Indian J Med Res 136, July 2012, pp 98-101

Correspondence

Emergence of fluoroquinolone resistance in *Salmonella enterica* serovar Typhi in Andaman and Nicobar Islands, India

Sir,

Enteric fever, due to infection with *Salmonella enterica* serotype Typhi and Paratyphi A, is estimated to cause more than 27 million infections each year worldwide with 216,000 deaths[1,2]. Almost 80 per cent of cases and deaths occur in Asia. Annual incidence as high as 1,100 cases per 100,000 population has been documented in developing countries[3,4].

It is a major public health problem in India, accounting for more than 300,000 cases per year[5]. Decreased susceptibility of *Salmonella enterica* serovar Typhi isolates to fluoroquinolones and treatment failure with these drugs have been reported frequently in the past several years[6-10]. In India, resistance of *S.* Typhi to chloramphenicol was first reported from Kerala in 1972[11]. According to a recent report, re-emergence of chloramphenicol susceptible *Salmonella enterica* serovar Typhi and Paratyphi A was observed in Chennai[12].

Enteric fever cases occur regularly at Port Blair and at a few other endemic pockets in the rural areas in the archipelago of Andaman and Nicobar Islands, a Union Territory of India. Though treatment failure with fluoroquinolones is frequently reported by clinicians, the drug sensitivity status of *Salmonella* Typhi isolates is not known. A study was, therefore, initiated to assess this, and this communication presents the initial results of this study.

The study was conducted during May 2009 - October 2010. Blood and stool samples were collected from suspected patients (both adult and children) of typhoid fever attending the outpatient departments or admitted to two referral hospitals *viz*., G.B. Pant Hospital and INHS Dhanwantari, in Port Blair, prior to the administration of antimicrobial drugs. Written consent was obtained from the patient/guardian prior to collection of samples. The study protocol was approved by the ethical committee of RMRC (ICMR), Port Blair.

Blood samples were inoculated into brain heart infusion broth and incubated at 37°C. Subcultures were made onto MacConkey agar and Hektoen Enteric Agar plates (Hi-Media, Mumbai, India), first after overnight incubation of the broth and thereafter daily for seven days. Stool samples were collected in sterile containers (Hi-media, Mumbai), inoculated into selenite-F broth and subcultured onto MacConkey agar and Hektoen Enteric Agar plates after 18 h incubation[13]. The isolates obtained were identified by conventional biochemical tests[14] and confirmed by agglutination with *Salmonella* antisera (Denka Seiken Co, Ltd., Japan). The antibiotic susceptibility tests were done by Kirby-Bauer disk diffusion method[15] according to Clinical and Laboratory Standards Institute (CLSI) guidelines[16] using commercially available disks (Hi-media Laboratories, Mumbai) of ampicillin (10 µg, AMP), carbenicillin (100 µg, CAR), imipenem (10 µg, IMP), amikacin (30 µg, AMK), chloramphenicol (30 µg, CHL), co-trimoxazole (25 µg, CoT), nalidixic acid (30 µg, NAL), norfloxacin (10 µg, NOR), ciprofloxacin (5 µg, CIP), ofloxacin (5 µg, OFX), gatifloxacin (5 µg, GAT), gentamycin (10 µg, GEN), nitrofurantoin (300 µg, NIT), and tetracycline (30 µg, TET), azithromycin (30 µg, AZT), cephalothin (30 µg, CEF), cefuroxime (5 µg, CXM), cefixime (30 µg, CFM), ceftriaxone (30 µg, CRO). *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 strains were used in the assay as quality control strains. Minimum inhibitory concentration (MIC) for fluoroquinolone of the resistant isolates was assessed by E-Test (AB BIODISK, Sweden). Carbenicillin, imipenem and nitrofurantoin were included though these are not recommended for treatment of typhoid fever, because

resistance to these drugs could be used as a phenotypic characteristic to study the clonal relatedness of the isolates.

