ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Detection of Mutations in Ciprofloxacin Resistant *Salmonella Enterica serotypes typhi* and *Paratyphi A*

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ABSTRACT

Background: Typhoid fever is a systemic infection caused by *Salmonella* species. Fluoroquinolones are mostly the antibiotic of choice for treatment. Resistance to ciprofloxacin has been noticed to increase due to the emergence of new mutations in bacterial DNA. **Aims:** Analyse the plasmid DNA profile and to detect any mutations in DNA gyrase responsible for ciprofloxacin resistance. **Methods:** In clinical isolates 160 were *Salmonella typhi* and 132 were *Salmonella paratyphi A* collected over five years. The antimicrobial susceptibility was studied. DNA sequencing of *gyrA*, *gyrB*, and *parC* genes was performed in 8 isolates. **Results:** Four enteric fever *Salmonella* isolates in the present study showing ciprofloxacin MIC of 1µg/ml and 4 other randomly chosen isolates with MIC < 1µg/ml were subjected for molecular sequencing by PCR. The 4 ciprofloxacin resistant strains showed a point mutation (C→T or C→A) at 128th base corresponding to amino acid sequence change of Ser→Tyr or Ser→Phe at 83rd position, which was not detected in other 4 strains. Plasmid DNA analysis did not reveal any abnormal R-Plasmid. **Conclusions:** It is very important to keep searching for new mutations and continuously monitor drug resistance in order to efficiently manage cases with enteric fever.

Keywords: Gyrase, *gyrA*, Nalidixic acid resistant *Salmonella paratyphi A*, Nalidixic acid resistant *Salmonella typhi*, Polymerase chain reaction.

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ISSN: 0975-8585

INTRODUCTION

Enteric fever is a worldwide problem and widely prevalent in the developing countries of the tropics [1]. For every ten cases of *Salmonella typhi* infection, there are one or two cases of paratyphoid fever, caused by the human-adapted *Salmonella enterica serovars Paratyphi A, B* and *C*. Since paratyphoid fever is indistinguishable from typhoid fever in its clinical course, *Salmonella enterica serovars Typhi, Paratyphi A*, *B* and *C* are collectively referred to as typhoidal *Salmonella serovars [*2].

Resistance to commonly used antibiotics, such as chloramphenicol, ampicillin, and co-trimoxazole has been reported from different parts of India in the last two decades. In the recent past, fluoroquinolones and cephalosporins have gained importance for the treatment of enteric infections [1]. The problem worsened with the advent of nalidixic acid resistant *Salmonella typhi* (NARST), soon it was found that *Salmonella paratyphi A* clinical isolates world over also showed similar resistance pattern and these were named as nalidixic acid resistant *Salmonella paratyphi A* (NARSPT) [3]. From 1993 onwards *Salmonella* has shown decreased susceptibility to ciprofloxacin and genetic analysis has shown that mutation in the gene *gyrA* is responsible for this [4].

Ciprofloxacin targets the DNA gyrase enzyme of the bacterium and inactivates it. Recently there have been reports of treatment failure with this antibiotic, and a concurrent rise in Minimum Inhibitory Concentration (MIC). Although outright resistance has not yet been reported, the steady increase in MIC indicates that this may occur in the near future [5].

A number of mechanisms have been postulated to account for drug resistance in bacteria. The commonest mode of resistance to quinolones is by a mutation in the gene coding for the A subunit of the gyrase enzyme, *gyrA*. Maximum mutations occur in the Quinolone Resistance Determining Region (QRDR), usually a point mutation at position 83 or 87. The mar locus has been associated with multiple antibiotic resistance in *Enterobacteriaceae* bacteria in which the mar locus is constitutively expressed are protected from the bactericidal effects of fluoroquinolones and mutate more easily to confer resistance to a higher flouroquinolone concentration. Homologous sequences have been detected in *Salmonella typhimurium*, though its role in fluoroquinolone resistance has not been extensively studied [6].

