MAK HILL Publications International Journal of Tropical Medicine

Research Article
doi: 10.59218/makijtm.2023.2.6.11

Mutational Analysis of gyrA, gyr B and par C genes Encoding for Fluoroquinolones Resistance in *Salmonella typhi* and *Salmonella paratyphi* a Isolated from Blood Culture of Enteric Fever Patients in a Tertiary Care Hospital

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OPEN ACCESS

Key Words
Salmonellae, fluoroquinolones, gyrA gene, parC gene

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Received: 12 August 2023
Accepted: 23 August 2023
Published: 24 August 2023

Citation: M. Banumathy, S.Viji and V.M. Theeba, 2023. Mutational Analysis of gyrA, gyr B and par C Genes Encoding for Fluoroquinolones Resistance in *Salmonella typhi* and *Salmonella paratyphi* a Isolated from Blood Culture of Enteric Fever Patients in a Tertiary Care Hospital. Int. J. Trop. Med., 18: 6-11, doi: 10.59218/makijtm.2023.2.6.11

ABSTRACT

Emergence of multi-drug resistant *Salmonella* strains, made the fluoroquinolones like Ciprofloxacin being the drug of choice in the treatment of typhoid feverearlier. But indiscriminate use of fluoroquinolones led to the development of resistance against these agents also. Present study was aimed to study mutational analysis of gyrA, gyr B and par C genes encoding for fluoroquinolones resistance in *Salmonella typhi* and *Salmonella paratyphi* A isolated from blood culture of enteric fever patients in a tertiary care hospital. Present study was single-center, prospective, observational study, conducted in patients with signs and symptoms of enteric fever. Study on molecular characterization of anti-microbial resistance in *Salmonella enterica* serovar typhi and paratyphi was carried out. 250 patients of clinically suspected enteric fever cases were analysed. A total of 28 (11.2 %) blood culture positive *Salmonella enterica* were isolated from 250 blood culture samples, serovars identified were 24 *Salmonella typhi* (86%) and 4 *Salmonella paratyphi* A (14%). Male: Female ratio of the patients included in this study was 1.03: 1.Majority of the *Salmonella enterica* species was isolated from the age group of 21-30 years (33%), followed by 11-20 years and 31-40 years (25% each). The antimicrobial susceptibility pattern of *Salmonella* typhi showed 100% sensitivity to Chloramphenicol, Cotrimoxazole, Ampicillin, Cefotaxime, Ceftriaxone and Azithromycin whereas 50% of the isolates were resistant to Nalidixic acid, Pefloxacin and Ciprofloxacin. The quinolone resistance was found to be high in *S. paratyphi* A (50%) than in *S. typhi* (25%). All the 25% (n = 24), of quinolone resistant *S. typhi* isolates and 50% (n = 4) of *S. paratyphi* A isolates were found to be negative for qnrA, qnrB and qnrS which are plasmid mediated quinolone resistance genes. The present study reveals that, typhoidal *Salmonellae* isolated were mostly resistant to fluoroquinolones, which was due to mutations in the quinolone resistance determining region (QRDR) of gyrA and parC gene.

INTRODUCTION

Enteric fever is a world health problem, which occurs predominantly in the developing countries like India[1]. The term enteric fever comprises of both typhoid fever and paratyphoid fever. Typhoid fever is caused by *Salmonella enterica* subspecies enterica serovar typhi.The organism that causes Paratyphoid fever can be either of the three serovars *Salmonella* paratyphi A, *Salmonella* paratyphi B (known as scholtmuelleri) or *Salmonella* paratyphi C[2,3].

Enteric fever is an acute systemic illness characterized by step ladder pattern type of remittent fever, rose spots, coated tongue, hepatosplenomegaly and relative bradycardia. The complications of enteric fever are gastrointestinal bleeding, intestinal perforation and the neurological manifestations that occurs rarely like meningitis, cerebellar ataxia and neuropsychiatric symptoms[1,2].

Antibiotics are the mainstay for treatment of enteric fever. Prompt treatment of the disease with appropriate anti-microbials in appropriate time is a major criteria in reducing the mortality from 30% to 0.5%[4]. The emergence of multi-drug resistant *Salmonella* strains, made the fluoroquinolones like Ciprofloxacin being the drug of choice in the treatment of typhoid fever. But indiscriminate use of fluoroquinolones led to the development of resistance against these agents also. This condition is further complicated by the emergence of quinolone resistant strain and there are studies that show, a steady increase in minimum inhibitory concentration (MIC) of Ciprofloxacin[5,6].