DNA was extracted from the isolates following standard protocol and the quinolone resistance determining region (QRDR; *gyrA, gyrB, parC* and *parE*) was amplified using specific primers[17]. The amplified products were then cycle sequenced (PCR machine: ABI, 9700). The products were sequenced (Genetic Analyzer, ABI 3130, USA) and analyzed using SeqScape software (Applied Biosystem, USA). MEGA5 software[18] was used to determine mutations in these DNA gyrase and topoisomerase IV genes. All isolates were screened for the presence of plasmid mediated quinolone resistant determinants (PMQR), *qnrA, qnrB, qnrC, qnrS, qepA* and *aac (6')-Ib-cr* genes by polymerase chain reaction[19].

Blood and stool samples from 68 patients were cultured during the study period. Six (8.82%, 95% CI: 3.31, 18.22) isolates of *S.* Typhi were obtained, four from stool cultures and two from blood cultures. These isolates showed five different drug resistance patterns. All the isolates were (100%, 95% CI: 54.07, 100.0) resistant to co-trimoxazole, ampicillin, carbenicillin, azithromycin and nalidixic acid (Table).

Table. Antibiotic resistance pattern and minimum inhibitory concentration (MIC) for quinolones of six isolates of *Salmonella enterica* serovar Typhi obtained from Andaman and Nicobar Islands, India

Drug	*S.* Typhi isolates (n=6)					
	DS-560	DN-025	BC-213	ES-B43	ES-B148	DS-533
CoT	R	R	R	R	R	R
AMP	R	R	R	R	R	R
CAR	R	R	R	R	R	R
CEF	R	S	S	R	R	R
CXM	S	S	S	S	S	R
CFM	S	S	S	S	S	S
CRO	S	S	S	S	S	S
AZT	R	R	R	R	R	R
TET	S	S	R	R	R	R
CHL	S	S	S	S	S	S
GEN	R	S	S	S	S	R
AMK	S	S	R	R	R	R
NAL	R	R	R	R	R	R
NOR	I	I	R	I	I	I
CIP	I	I	R	I	I	I
OFX	S	I	R	I	I	I
GAT	S	S	R	S	S	S
NIT	S	S	R	R	I	R
IMP	S	S	S	S	S	S
	MIC (µg/ml)					
NAL	>256	>256	>256	>256	>256	>256
NOR	1	1	>256	1	1	1
CIP	0.25	0.25	>256	0.25	0.25	0.25

S, sensitive; R, resistant; I, intermediate resistance; AMP, ampicillin; CAR, carbenicillin; IMP, imipenem; AMK, amikacin; CHL, chloramphenicol; CoT, co-trimoxazole; NAL, nalidixic acid; NOR, norfloxacin; CIP, ciprofloxacin; OFX, ofloxacin; GAT, gatifloxacin; GEN, gentamycin; NIT, nitrofurantoin; TET, tetracycline; AZT, azithromycin; CEF, cephalothin; CXM, cefuroxime; CFM, cefixime; CRO, ceftriaxone

One of the six isolates (95% CI: 0.42, 64.12) was resistant to all the four fluoroquinolones tested *viz.* norfloxacin, ciprofloxacin, ofloxacin and gatifloxacin. The MICs of NAL, NOR and CIP for this isolate were >256 µg/ml. The remaining five isolates showed intermediate resistance to norfloxacin and ciprofloxacin. The MICs of ciprofloxacin and norfloxacin for all these isolates were 0.25 and 1 µg/ml, respectively. While these five isolates were sensitive to gatifloxacin, only one was sensitive to ofloxacin, the remaining four showing intermediate level resistance. All *Salmonella enterica* serovar Typhi isolates were sensitive to third generation cephalosporins, chloramphenicol and imipenem.

All six isolates had double mutations in *gyrA* gene, one at position 83 that resulted in the substitution of serine with lysine and the other one at position 87 that led to the substitution of aspartic acid with asparagine (Genebank Accession Number- HQ318777). All isolates also had a functional mutation at position 80 of *parC* gene resulting in the substitution of serine with isoleucine (Genebank Accession Number- HQ318779). No mutations were detected in *gyrB* and *parE* genes of the six isolates. None of the isolates were found to harbour the PMQR determinants.

Fluoroquinolones, particularly ciprofloxacin, ofloxacin and gatifloxacin are used as the first line drugs for the treatment of enteric fever in Andaman and Nicobar Islands. All the isolates obtained so far were either fully resistant or showed intermediate level resistance to the most common drug used to treat enteric fever *viz.* ciprofloxacin with one showing an MIC above the break-point for resistance and the remaining above the level (0.125 µg/ml) that is considered to confer intermediate level resistance. This corroborates with the frequent treatment failures observed in the islands.

