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Antimicrobial Susceptibility Pattern and Characterization of Ciprofloxacin Resistant *Salmonella enterica* Serovar Typhi Isolates in Kerala, South India

N. Ayana and K. Surekha
Department of Biotechnology and Microbiology, School of Life Sciences,
Kannur University, Kerala, India

Abstract: The aim of the study was to determine antimicrobial susceptibility pattern and to examine the mechanism of increasing quinolone resistance of *S. typhi* isolates in Kerala, South India. Ciprofloxacin resistant isolates were characterized by mutation analysis of quinolone resistance determining region (QRDR) of *gyrA*, *gyrB*, *parC* and *parE* genes and the results showed that majority of the isolates were resistant to nalidixic acid. There was an increase in the number of isolates showing resistance to ciprofloxacin, ceftriaxone and ofloxacin. Multi drug resistant (MDR) *S. enterica* serovar Typhi were not isolated. Majority of ciprofloxacin resistant isolates carried mutation in quinolone resistance determining region (QRDR) of *gyrA* and strains with high level ciprofloxacin resistance carried double mutation in quinolone resistance determining region (QRDR) of *gyrA* and single mutation in quinolone resistance determining region (QRDR) of *parC*. In conclusion we reported that there was an increase in the number of *S. typhi* isolates showing resistance to flouroquinolones and ceftriaxone. We have found out that flouroquinolone resistance in *S. typhi* isolates was not plasmid encoded. We had also found out that mutations in quinolone resistance determining region (QRDR) region of *gyrA* play an important role in flouroquinolone resistance of *S. typhi* isolates and *parC* mutation facilitate increasing level of resistance.

Key words: Flouroquinolones, drug resistance, typhoid fever, Salmonella, mutation

INTRODUCTION

Salmonella enterica serovar Typhi is the causative agent of typhoid fever which cause 16 million cases with 6,00,000 deaths worldwide annually (Crump *et al*., 2004). Majority of these cases were reported in developing countries (Zulfiqar, 2006). Until the development of multi drug resistant (MDR) *S. enterica* serovar Typhi strains in 1989, chloramphenicol was the drug of choice for treating typhoid fever (Rowe *et al*., 1997). Since then, flouroquinolones particularly ciprofloxacin was the drug of choice in the treatment of typhoid fever (Rowe *et al*., 1991). But reports have indicated the emergence of *Salmonella enterica* serovar Typhi strains that exhibited decreased susceptibility to flouroquinolones (Kenji *et al*., 2001; Parry *et al*., 1998). The emergence of *S. enterica* serovar Typhi strains with decreased flouroquinolone susceptibility and nalidixic acid resistance have been reported in India (Tamilarasu *et al*., 2005). High level ciprofloxacin resistance in *S. enterica* serovar Typhi has also been recently reported by Hasan *et al*. (2005). Since, physicians still administer ciprofloxacin for the treatment of typhoid fever ciprofloxacin resistance and reduced susceptibility to ciprofloxacin is a matter of concern.

Resistance to quinolones arise mainly due to a number of mechanisms, including point mutations that result in amino acid substitutions in DNA gyrase (*gyrA* and *gyrB*) and topoisomerase IV

Corresponding Author: N. Ayana, Department of Biotechnology and Microbiology, School of Life Sciences, Kannur University, Thalassery Campus, Palayad Kannur, Kerala, 670 661, India

(*parC* and *parE*) which maintain the level of super coiling of bacterial DNA, reduced outer membrane permeability, increased efflux of drugs and plasmid encoded gene (Katie *et al*., 2005; George, 2005; Piddock, 2002; John *et al*., 2005; George *et al*., 2006). Since, there is an increase in the occurrence of antibiotic resistant *S. typhi* isolates routine surveillance should be performed to determine the extent of antibiotic resistance and to provide suitable guidelines for treatment. The aim of the study was to examine the mechanism of increase in ciprofloxacin resistance in *S. typhi* isolates in Kerala by testing antimicrobial susceptibility, MIC determination, plasmid analysis and mutation analysis of quinolone resistance determining region (QRDRs) of *gyrA*, *gyrB*, *parC* and *parE* gene of flouroquinolone non-susceptible isolates.