Fluoroquinolones target DNA gyrase and topoisomerase IV, which are bacterial enzymes that are a part of a complex which uncoils and recoils the bacterial DNA for transcription[4]. *Salmonella typhi* commonly develops fluoroquinolone resistance through specific mutations in gyr A and par C, that codes for binding region of DNA gyrase and topoisomerase IV, respectively.Present study was aimed to study mutational analysis of gyrA, gyr B and par C genes encoding for fluoroquinolones resistance in *Salmonella typhi* and *Salmonella paratyphi* A isolated from blood culture of enteric fever patients in a tertiary care hospital

MATERIALS AND METHODS

Present study was single-center, prospective, observational study, conducted in department of microbiology, at Coimbatore Medical College and Hospital, Coimbatore, India. Study duration was of 1 year (July 2016 to June 2017). Ethical clearance was obtained from Institutional Ethics Committee and informed consent was obtained from the patients and the parents or guardians of the accompanying children.

Inclusion criteria: Patients with signs and symptoms of enteric fever like (step ladder pattern type of remittent fever, relative bradycardia, hepatosplenomegaly and rose spots) of all age groups attending on an outpatient or inpatient basis, willing to participate in present study.

Exclusion criteria: Those patients who had taken antibiotics within a week.

Study on molecular characterization of anti-microbial resistance in *Salmonella enterica* serovar typhi and paratyphi from blood culture isolates in a tertiary care hospital was carried out in fever op and in medicine/paediatric department. A detailed history regarding the patients name, age, address, duration and history of presenting illness, treatment history about antibiotics taken were obtained.

Under aseptic precautions, 5 and 10 mL of venous blood was collected from children and adults respectively in patients of acute febrile illness. Blood samples collected were inoculated aseptically into blood culture bottle containing 25-50 mL of Brain heart infusion broth and incubated aerobically at 37°C. The broth was examined regularly for bacterial growth like turbidity and subculture was done on Nutrient agar, Mac Conkey agar and on Blood Agar plate. The plates were incubated for 24 hrs at 37°C. Any growth was further processed for identification as per the standard procedure. The organisms were identified by their colony morphology, Gram staining methods, motility test and biochemical reactions with suitable controls. (Catalase, Oxidase, Indole test, Citrate test, Urease test, Triple sugar iron test, Nitrate reduction test, Methyl red and voges-Proskauer test, Sugar fermentation tests, Decarboxylation test, Slide Agglutination test)

Antibiotic susceptibility testing was done by modified Kirby-Bauer disc diffusion technique using Mueller Hinton agar as per CLSI guidelines. The result was compared with McFarlands turbidity standard. The Quinolone resistant *Salmonella enterica* species were subjected for the plasmid mediated quinolone resistance genes qnrA, qnrB and qnrS, as well as chromosomal mediated resistance genes gyrA, gyrB and parC by Multiplex PCR.

The statistical analysis was done using R version 3.2 and p- value was calculated using Chi-Square test and graphs were made on Microsoft Excel sheet.

RESULTS

250 patients of clinically suspected enteric fever cases were analysed. A total of 28 (11.2%) blood culture positive *Salmonella enterica* were isolated from 250 blood culture samples, serovars identified were 24 *Salmonella typhi* (86%) and 4 *Salmonella* paratyphi A (14%) (Table 1).

Table 1: Distribution of *Salmonella* species

Salmonella species	No. of Isolates	Percentage
Salmonella typhi	24	86
Salmonella paratyphi A	4	14

Table 2: Gender distribution

Gender	Suspected enteric fever cases (n = 250)	Culture positive enteric fever (n = 28)	Percentage
Male	127	19	15
Female	123	9	7

Table 3: Age wise distribution

	Salmonella typhi Isolated (n = 24)		*Salmonella paratyphi* A Isolated (n = 4)		Percentage (n = 28)	
Age (years)	No.	Percentage	No.	Percentage	No.	Percentage
1-10	1	4	-	-	1	4
11-20	7	29	-	725		
21-30	6	25	3	75	9	32
31-40	6	25	1	25	7	25
41-50	3	13	-	-	3	11
>50 years	1	4	-	-	1	4

Table 4: Correlation between neutrophil count and blood culture positive enteric fever

Neutrophil counts	No. of blood culture positive (n = 28)	Percentage
Neutropenia	26	93
Neutrophilia	1	3.5
Normal	1	3.5