Mutations in *gyrA* are seen almost invariably in isolates with MIC >0.125 µg/ml[20], which is also seen in the present study. All these isolates had multiple mutations in topoisomerase gene, which is considered as a pre-requisite for conferring high level of resistance to quinolones[21]. The present preliminary data show that multiple mutations in topoisomerase genes, including double mutations in *gyrA,* need not always confer high level of resistance to ciprofloxacin as reported earlier[22]; rather it may result only in reduced susceptibility to the drug. In a study conducted in Hong Kong, it was reported that 19 isolates of *S. enterica* that had the same double mutations in *gyrA* as seen in our isolates, along with a mutation in *parC* had ciprofloxacin MIC in the range of 0.12 - 0.5 µg/ml, though the mutation in *parC* was different from the one observed here[23]. *Vibrio cholerae* with reduced susceptibility towards ciprofloxacin (0.25 to 0.5 mg/l) harboured one mutation each in *gyrA* and *parC*, respectively[24].

Enteric fever is a common occurrence in Andaman and Nicobar Islands with an estimated 1,100 cases occurring every year among the 350,000 population giving an incidence of > 450 cases/100,000 (unpublished data). Strains with genetic characteristics that confer resistance to fluoroquinolones appear to be the dominant clone of *Salmonella enterica* serovar Typhi causing enteric fever in the Islands. Resistance to third generation cephalosporins does not appear to have emerged in the Islands yet. However, there is every possibility that the bacteria may acquire such properties in near future, leaving little options for the physician for specific therapy for enteric fever. Routine monitoring of drug resistance among *Salmonella enterica* serovar Typhi is essential from public health point of view.

Acknowledgment

The authors thank the Indian Council of Medical Research, New Delhi, for providing financial grant for the study.

R. Thamizhmani[1], Debdutta Bhattacharya[1], Sayi D.S.[1], Haimanti Bhattacharjee[1], N. Muruganandam[1], S.R. Ghosal[1], Bharadwaj A.P.[2], M. Singhania[3], Subarna Roy[1] & Sugunan A.P.[1*]

[1]Regional Medical Research Centre (ICMR)
[2]Chirayu Child Care Centre,
[3]G.B. Pant Hospital, Port Blair 744101
Andaman & Nicobar Islands, India
[*]*For correspondence:*
sugunanap@icmr.org.in
pblicmr@sancharnet.in

References

1. Crump JA, Luby SP, Mintz ED. The global burden of typhoid fever. *Bull World Health Organ* 2004; *82* : 346-53.
2. Christopher MP, Ho VA, Phuong LT, Bay PVB, Lanh MN, Tung LT, *et al*. Randomized controlled comparison of ofloxacin, azithromycin, and an ofloxacin-azithromycin combination for treatment of multidrug-resistant and nalidixic acid-resistant typhoid fever. *Antimicrob Agents Chemother* 2007; *44* : 819-25.
3. Ivanoff B, Levine MM, Lambert PH. Vaccination against typhoid fever present status. *Bull World Health Organ* 1994; *72* : 957-71.