MATERIALS AND METHODS

Bacterial Strains

A total of 48 strains of *S. enterica* serovar Typhi were isolated from blood cultures of febrile patients from various hospitals in Kerala, India from the period 2006-2007. Strains were tested biochemically and confirmation was done using agglutinating O antisera.

Antimicrobial Susceptibility Testing

S. enterica serovar Typhi isolates were tested for susceptibility to antimicrobials by disc diffusion technique according to guidelines provided by the Clinical and Laboratory Standards Institute, CLSI (Formerly National Committee for Clinical Laboratory Standards) (NCCLS, 2006) using commercially available antimicrobial discs (Hi Media Laboratories, India). Following antimicrobials were tested: chloramphenicol (30 μg), ampicillin (10 μg), amoxycillin (30 μg), gentamycin (10 μg), co-trimoxazole (25 μg), tetracycline (30 μg), nalidixic acid (10 μg), ciprofloxacin (5 μg), ofloxacin (5 μg) and ceftriaxone (25 μg). *Escherichia coli* ATCC 25922 was used as control strain for antimicrobial susceptibility testing.

Determination of Minimum Inhibitory Concentration (MIC)

Minimum Inhibitory Concentration (MIC)s of ampicillin, amoxycillin, tetracycline, gentamycin, chloramphenicol, ciprofloxacin, ofloxacin, nalidixic acid and ceftriaxone were determined for each strain by broth macrodilution method according to guidelines provided by CLSI (NCCLS, 2000).

Plasmid DNA Analysis

All isolates of *S. enterica* serovar Typhi isolates were subjected to plasmid profiling. Plasmid DNA was extracted by rapid procedure using alkaline lysis method (Kado and Len, 1981). Plasmid DNA was analyzed on its electrophoretic movement on 0.8% agarose gel at 100 V for 2 h. Molecular mass of plasmid DNA was estimated by Hind III digested Lambda DNA marker.

Transformation Experiments

Transformation experiments with plasmid DNA from ciprofloxacin resistant *S. enterica* serovar Typhi isolates were done to study whether genes encoding flouroquinolone resistance is plasmid borne. Plasmid DNA isolated from ciprofloxacin resistant isolates was transformed in to *E. coli* DH5α using standard protocol (Sambrook *et al*., 1989). Transformants were grown on LB plates containing 5 mg L^{-1} of ciprofloxacin.

Amplification of Quinolone Resistance Determining Region of *gyrA*, *gyrB*, *parC* and *parE* Genes

Polymerase chain reaction (PCR) primers were designed using Primer 3 programme. (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_results.cgi). Primers were synthesized using DNA

Table 1: Primers used for amplification and sequencing of *gyrA*, *gyrB*, *parC* and *parE* genes

Primer	Sequence	Length	Amplicon size
gyrA1	5' GGTACACCGTCGCGTACTTTT3'	21	580 bp
gyrA2	5' ACCGGTACGGTAGGCTTCTT 3'	20	
gyrB1	5' TACCTGCTGGAAAACCCATC3'	20	550 bp
gyrB2	5' CCTGCTTGCCTTTCTTCACT 3'	20	
parC1	5' CGCCTTGCGCTACATGAAT 3'	19	425 bp
parC2	5' GAAGTTTGGCCACCCAGTCC 3'	20	
parE1	5' GTACCGCGCAGGATCTTAATC 3'	21	410 bp
parE2	5' CGTCAGCGGTAATAGACCT 3'	19	

synthesizer (AB1 3900 DNA synthesizer). Chromosomal DNA from ciprofloxacin resistant isolates was prepared by boiling the strains for 10 min, followed by centrifugation at 13,000 rpm for 2 min to obtain the supernatant. PCR was performed on MJ Research thermal cycler™ with the four primer pairs shown in Table 1.