Alteration in outer membrane proteins have been linked to increased resistance to the bactericidal effects of ciprofloxacin. Correlation has been documented between decreased fluroquinolone uptake and decreased production of an outer membrane protein in members of Enterobacteriaceae [7, 8]. Transferable plasmids were found to be responsible for the spread of multi drug resistance in *Salmonella typhi*. These plasmids are of two major types. First is pHCM2 a cryptic plasmid found in Asia and the second larger self transferable plasmid [3, 4, 9]. Hence this study was done to analyse the plasmid DNA profile and to detect any mutations in DNA gyrase responsible for ciprofloxacin resistance.

ISSN: 0975-8585

MATERIALS AND METHODS

Patients attending the outpatient department or admitted at JSS Hospital during Jan 2006-Dec 2010, who are clinically suspected as enteric fever cases were included in the study. Isolates of *Salmonella typhi* and *paratyphi A* from blood cultures were identified on the basis of biochemical reactions and serology [10]. Susceptibility to the quinolones like ciprofloxacin and nalidixic acid was determined by Kirby Bauer disk diffusion method as per Clinical Laboratory Standards Institute (CLSI) guidelines. Additionally the Minimum Inhibitory Concentration (MIC) of ciprofloxacin was determined by agar microdilution [11]. The strains with MIC of 1µg/ml were subjected PCR to detect plasmid DNA profile and mutations in DNA gyrase.

The plasmid DNA was extracted by alkaline lysis method [12]. PCR amplification of QRDR in *gyrA* was done to identify mutations responsible for high level resistance to ciprofloxacins (HLRC). A 290 bp region corresponding to the QRDR in DNA *gyrA* gene of *Salmonella* was targeted using degenerate primers. The primers used in the study were forward primer: 5' CTGAAGCCGGTACACCGTCG '3 and reverse primer: 5' TCGGCCATCAGTTCGTGGGC '3. The constituents of the DNA mixture was 1 µl DNA, 1 µl each of 10pM forward and reverse primers (Eurofins, Bangalore), 2 µl 10 x buffer ,0.6 µl of 2mM dNTPs mix, 1.5 mM MgCl2 and1 U of Taq polymerase enzyme. The volume was made up to 20 µl with sterile distilled water. Amplification was performed in a thermal cycler (eppendorf) with an initial denaturation step at 950C for 5 min, followed by 35 cycles comprising of 1 minute at 940C (denaturation), 1 minute at 520C (annealing),1 minute at 720C (extension) and 5 minutes at 720C (final extension). The PCR product was run in 1% agarose gel and observed under UV transillumination. The band at 290 bp was gel eluted employing Gel extraction method of Nucleospin. The pure eluted product was sequenced at Chromous Biotech, Bangalore. *Salmonella typhimurium* ATCC-13311 was used as the control for DNA sequencing studies.