4. Kumar R, Gupta N, Shalini. Multidrug-resistant typhoid fever. *Indian J Pediatr* 2007; *74* : 39-42.
5. Gupta V, Kaur J, Chander J. An increase in enteric fever cases due to *Salmonella Paratyphi* A in and around Chandigarh. *Indian J Med Res* 2009; *129* : 95-8.
6. Kadhiravan T, Wig N, Kapil A, Kabra SK, Renuka K, Misra A. Clinical outcomes in typhoid fever: adverse impact of infection with nalidixic acid-resistant *Salmonella typhi. BMC Infect Dis* 2005; *5* : 37.
7. Asna SM, Haq JA, Rahman M. Nalidixic acid-resistant *Salmonella enterica* serovar Typhi with decreased susceptibility to ciprofloxacin caused treatment failure a report from Bangladesh. *Jpn J Infect Dis* 2003; *56* : 32-3.
8. Threlfall EJ, Ward LR. Decreased susceptibility to ciprofloxacin in *Salmonella enterica* serotype Typhi, United Kingdom. *Emerg Infect Dis* 2001; *7* : 448-50.
9. Kownhar H, Muthu Shankar E, Rajan R, Anand Rao U. Emergence of nalidixic acid-resistant *Salmonella enterica* serovar Typhi resistant to ciprofloxacin in India. *J Med Microbiol* 2007; *56* : 136-7.
10. Kumar S, Rizvi M, Berry N. Rising prevalence of enteric fever due to multidrug resistant *Salmonella*: an epidemiological study. *J Med Microbiol* 2008; *57* : 1247-50.
11. Paniker CKJ, Vimla KN. Transferable chloramphenicol resistance in *Salmonella* Typhi. *Nature* 1972; *239* : 109-10.
12. Krishnan P, Stalin M, Balasubramanian S. Changing trends in antimicrobial resistance of *Salmonella enterica* serovar Typhi and *Salmonella enterica* Paratyphi A in Chennai. *Indian J Pathol Microbiol* 2009; *54* : 505-8.
13. World Health Organization. *The diagnosis, treatment and prevention of typhoid fever WHO 2003*. Available from: *http://whqlibdoc.who.int/hq/2003/WHO_V&B_03.07.pdf*, accessed on May 7, 2011.
14. World Health Organization. *Manual for the laboratory identification and antimicrobial susceptibility testing of bacterial pathogens of public health importance in the developing world.* World Health Organization. 2003. Available from: *http://www.ritm.gov.ph/arsp/Manual%20for20Lab%20ID,%20AST-cdc,%20who.pdf*, accessed on May 7, 2011.
15. Bauer AW, Kirby WM, Sherris JC, Turck M. Antibiotic susceptibility testing by a standardized single disk method. *Am J Clin Pathol* 1966; *45* : 493-6.
16. Clinical and Laboratory Standards Institute. *Performance standards for antimicrobial disk susceptibility tests*. Approved standard M2-A10. Wayne, Pa: Clinical and Laboratory Standards Institute; 2007.
17. Dutta S, Kawamura Y, Ezaki T, Nair GB, Lida KI, Yoshida SI. Alteration in the GyrA Subunit of DNA Gyrase and the ParC Subunit of Topoisomerase IV in quinolone-resistant *Shigella dysenteriae* serotype 1 clinical isolates from Kolkata, India. *Antimicrob Agents Chemother* 2005; *49* : 1660-1.
18. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* 2011; *28* : 2371-9.
19. Bhattacharya D, Bhattacharjee H, Thamizhmani R, Sayi DS, Bharadwaj AP, Singhania M, *et al*. Prevalence of the plasmid-mediated quinolone resistance determinants among clinical isolates of *Shigella* sp. in Andaman & Nicobar Islands, India. *Lett Appl Microbiol* 2011; *53* : 247-51.
20. Hakanen A, Kotilainen P, Jalava J, Siitonen A, Huovinen P. Detection of decreased fluoroquinolone susceptibility in Salmonellas and validation of nalidixic acid screening test. *J Clin Microbiol* 1999; *37* : 3572-7.
21. Renuka K, Sood S, Das BK, Kapil A. High-level ciprofloxacin resistance in *Salmonella enterica* serotype Typhi in India. *J Med Microbiol* 2005; *54* : 999-1000.
22. Gaind R, Paglietti B, Murgia M, Dawar R, Uzzau S, Cappuccinelli P, *et al*. Molecular characterization of ciprofloxacin-resistant *Salmonella enterica* serovar Typhi and Paratyphi A causing enteric fever in India. *J Antimicrob Chemother* 2006; *58* : 1139-44.
23. Ling JM, Chan EW, Lam AW, Cheng AF. Mutations in topoisomerase genes of fluoroquinolone-resistant *Salmonellae* in Hong Kong. *Antimicrob Agents Chemother* 2003; *47* : 3567-73.
24. Quilici M, Massenet D, Gake B, Bwalki B, Olson DM. *Vibrio cholerae* O1 variant with reduced susceptibility to ciprofloxacin, Western Africa. *Emerg Infect Dis* 2010; *16* : 1804-5.