Table 5: Correlation between hepatosplenomegaly and blood culture positive enteric fever

Liver and spleen	No. of. Blood culture positive (n = 28)	Percentage
Enlarged	28	100
Normal	0	0

Table 6: Antimicrobial susceptibility pattern of *Salmonella typhi*

	Salmonella typhi (n = 24)	
Antibiotic disc	Sensitive (%)	Resistant (%)
Chloramphenicol (30 µg)	23 (95)	1 (4)
Ampicillin (10 µg))	22 (91)	2 (8)
Trimethoprim/sulfamethoxazole (1.25/23.75 µg)	22 (91)	2 (8)
Nalidixic Acid (30µg)	18 (75)	6 (25)
Ciprofloxacin (5µg)	18 (75)	6 (25)
Pefloxacin (5 µg)	18 (75)	6 (25)
Ceftriaxone (30µg),	24 (100)	-
Cefotaxime (30µg)	24 (100)	-
Azithromycin (15 µg)	24 (100)	-
Multi-drug resistance	-	1 (4)

Table 7: Antimicrobial susceptibility pattern of *Salmonella paratyphia* (n = 4)

	Salmonella paratyphia (n = 4)	
Antibiotic disc	Sensitive (%)	Resistant (%)
Chloramphenicol (30 µg)	4 (100)	-
Ampicillin (10 µg)	4 (100)	-
Trimethoprim/sulfamethoxazole (1.25/23.75 µg)	4 (100)	-
Nalidixic Acid (30 µg)	2 (50)	2 (50)
Ciprofloxacin (5 µg)	2 (50)	2 (50)
Pefloxacin (5 µg)	2 (50)	2 (50)
Ceftriaxone (30 µg)	4 (100)	-
Cefotaxime (30 µg)	4 (100)	-
Azithromycin (15 µg)	4 (100)	-

Male: Female ratio of the patients included in this study was 1.03: 1, the ratio of culture positives was higher among the males than females (2.1: 1), was statistically significant (p<0.05) (Table 2).

Majority of the *Salmonella enterica* species was isolated from the age group of 21-30 years (33%), followed by 11-20 years and 31-40 years (25% each) (Table 3).

26 (93%) of blood culture positive cases showed neutropenia, whereas neutrophilia and normal neutrophil count was observed in 1 (3.5%) case of enteric fever. The statistical p<0.001 that is significant (Table 4).

Hepatosplenomegaly was observed in all the 28 (100%) blood culture positive cases. The statistical p<0.001 being significant (Table 5).

The antimicrobial susceptibility pattern of *Salmonella typhi* showed 100% sensitivity to Cefotaxime, Ceftriaxone and Azithromycin. 95% of S.typhi isolates were sensitive to Chloramphenicol and 91% of S.typhi isolates were sensitive to Ampicillin and Cotrimoxazole. Whereas the 25% of the isolated S.typhi strains were resistant to Nalidixic acid (surrogate marker for fluoroquinolone resistance) and to Pefloxacin (a surrogate marker, as per CLSI 2015). The resistance to Ciprofloxacin was also found to be 25%. Only a single strain (4%) out of the 24 isolated Salmonella typhi was found to be multi-drug resistant and this isolate was also resistant to fluoroquinolones (Table 6).

The (100%) sensitivity was observed in Chloramphenicol, Cotrimoxazole, Ampicillin, Cefotaxime, Ceftriaxone and Azithromycin whereas 50% of the isolates were resistant to Nalidixic acid, Pefloxacin and Ciprofloxacin (Table 7).

In present study, 25% of the S.typhi isolates and 50% of S.paratyphi A showed resistance to fluoroquinolones. The quinolone resistance was found to be high in *S. paratyphi* A (50%) than in *S. typhi* (25%) (Table 8).

All the 25% (n = 24), of quinolone resistant S.typhi isolates and 50% (n = 4) of *S. paratyphi* A isolates were found to be negative for qnrA, qnrB and qnrS which are plasmid mediated quinolone resistance genes. All the 6-fluoroquinolone resistant *S. typhi* isolates showed mutations in gyrA gene in the quinolone resistance determining region, whereas only two of them showed mutations in parC gene. 2 of the fluoroquinolone

Table 9: analysis of resistant genes by molecular methods

Salmonella species	No. of resistant isolates	PMQR genes			Mutation of QRDR genes		
		qnrA	qnrB	qnrS	gyrA	gyrB	parC
Salmonella typhi	6	0	0	0	6 (100)	0	2 (33)
Salmonella paratyphi A	2	0	0	0	2 (100)	0	2 (100)