RESULTS

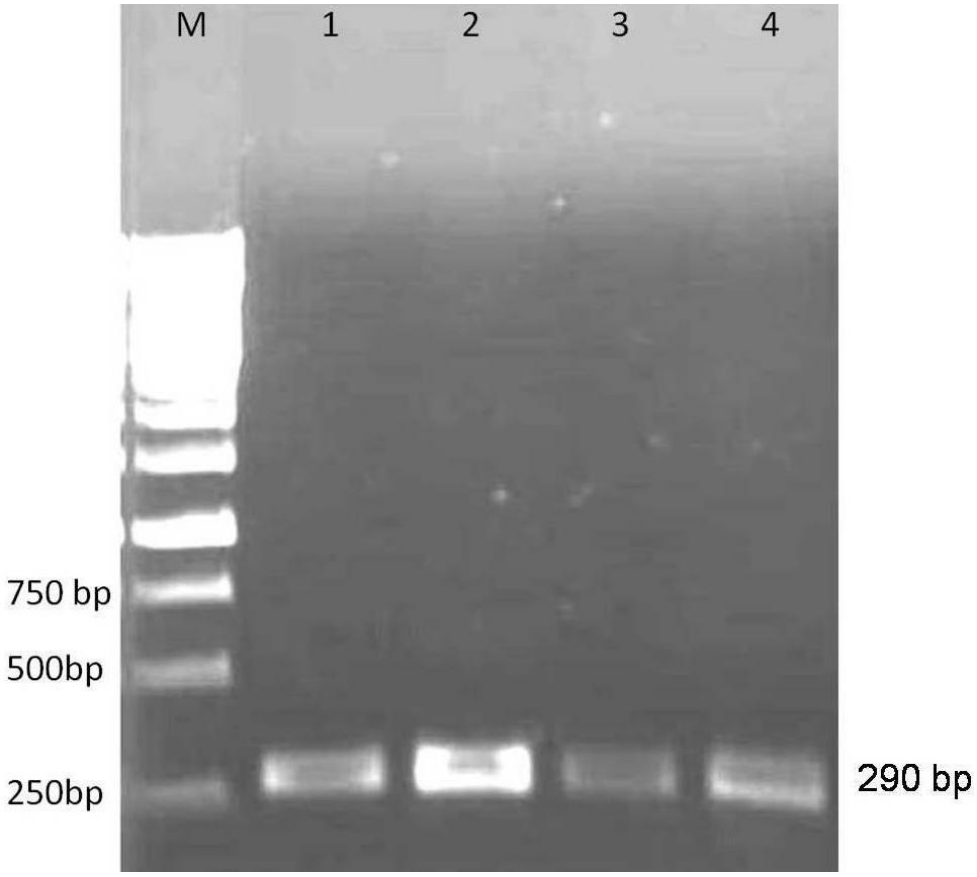
Out of 292 isolates of *Salmonellae* 160 were *Salmonella typhi* and 132 were *Salmonella paratyphi A*. Four enteric fever *Salmonella* isolates in the present study showing ciprofloxacin MIC of 1µg/ml and 4 other randomly chosen *Salmonella* isolates were subjected for molecular sequencing by PCR. Figure 1 shows the QRDR in *gyrA* of *Salmonellae* Lane M-250bp ladder, Lane 1 and 2 *Salmonella typhi*, Lane 3 and 4 *Salmonella paratyphi A*. Gene sequencing and protein sequencing of the isolates showed a point mutation (C→T or C→A) at 128th base corresponding to amino acid sequence change of Ser→Tyr or Ser→Phe at 83rd position. R-Plasmids were not detected in any of the strains (Figure 2).

DISCUSSION

Enteric fever continues to be a public health related problem in all the developing countries. National enteric fever data is found to be 2 to 3 % in India. The present study also found similar rate (2%) of enteric. Antibiotic susceptibility/resistance profile is also found to be similar in these two agents. The antimicrobials used for treatment of enteric fever used to be