PCR was performed in a total volume of 25 μL which contained 5 μL of supernatant, 25 pmol of each primer, 200 μM dNTPs, 1.5 mM $MgCl_2$ and 0.5 U of Taq polymerase (Bangalore Genei, India). After an initial denaturation step of 3 min at 94°C, amplification was performed over 30 cycles, each consisting of 1 min at 94°C, 1 min at hybridization temperature (56°C for *gyrA,* 58°C for *gyrB* and 53°C for *parC* and *parE*) and 1 min at 72°C, with a final extension step of 10 min at 72°C. PCR products were purified according to standard procedures (Sambrook *et al*., 1989).

Sequencing and Analysis of Quinolone Resistance Determining Region of *gyrA*, *gyrB*, *parC*, *parE* Genes

Sequencing of amplified quinolone resistance determining region of *gyrA*, *gyrB*, *parC* and *parE* genes was carried out by the method of Sanghavi *et al*. (1999) in an automated DNA sequencer (ABI 3100 Genetic Analyzer) with primers given in Table 1. DNA sequences of amplified regions were analyzed using commercial software (Chromas software). Sequences were compared using BLAST analysis with nucleotide sequence database of *gyrA*, *gyrB*, *parC* and *parE* under the following accession numbers: *S. enterica* serovar Typhi *gyrA* accession No: AB071870, *S. enterica* serovar Typhi *gyrB* accession No: AB072396, *S. enterica* serovar Typhi *parC* accession No: AB071987 and *S. enterica* serovar Typhi *parE* accession No: AB072701.

RESULTS

The study revealed that 68.7% *S. enterica* serovar Typhi isolates were resistant to nalidixic acid (Fig. 1). *S. typhi* isolates were categorized in to ciprofloxacin sensitive, intermediately susceptible and resistant isolates by disk diffusion technique. Fifty percent isolates showed susceptibility to ciprofloxacin, 50% were intermediately susceptible and none were resistant. 16.7% of isolates showed resistance to ofloxacin. None of the isolates exhibited multiple drug resistance. All strains were sensitive to chloramphenicol. Fifty percent strains were resistant to tetracycline. 8.3% isolates showed resistance to ceftriaxone and 23% showed intermediate resistance to ceftriaxone. Antibiotyping of *S. typhi* strains yielded 18 biotypes (Table 2).

A summary of MIC values and number of strains is shown in Table 3. MIC values of ciprofloxacin ranged between 0.25-16 mg L^{-1}. According to NCCLS break point the criteria for ciprofloxacin resistance was an MIC value of ≥ 4 mg L^{-1}, for decreased susceptibility MIC value was in range between 1 and <4 mg L^{-1} and for susceptible strains MIC value was <1 mg L^{-1}. So, in this study out of 48 isolates, 23 (47.9%) isolates showed ciprofloxacin resistance and 10 (20.8%) isolates showed decreased susceptibility to ciprofloxacin. 36 (75%) isolates showed MIC value of >4 mg L^{-1} for ofloxacin. One strain had MIC value of 16 mg L^{-1} for ciprofloxacin and 64 mg L^{-1} for ofloxacin.

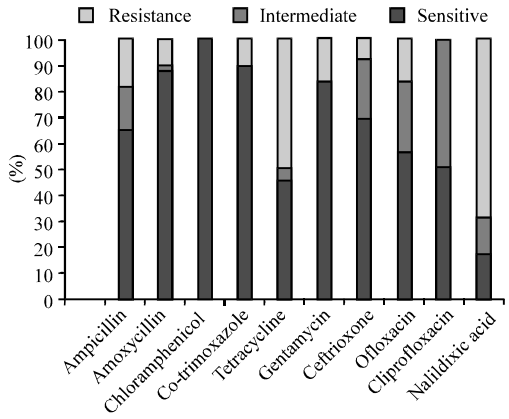

Fig. 1: Antimicrobial susceptibility pattern of *Salmonella enterica* serotype Typhi isolates from Kerala during 2006-2007 (n = 48)

Table 2: Antibiotyping of *S. typhi* isolates from Kerala (n = 48)

