Molecular Analysis of Fluoroquinolone-resistant Salmonella Paratyphi A Isolate, India

Satheesh Nair,* Madhulika Unnikrishnan,† Keith Turner,* Subash Chandra Parija,† Carol Churcher,* John Wain,* and Belgode Narasimha Harish†

Salmonella enterica serovar Paratyphi A is increasing-ly a cause of enteric fever. Sequence analysis of an Indian isolate showed a unique strain with high-level resistance to ciprofloxacin associated with double mutations in the DNA gyrase subunit gyrA (Ser83→Phe and Asp87→Gly) and a mutation in topoisomerase IV subunit parC (Ser80→Arg).

Salmonella enterica serovar Paratyphi A is the second most common cause of enteric fever after S. Typhi. Approximately 0.25 S. Paratyphi A infections (paratyphoid fever) occur for each S. Typhi infection (typhoid fever) (7). Given global estimates of >21 million cases of typhoid fever in the year 2000, >5 million cases per year of S. Paratyphi A probably occur. Paratyphoid fever is a major clinical problem in India, but large outbreaks were not reported until 1996 (2). Elsewhere, in southern China for example, extensive outbreaks are also occurring (3).

Since 1998, plasmid-mediated multidrug resistance in S. Paratyphi A, associated with chromosomally mediated reduced susceptibility to ciprofloxacin, has caused concern (4). Reduced susceptibility to fluoroquinolones results in a poor response of salmonellosis patients to treatment and may allow prolonged bacterial shedding (5). Rising resistance to fluoroquinolones is likely to be driving an increase in cases of paratyphoid fever in regions where fluoroquinolones are used empirically to treat enteric fever. We must monitor the emergence of resistance in this enteric pathogen to differentiate between the acquisition of resistance during treatment (mutations occurring in different bacterial strains) or clonal expansion of a successful strain by person-to-person spread (identical mutations associated with a single strain). To do so, we need to describe the molecular basis of resistance and the genotype of the resistant strains. High-level ciprofloxacin-resistant S. Paratyphi A (MIC 8 µg/mL) is present in India (6) and Japan (MIC ≥128 µg/mL) (7). However, because S. Typhi, the major cause of enteric fever in India, has not yet developed high-level resistance to fluoroquinolones, enteric fevers are often treated empirically with fluoroquinolones. If this trend continues, fluoroquinolone-resistant strains of S. Paratyphi A are almost certain to become a major cause of enteric fever in many areas.

We analyzed, by DNA sequencing, the DNA gyrase and topoisomerase IV genes of the first reported highly fluoroquinolone-resistant S. Paratyphi A isolate (6). We looked at the full coding sequence, including the quinolone resistance–determining region (QRDR), of both subunits of DNA gyrase and topoisomerase IV for mutations associated with resistance to fluoroquinolones. We also used multilocus sequence typing (MLST) to confirm the identity of the isolate.

The Study

The strain described here (Pond1) was first isolated in Pondicherry, India, in November 2002 from the blood of a 23-year-old man admitted with fever and with no history of having received antimicrobial chemotherapy (6). The isolate was resistant to ciprofloxacin and nalidixic acid, and the MIC of ciprofloxacin was 8 mg/L. It was sensitive to all other antimicrobial drugs tested by disk diffusion: ampicillin, chloramphenicol, cotrimoxazole, gentamicin, and ceftriaxone. Repeat testing showed that zones of inhibition indicating susceptibility were seen around both ofloxacin (17 mm) and ciprofloxacin (21 mm) 5-µg disks; however, in light of the ciprofloxacin MIC and resistance to nalidixic acid, Pond1 was considered resistant to fluoroquinolones. No similar isolates have been seen in this area since the initial report, although the total number of paratyphoid fever cases has increased.