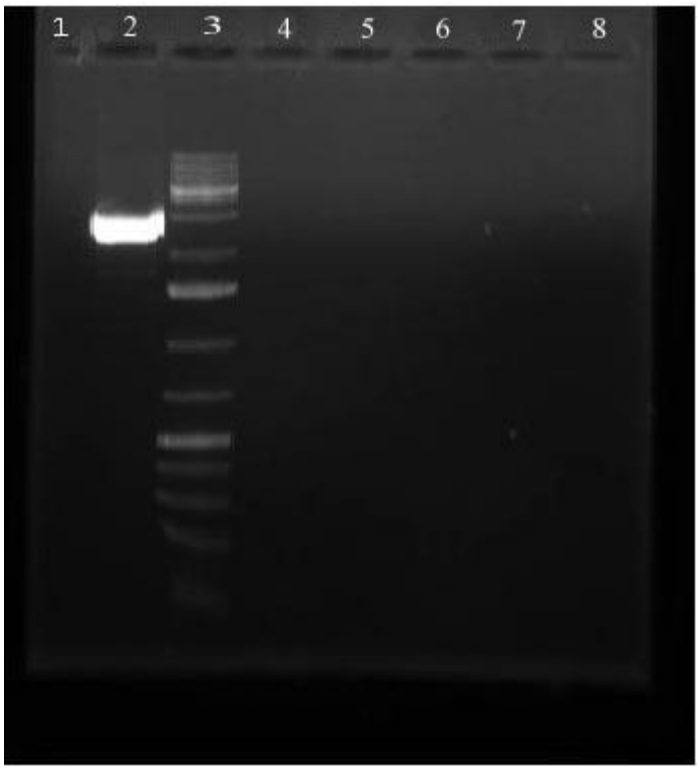
chloramphenicol, trimethoprim-sulfamethoxazole and ampicillin. However, after the development of resistance to these agents, fluoroquinolones such as ciprofloxacin, became the drug of choice for the treatment of this infection [13, 14]. However, there have been several reports, including from India, of therapeutic failure of ciprofloxacin in patients with enteric fever [15, 16]. All these strains were interpreted as ciprofloxacin susceptible by the standard antibiotic susceptibility tests done in a clinical laboratory using the CLSI guidelines but showed a gradual increase in the MIC of ciprofloxacin, although the MIC values were still below the breakpoint of resistance [17, 18]. The present study found that enteric fever *Salmonellae* has regained susceptibility to the drugs to which they had become resistant earlier such as ampicillin, chloromycetin & co-trimoxazole. Similar observations have been made by several workers and other developing nations [19, 20]. Nalidixic acid resistant *Salmonella typhi* are being increasingly isolated in recent years. But all these isolates are found to be susceptible to ciprofloxacin by disc diffusion and by MIC detection [17, 21].

Figure 1: PCR Amplification of QRDR in *gyrA* gene of *Salmonellae.*
Lane M: 250 bp ladder, Lane 1 & 2: *Salmonella typhi*, Lane 3 & 4: *Salmonella paratyphi A*



ISSN: 0975-8585

Figure 2: Plasmid extraction by alkaline lysis method.
Lane 1: Water, Lane 2: pUC 19 plasmid, Lane 3: DNA ladder,
Lane 4 to 6: *Salmonella typhi*, Lane 7 & 8: *Salmonella paratyphi* A



The chromosomal-mediated drug resistance phenomenon against fluoroquinolones has been reported recently as a result of selective pressure on the bacterial population due to their uncontrolled use. This has been attributed to a single point mutation in the quinolone resistance determining region of the topoisomerase gene *gyrA*, which encodes DNA gyrase, other mechanisms such as decreased permeability and active efflux of the antimicrobial agent may also be involved [22]. DNA gyrase is the primary target of quinolone action; a single point mutation in the quinolone resistance-determining region of *gyrA* can mediate resistance to the nonfluorinated quinolone nalidixic acid and reduced susceptibility to fluoroquinolones such as ciprofloxacin. Resistance to quinolones is known to arise due to a number of mechanisms, including point mutations that result in amino acid substitutions in DNA gyrase (*gyrA* and *gyrB*)and topoisomerase IV (*parC) [*22]. During the present study all isolates were susceptible to chloromycetin, co-trimoxazole and only 11 showed zone of inhibition falling into intermediate susceptibility pattern. Hence the Plasmid DNA analysis did not reveal any abnormal R-Plasmid, which is mobile extrachromosomal genetic element having a tendency to be present or absent in bacteria.

Earlier studies reported that strains had a significantly higher range of MIC of ciprofloxacin (0.023 mg/l to 1.0 mg/l) compared to the nalidixic acid sensitive strains (0.0016 mg/l to 0.125 mg/l) 21. We observed that MIC of ciprofloxacin was 1 µg/ml. The 4 ciprofloxacin

resistant strains showed a point mutation (C→T or C→A) at 128th base corresponding to amino acid sequence change of Ser→Tyr or Ser→Phe at 83rd position, which was not detected in other 4 strains. Mutation at 87th position has been reported in several other studies [4, 6, 19]. Other mutations were not observed during the study.