Antibiotype	Sensitivity pattern	No. of strains with pattern
ABT1	Amp Chl Tet Amx Gen Sxt Cro	7(14.6)
ABT2	Amp Chl Tet Amx Gen Sxt Cro Ofl	3(6.3)
ABT3	Amp Chl Amx Gen Sxt Cro	6(12.5)
ABT4	Amp Chl Amx Sxt Cro Nal Ofl Cip	1(2.1)
ABT5	Amp Chl Tet Amx Gen Sxt Cro Nal Ofl Cip	2(4.2)
ABT6	Chl Amx Sxt	4(8.3)
ABT7	Chl Gen Sxt Nal Ofl Cip	3(6.3)
ABT8	Amp Chl Tet Amx Gen Sxt	1(2.1)
ABT9	Amp Chl Gen Sxt Cro Nal Ofl Cip	1(2.1)
ABT10	Amp Chl Tet Sxt Cro Ofx	1(2.1)
ABT11	Chl Tet Amx Gen Sxt Nal Ofl Cip	1(2.1)
ABT12	Amp Chl Amx Gen Sxt Cro Ofl Cip	4(8.3)
ABT13	Chl Tet Amx Gen Sxt Cro Ofl Cip	2(4.2)
ABT14	Amp Chl Tet Amx Gen Sxt Ofl Cip	4(8.3)
ABT15	Amp Chl Tet Amx Gen Cro Ofl Cip	1(2.1)
ABT16	Chl Amx Gen Cro Ofl Cip	4(8.3)
ABT17	Chl Tet Ofl Cip	1(2.1)
ABT18	Chl Amx Sxt Cro	2(4.2)

n = Total number of strains analyzed. Values in parentheses indicates percentage; Amp: Ampicillin, Chl: Chloramphenicol, Tet: Tetracycline, Amx: Amoxycillin, Gen: Gentamycin, Sxt: Co-trimoxazole, Cro: Ceftriaxone, Nal: Nalidixic acid, Ofl: Ofloxacin, Cip: Ciprofloxacin

Table 3: Minimum inhibitory concentration of *S. typhi* isolates (n = 48)

	MIC values (mg L^{-1})										
	<1	1	2	4	8	16	32	64	128	256	>256
Antibiotics	No. of strains										
Ampicillin	8	12	3	3	3	5	3	2	3	4	2
Amoxycillin	10	8	2	7	10	3	2	1	3		0
Tetracycline	19	4	0	1	1	2	0	9	9	1	2
Gentamycin	14	24	3	1	0	3	1	1	1	0	0
Chloramphenicol	14	15	7	7	4	1	0	0	0	0	0
Ciprofloxacin	15	3	7	17	5	1	0	0	0	0	0
Ofloxacin	10	1	1	18	11	6	0	1	0	0	0
Ceftriaxone	16	10	9	4	2	3	1	3	0	0	0
Nalidixic acid	0	3	2	1	1	2	2	4	8	1	15

n = Total number of strains analyzed

Plasmid profile analysis revealed that none of the isolates carried high molecular weight plasmids. Eighty five percent of isolates carried plasmid with 23 Kb molecular weight and all the isolates had smaller bands of plasmids.

To find out whether flouroquinolone resistance associated with each strain was located on a transformable plasmid, plasmid DNA from ciprofloxacin resistant *S. typhi* isolates was transformed to *E. coli* DH5α. The transformants was not able to grow on media containing 5 mg L^{-1} of ciprofloxacin.

Quinolone resistance determining region (QRDR) of *gyrA*, *gyrB*, *parC* and *parE* genes of 20 ciprofloxacin resistant isolates (13 strains that showed resistance to ciprofloxacin and 7 strains that showed decreased susceptibility to ciprofloxacin) and two ciprofloxacin susceptible strains were amplified and sequenced. DNA sequences were compared using BLAST analysis with that of nucleotide sequence database of 4 genes. Out of 20 ciprofloxacin resistant isolates 18(90%) isolates contained single amino acid substitution at 83 position in quinolone resistance determining region of *gyrA* and 60% isolates with high level ciprofloxacin resistance (MIC≥ 4 mg L^{-1}) contained a double amino acid alteration at 83 and 87 position in quinolone resistance determining region of *gyrA* and single alteration at 84 position in quinolone resistance determining region of *parC*. Substitutions were Ser 83→Phe and Asp87→Asn, Tyr in gyrA, or Glu84→Lys in *parC*. Amino acid substitutions were not detected in quinolone resistance determining region of *gyrB* and *parE*. No alteration in quinolone resistance determining region of *gyrA*, *gyrB*, *parC* and *parE* were detected in two ciprofloxacin susceptible strains.