Polymerase chain reaction (PCR) amplification (Table 1) and direct DNA sequencing of both strands of the full length of gyrA and gyrB and topoisomerase IV (parC and parE) subunit genes was performed with an ABI Prism dye terminator cycle sequencing kit (Perkin Elmer, Foster City, CA, USA) on an ABI 3730 automated sequencer. Results showed 3 mutations: 2 in gyrA and 1 in parC. A comparison of these mutations with those previously described in fluoroquinolone-resistant S. Paratyphi A is shown in Table 2. A rise of fluoroquinolone resistance over time is apparent, and although the point mutations do not fully explain the MIC data, we noted general associations: a single mutation in gyrA is always associated with resistance to nalidixic acid and reduced susceptibility to ciprofloxacin and ofloxacin, and a double mutation in gyrA is always accompanied by mutations in parC and is asso-

*The Wellcome Trust Sanger Institute, Cambridgeshire, United Kingdom; and †Jawaharlal Institute of Postgraduate Medical Education and Research, Pondicherry, India
associated with high-level (>4 µg/mL) resistance. Although this sample is limited, heterogeneity in mutations found at all sites suggests that this finding is not the result of a single successful strain’s sequentially acquiring mutations but rather that resistance is arising in several different strains.

Conclusions

The QRDR within topoisomerases contains hotspots for mutations around the active site, which are associated with raised MIC values for fluoroquinolones (10). For gyrA from nalidixic acid–resistant Salmonella isolates, 2 mutations are most frequently observed in clinical isolates: Ser83→Phe and Ser83→Tyr (8,9,11). The association with resistance of mutations seen in parC, however, is less clear (12). Mutations in parC of gram-negative bacteria are usually within the QRDR at amino acids 80 and 84 (Ser 80→Ile, Glu 84→Gly, Lys). The first reported mutation was Thr57→Ser, and other mutations have been described: Asp 79→Ala, Gly 78→Asp (9). Each mutation, in gyrA or parC, can give rise to different MICs in different isolates, which means that other factors must also influence the resistance phenotype in S. Paratyphi A. The most likely cause is changes in expression levels of proteins involved with permeability barriers and efflux pumps. These changes could be the result of either point mutations in transcription promoters and regulators or downstream effects of mutations in topoisomerases. Known mechanisms of fluoroquinolone resistance that we were able to screen for include transmissible plasmidborne resistance and efflux pumps. The qnr-containing plasmid was not detected with PCR using primers 5′ GGG TAT GGA TAT TAT TGA TAA 3′ and 5′ CTA ATC CGG CAG CAC TAT TA 3′ (13) in Pond1, and sensitivity testing gave the same zone size around both tetracycline and chloramphenicol discs when compared with a nalidixic acid–susceptible isolate. This finding, combined with the ciprofloxacin MIC of 8 µg/mL, argues against the presence of multiple antimicrobial drug resistance efflux pumps. Thus the high-level resistance seen in Pond1 appears to be associated directly with 3 point mutations in the topoisomerase genes.

To confirm the identity of the isolate as S. Paratyphi A, we used MLST. The primer sequences and MLST data are available from the MLST database at the Max-Planck-Institut für Infektionsbiologie (http://web.mpib-berlin.mpg.de/mlst/). Six of 7 sequenced loci matched exactly the previously described sequences, and 1 was a unique allele. This finding means that the isolate described here is within the clonal MLST group described as S. Paratyphi A but is a recognizable variant. This finding supports previous typing data that show very little variation (14); typing S. Paratyphi A is problematic because genomic restriction analyses (pulsed-field gel electrophoresis) of isolates from an outbreak are not always identical, and susceptible and resistant strains cannot be differentiated. For molecular epidemiologic studies to be carried out, several methods need to be used (15). A broader study of single base-pair differences between strains of S. Paratyphi A could provide a usable typing scheme.

### Table 1. Primer sequences for amplification of topoisomerases

| Primer  | Sequence 5′–3′ | Amplicon | Gene (coding sequence length) |
|---------|---------------|----------|------------------------------|
| gyrA7 (F) | 5′ GGG TCG ACT GTG ATT GTT GGT TGC 3′ | 3199 | 2637 |
| gyrA25 (R) | 5′ GAG ACG TCT CAG GCT 3′ | 2762 | 2415 |
| gyrB1 (F) | 5′ TGC CTC TGA ACT TGG 3′ | 2616 | 2259 |
| gyrB9 (R) | 5′ GAA GTG CGT GAC CTG CCT 3′ | 2183 | 1893 |
| parC3 (F) | 5′ CGA TCT CCG GTT CTC TCC 3′ | 2616 | 2259 |
| parC10 (R) | 5′ GCA ACG AGA TAA AAC CAA GGC 3′ | 2183 | 1893 |
| parE3 (F) | 5′ CGT ATG TGG GCT ACG CAC G 3′ | 2616 | 2259 |
| parE8 (R) | 5′ ATGCC GAA GTG TCCGC CACT 3′ | 2183 | 1893 |