PMQR: Plasmid mediated quinolone resistance and QRDR: Quinolone resistance determining region

Table 10: mutation analysis of gyrA, gyrB and parC genes

Resistant Strain	Mutations in gyrA	Mutations in gyrB	Mutations in parC
Salmonella typhi 1	Serine 83-Phenylalanine	No mutation	No mutation
Salmonella typhi 2	Serine 83-phenylalanine aspartic acid 87-Asparagine	No mutation	No mutation
Salmonella typhi 3	Serine 83-tyrosine	No mutation	No mutation
Salmonella typhi 4	Serine 83-tyrosine	No mutation	No mutation
Salmonella typhi 5	Serine 83-tyrosine	No mutation	Threonine -57 Serine
Salmonella typhi 6	Serine 83-phenylalanine aspartic acid 87-asparagine	No mutation	Threonine -57 Serine
Salmonella paratyphi A1	Serine 83-phenylalanine aspartic acid 87-asparagine	No mutation	Serine 80 - Isoleucine
Salmonella paratyphi A 2	Serine 83-phenylalanine aspartic acid 87-asparagine	No mutation	Serine 80- Isoleucine

resistant *S. paratyphi* A isolates showed mutations in gyrA and parC gene. None of the fluoroquinolone resistant isolates of *S. typhi* and *S. paratyphi* A showed mutations in gyrB gene. Some of the isolates showed single point mutation and few others showed two to three mutations in gyrA and parC gene (Table 9).

The mutations observed in gyrA gene was at position 83 where serine was replaced by phenyl alanine in all the six quinolone resistant *S. typhi* isolates and two of those isolates showed mutation at position 87, Aspartic acid being replaced by asparagine. Whereas in parC gene mutation was detected at position 57 threonine replaced by serine and at position 80 serine was replaced by isoleucine (Table 10).

DISCUSSIONS

Enteric fever remains a global health problem, especially in developing countries like India. In the past with the widespread emergence and spread of multi-drug resistance Salmonella typhi strains, Ciprofloxacin became the first line drug in treatment of enteric fever[7]. Later Salmonella enterica species developed resistance to quinolones by various mechanisms[7]:

- Mutations in the gyrA and gyrB genes encoding DNA gyrase, parC and parE genes that encodes topoisomerase
- Plasmid mediated quinolone resistance genes qnrA, qnrB and qnrS that are present in Salmonella enterica species being transmitted by plasmids or transposon
- Decreased permeability to quinolones
- Overexpression of efflux pumps

Then third generation Cephalosporins, Cefotaxime and Ceftriaxone were effective in treatment of enteric fever. Later, due to production of Extended spectrum beta lactamases *S. typhi* develops resistance to these group of drugs. Recently there are studies showing S.typhi strains developing resistance to Azithromycin also.

In this study, 250 clinically suspected cases of enteric fever were investigated and 28 (11.2%) were found to be blood culture positive for Salmonella enterica species. This isolation rate (11.2%) is comparable with the 14% isolation by Suresh *et al*.[9].

Among 28 isolated Salmonella enterica species the serovars identified were 24 (85.7%) S. typhi and 4 (14.2%) *S. paratyphi* A which is consistent with the studies of Veeraraghavan *et al*.[10] with the isolation of *S. typhi* (84%) and *S. paratyphi* A (15%). Another study by Shoorashetty *et al*.[11] also identified 80% S.typhi and 20% *S. paratyphi* A. Girotra *et al*.[12] showed an isolation rate of *S. typhi* (78%) and *S. paratyphi* A(22%). The sex predilection is more in males than in females with a ratio of 2.1 : 1, which is comparable with the other studies of Shoorashetty *et al*.[11] Riyazchungathu *et al*.[13] and Jain and Chugh[6].

In the present study, Salmonella enterica species in blood culture were isolated predominantly in young adults, 21-30 years (32%) and then, in the age group of, 11-20 years (25%), while the prevalence was low in extremes of age like <10 years and >50 years (i.e., 1%). This correlates with the study by Lovely Akter *et al*.[2].

The increased prevalence of enteric fever in males and in the age group of young adults can be attributed to the fact that they are more vulnerable to stay outside the home for various reasons like study, occupation and are likely to take outside food, with the possibility of contamination. Personal hygiene, proper sanitation and vaccination will prevent these situations.