DISCUSSION

Recently, there is an increase in the occurrence of antibiotic resistant bacteria of all genera. So, scientific community should perform routine surveillance of microbial population to determine the extent of antibiotic resistance to provide suitable guidelines for treatment. In the present study, 48 clinical isolates of *S. typhi* from Kerala were tested for their susceptibility to 10 antibiotics used in the treatment of typhoid fever. MDR *S. enterica* serovar Typhi were not isolated in this study. Studies conducted at various regions in India also shows there is a decrease in the occurrence of MDR *S. typhi* (Madhulika *et al*., 2004; Sanghavi *et al*., 1999; Saha *et al*., 2002). Reoccurrence of resistant strains has also been reported by Kumar *et al*. (2002). Studies in US reported an increase in the number of MDR nalidixic acid resistant *S. typhi* (Marta-Louise *et al*., 2000; Jennifer *et al*., 2007) whereas studies in Bangladesh reported an decrease in MDR isolates with no corresponding increase in sensitive strains (Rahman *et al*., 2002).

There was an increase in the number of strains showing resistance to nalidixic acid, ciprofloxacin and ceftriaxone. Fifty percent of isolates were identified as intermediately susceptible to ciprofloxacin by disk diffusion technique. There was an increased value of MICs of ciprofloxacin. MIC values of ciprofloxacin of isolates ranged between 0.25-16 mg L^{-1}. This finding was similar to earlier studies in UK and India (Threlfall and Ward, 2001; Jesudason *et al*., 1996). MIC values showed that 48% isolates were ciprofloxacin resistant and 21% showed decreased susceptibility to ciprofloxacin. 8.3% strains showed resistance and 23% strains showed intermediate resistance to ceftriaxone which was in contrast to earlier study in which all strains were reported as ceftriaxone sensitive (Pallab *et al*., 2006).

The present study also focused to examine the genetic locus of flouroquinolone resistance. Flouroquinolone resistance usually arises spontaneously due to mutations within the Type II topoisomerase genes often in combination with decreased expression of multidrug efflux pump. Some studies reports that quinolone resistant gene is located on plasmids. Plasmid mediated quinolone resistance was first reported in a clinical isolate of *Salmonella enterica* strain in UK in 2007 (Katie *et al*., 2007). To find out whether flouroquinolone resistance associated with *S. typhi* isolates

was located on a transformable plasmid we conducted transformation experiment. The result showed that none of the isolates carry ciprofloxacin resistance genes on plasmid.

Earlier studies reported that flouroquinolone resistance arises due to mutations in quinolone resistance determining region of genes encoding DNA gyrase (*gyrA*, *gyrB)* or DNA topoisomerase IV (*parC*, *parE*) (Katie *et al*., 2005; George, 2005; Piddock, 2002). We tried to characterize *gyrA*, *gyrB*, *parC* and *parE* mutations in ciprofloxacin resistant isolates in Kerala. Ninety percent of ciprofloxacin resistant isolates had single mutation in the quinolone resistance determining region of *gyrA*. Sixty percent isolates with high level of ciprofloxacin resistance carried double mutation in *gyrA* and single mutation in *parC*. The result is in consistent with earlier findings that *gyrA* mutations are necessary for quinolone resistance. *parC* mutations facilitate increasing levels of resistance (Piddock, 2002).

CONCLUSION

There was an increase in the number of isolates showing resistance to flouroquinolones and ceftriaxone. We have found out that flouroquinolone resistance in *S. typhi* isolates was not plasmid encoded. We had also found out that mutations in quinolone resistance determining region of *gyrA* play an important role in flouroquinolone resistance of *S. typhi* isolates and *parC* mutation facilitate increasing level of resistance.

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