB 1 and *Salmonella* Typhi MUB 34 bore plasmids coding for resistance to nalidixic acid. This is not surprising as it has been reported that naturally occurring plasmids gene *qnrB* and *qnrS* borne by some strains of *Salmonella* Typhi codes for quinolone resistance (Geetha *et al*., 2014). PMQR 7.1% reported by Qian *et al.* (2020) had reported that only 7.1% of isolates from their study born plasmid coding for resistance to quinolones.

These finding goes to show that plasmid carriage does not play major roles in the resistance to quinolone antibiotics in the study area but since they are transferable if not handled properly, the plasmid mediated resistance may soon spread among the population of organisms in the study area and even beyond.

The other critical observation from the study is that Six 6 (2.7%) S. Typhi isolates from this study demonstrated resistance to ciprofloxacin but were susceptible to Nalidixic acid. These *Salmonella enterica* serotype Typhi strains with nonclassical quinolone resistance phenotype (decreased susceptibility to ciprofloxacin but with susceptibility to nalidixic acid) are not detected by the nalidixic acid disk screening test and can result in fluoroquinolone treatment failure.

Results from thus study showed that 56% of the isolates had point mutations at serine 83 while 24% had mutation at point 87. This is not surprising as other author have reported similar mutation among *Salmonella* typhi isolates in different places. Gopal *et al.* (2016) have reported that 94% of *Salmonella* typhi isolates from their study had point mutations in *gyrA* position 83. in another study, Onyenwe *et al*., (2012) reported that 64% of the is *S* Typhi Isolates possessed mutations in the *gyrA* gene. This is higher that the occurrence of point mutations in our study. Furthermore, Qian et al 2020 had reported 45 of isolates showing mutation at codon 83 while 61 had mutation at position 133 of the QRDR in the Jiangsiu province of china, These findings are instructive as it has been reported that a single point mutation in *gyrA* between amino acids 67 and 106 (known as the quinolone resistance-determining region or QRDR) can give rise to nalidixic acid (a first-generation quinolone) resistance among isolates of *Salmonella*. This resistance is usually accompanied by a reduction in the susceptibility (MIC 0.125-1.0 mg/L) of these *isolates* to ciprofloxacin. Fluoroquinolones mechanism of action in *Salmonella* include inhibition of tertiary super coiling of bacterial DNA, primarily by inhibiting the action of *gyrA* and *gyrB*, respectively (Anderson, 2007). It has also been shown that a single mutation in *gyrA* on its own is not sufficient for clinical resistance to fluoroquinolones, but a *gyrA* mutation is a

good marker indicating that fluoroquinolones should not be chosen for treating the respective infection Randall *et al.,* 2005).

CONCLUSION

BLAST sequence analysis of the *GyrA* gene with reference *S.* Typhi isolate from the NCBI website revealed that 14 (56%) of the isolates had point mutations at position 83-serine while 6 (24%) had mutation at position 87. Further observation of the mutation pattern indicated that 12 (48%) of isolates had single point mutation at position ser 83 while 4(16%) had double mutation at points 83 and 87 of QRDR of *gyrA* gene This confirms earlier reports that quinolone resistance is mostly attributed to point mutations in the gene coding for DNA gyrase and topoisomerase IV. From this study, it was observed that only two isolates bore plasmids coding for resistance to nalidixic acid which means that plasmid mediated resistance to quinolone in the study area is very low. Continuous surveillance for plasmids and *GyrA* gene mutation of *Salmonella* Typhi is very important towards ensuring that the treatment regimen for enteric fever is made more effective thereby maintaining the useful life of the few remaining antimicrobials available to treat enteric fever.

CONFLICT OF INTEREST: Authors declare no conflict of interest

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