The 24 isolates of S.typhi and four of S.paratyphi A isolates were 100% sensitive to Cefotaxime, Ceftriaxone and Azithromycin which correlates the study of Nata, Veeraraghavan *et al*.[10], Raveendaran *et al*.[14]. In contrast to this there are studies like Jain and Chugh[6]. Altayb[15] which shows that S.typhi strains have developed low level of resistance to ceftriaxone. The development of resistance to III generation Cephalosporins and Azithromycin can be attributed to the facts like inappropriate prescription and irrational use of these drugs by clinicians.

The irrational use of fluoroquinolones following emergence of MDR *S. typhi* strains and the rampant use of Ciprofloxacin not only for enteric fever but also for other infections has led to the development of resistance for these drugs. The antimicrobials like Ceftriaxone, Cefixime and Azithromycin are effective against fluoroquinolone resistant isolates, which showed 100% sensitivity in this study but are cost-effective. The other alternatives are Ampicillin, Cotrimoxazole and Chloramphenicol but the clinical outcome is questionable and the re-emergence of MDR isolates must be borne in mind.

The quinolone resistance is mediated by mutations in chromosomal genes (gyrA, gyrB and parC). The point mutations alter DNA gyrase and topoisomerase IV that targets the quinolone drugs. A single point mutation at the quinolone resistance determining region of the gyrA confers resistance to Nalidixic acid in S.typhi , whereas complete resistance to fluoroquinolones is usually associated with double mutation in gyrA gene.[3]The common mutations encountered in gyrA gene is a single point mutation at position 83 changing serine to phenylalanine and at position 87 changing aspartate to tyrosine or glycine as explained by the study of Raveendran *et al*.[14] and Dimitrov *et al*.[16].

In this study, fluoroquinolone resistant typhoidal Salmonella isolates were negative for plasmid mediated quinolone resistant genes like qnrA, qnrB and qnrS. But all these isolates were found to have mutations in these genes gyrA and parC which indicates chromosomal mediated resistance that correlates with the studies of Veeraraghavan *et al*.[10], Menezes *et al*.[17], Harish *et al*.[18] and Das *et al*.[19].

Most of the isolates in the present study had mutations in gyrA gene that were observed at position 83, Serine being replaced by phenylalanine and at position 87 Aspartic acid replaced by Asparagine , correlating with the studies of Das *et al*.[19] Godfred A Menezes *et al*.[17] Harish *et al*.[18] and Dimitrov *et al*.[16].

The mutations in par C gene are commonly encountered at position 57 Threonine being replaced by serine and at position 80 threonine being replaced by Isoleucine. In this study few isolates show mutation at position 57 threonine replaced by serine and at position 80 serine being replaced by isoleucine. This correlates with the studies of Dimitrov *et al*.[16], Menezes *et al*.[17] Harish *et al*.[18] and Das *et al.*[19]. The mutations in quinolone resistance determining region genes infers treatment failure if the patients are treated with Ciprofloxacin.

No mutations was observed in gyrB gene in the present study. This correlates with the study of Das *et al*.[19], Veeraraghavan *et al*.[6]. Studies by Altayb *et al*.[15] and Das *et al*.[19] showing mutations in gyrB gene, with following amino acid changes, Methionine-Asparagine, Proline-Arginine, Glutamic acid-Glycine. If mutations occur in all three genes of QRDR region, will lead to an increased in-vitro resistance. Such patients do not respond to treatment with fluoroquinolones and go in for complications resulting in increased morbidity and mortality.

Since *Salmonella* species are developing resistance to various categories of antibiotics, this study is aimed to determine the antimicrobial resistance pattern to design antibiotic policy that aids the clinicians to treat the patients earlier with appropriate antibiotics. This promotes complete cure of the patients and hence prevent them in developing complications of the disease. The patients going on to the carrier state is also eliminated that prevents the epidemics and endemics.

CONCLUSION

The present study reveals that, typhoidal Salmonellae isolated were mostly resistant to fluoroquinolones, which was due to mutations in the quinolone resistance determining region (QRDR) of gyrA and parC gene. Occurrence of mutations in many genes may lead to high–level of Ciprofloxacin resistance. As the isolates were 100% sensitive to Ceftriaxone, it can be considered as an alternative source in treatment of fluoroquinolone resistant strains. Furthermore, MIC of Ceftriaxone and its susceptibility pattern are to be monitored closely, because of their emerging resistance. Narrowing of therapeutic options warrants the control of diseases through proper sanitation, personal hygiene measures, safe water supply, detection and treatment of Typhoid carriers and adoption of vaccination.

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