CONCLUSION

Though enteric fever is a common disease in this part of our country, multidrug resistant *Salmonella typhi* and *Salmonella paratyphi A* are not found among the patients. Fluroquinolone resistance has been reported in many parts of the country, but it was found that enteric fever isolates are susceptible to fluroquinolones in and around Mysore. In order to manage better and prevent the spread of antimicrobial resistance, both clinicians and governments require accurate epidemiological information. At the present moment, this information tends to be lacking, especially in countries with large populations and unrestricted 'over the counter' prescription policies, such as in India. It is very important to keep searching for new mutations and continuously monitor drug resistance in order to efficiently manage cases with enteric fever.

REFERENCES

[1] Gautam V, Gupta NK, Chaudhary U, Arora DR. Braz J Infect Dis 2002;6(6):281-7.
[2] Raffatellu M, Wilson RP, Winter SE, Baumler AJ. J Infect Dev Ctries 2008;2(4):260-6.
[3] Butt T, Ahmad RN, Mahmood A, Zaidi S. Emerg Infect Dis 2003;9(12):1621-2.
[4] Wain J, Hoa NT, Chinh NT, Vinh H, Enerett MJ, Diep TS et al. Clin Infect Dis 1997;25(6):1404-10.
[5] Nath G, Tikoo A, Manocha H, Tripathi AK, Gulati AK. J Antimicrob Chemother 2000 Jul;46(1):149-50.
[6] Shanahan PM, Jesudason MV, Thomson CJ, Amyes SG. J Clin Microbiol 1998;36(6):1595-600.
[7] Aoyama H, Fujimaki K, Sato K, Fujii T, Inoue M, Hirai K et al. Antimicrob Agents Chemother 1988;32(6):922-4.
[8] Hasdemir UO, Chevalier J, Nordmann P, Pages JM. J Clin Microbiol 2004;42(6):2701-6.
[9] Wain J, Kidgell C. Trans R Soc Trop Med Hyg 2004;98(7):423-30.
[10] Old DC. *Salmonella*. In: Collee JG, Fraser AG, Marimon BP, Simmons A. *Salmonella*. Editors: Mackie & McCartney. Practical medical microbiology. 14th edition Edinburg: Churchill Livingstone 2006: P385-404.
[11] Clinical and Laboratory Standards Institute. M 100-S17. Performance standards for antimicrobial susceptibility testing; 17th informational supplement. Clinical and Laboratory Standards Institute, 2007. Wayne, PA
[12] Casin I, Breuil J, Darchis JP, Guelpa C, Collatz E. Emerg Infect Dis 2003;9(11):1482-3.
[13] Eykyn SJ, Williams H. Treatment of multiresistant *Salmonella typhi* with oral ciprofloxacin. Lancet 1987;330(8572):1407-8.
[14] Mandal BK. J Infect 1991;22(1):1–4.

[15] Aarestrup FM, Wiuff C, Molbak K, Threlfall EJ. Antimicrob Agents Chemother 2003;47(2):827–9.
[16] Kapil A, Sood S, Dash NR, Das BK, Seth P. Lancet 1999;354(9173):164.
[17] Jesudason MV, Malathy B, John TJ. Indian J Med Res1996;103:247–9.
[18] Kapil A, Renuka, Das B. Indian J Med Res 2002;115:49–54.
[19] Dimitrov T, Dashti AA, Albaksami O, Jadaon MM. J Clin Pathol 2010;63(1):83-7.
[20] Krishnan P, Stalin M, Balasubramanian S. Indian J Pathol 2009;52(4):505-8.
[21] Threlfall EJ, Skinner JA, Ward LR. J Antimicrob Chemother 2001;48(5):740-1.
[22] Zaki SA, Karande S. J Infect Dev Ctries 2011;5(5):324-37.