### Table 2. Presence of qnr and mutations in DNA gyrase and topoisomerase IV genes of *Salmonella enterica* serovar Paratyphi A strains associated with decreased susceptibility or resistance to ciprofloxacin

| Country | Year | MIC (µg/mL) | qnr | gyrA | parC | Reference |
|---------|------|-------------|-----|------|------|-----------|
| India   | 1999 | 0.38 >256 ND Ser83→Phe | ND  | (8)  |
| Bangladesh | 1999 | 0.5 >256 ND Asp87→Gly | ND  | (8)  |
| India   | 1999 | 0.5 >256 ND Ser83→Phe | ND  | (8)  |
| Hong Kong | 2000 | 0.5 >256 ND Ser83→Tyr | NM  | (9)  |
| Japan   | 2002 | ≥128 >256 ND Ser83→Phe, Asp87→Arg | Glu84→Lys | (7)  |
| India   | 2002 | 8 >256 NP Ser83→Phe, Asp87→Gly | Ser80→Arg† | This study |

*No mutations were detected in gyrB and parE. Cp, ciprofloxacin; Nal, nalidixic acid; ND, not determined; NP, not present; NM, no mutation within the quinolone resistance–determining region.
†European Molecular Biology Laboratory accession no. AM050347.
Resistance in *S. Paratyphi* A populations must be monitored because the acquisition of resistance to fluoroquinolones, coupled with the reduction in *S. Typhi* by the use of typhoid-specific vaccination, may cause *S. Paratyphi* A to become the main cause of enteric fever. Disc susceptibility testing does not always detect resistance, and screening with nalidixic acid and MIC testing remains the method of choice. The isolate described here, Pond1, contains a unique combination of mutations that provides a way to track the spread of this strain of *S. Paratyphi* A.

**Acknowledgments**

We thank the diagnostic microbiologists who isolated and characterized the strains described in this manuscript. Satheesh Nair, John Wain, and Keith Turner are funded by The Wellcome Trust of Great Britain.

Dr Nair is a research associate in tropical bacteriology at The Wellcome Trust Sanger Institute, The Wellcome Trust Genome Campus, United Kingdom. His research includes the use of molecular tools to detect genomic diversity of drug-resistant/drug-susceptible *Salmonella* spp. from different geographic and epidemiologic backgrounds and effects of drug resistance on the biology of the organism.

**References**

1. Crump JA, Luby SP, Mintz ED. The global burden of typhoid fever. Bull World Health Organ. 2004;82:346–53.
2. Sood S, Kapil A, Dash N, Das BK, Goel V, Seth P. Paratyphoid fever in India: an emerging problem. Emerg Infect Dis. 1999;5:483–4.
3. Yang J, Dong B, Wang M, Tang Z, Gong J, Li C, et al. An analysis of *S. Paratyphi* A and *S. Typhi* prevalence in Guangxi autonomous region between 1994–2002 [article in Chinese]. China Trop Med. 2004;4:177–18.
4. Chandel DS, Chaudhry R, Dhawan B, Pandey A, Dey AB. Drug-resistant *Salmonella enterica* serotype Paratyphi A in India. Emerg Infect Dis. 2000;6:420–1.
5. Wain J, Hoa NT, Chinh NT, Vinh H, Everett MJ, Diep TS, et al. Quinolone-resistant *Salmonella typhi* in Viet Nam: molecular basis of resistance and clinical response to treatment. Clin Infect Dis. 1997;25:1404–10.
6. Harish BN, Madhulika U, Parija SC. Isolated high-level ciprofloxacin resistance in *Salmonella enterica* subsp. *enterica* serotype Paratyphi A. J Med Microbiol. 2004;53:819.
7. Adachi T, Sagara H, Hirose K, Watanabe H. Fluoroquinolone-resistant *Salmonella Paratyphi* A. Emerg Infect Dis. 2005;11:172–4.
8. Walker RA, Skinner JA, Ward LR, Threlfall EJ. LightCycler *gyrA* mutation assay (GAMA) identifies heterogeneity in *gyrA* in *Salmonella enterica* serotypes Typhi and Paratyphi A with decreased susceptibility to ciprofloxacin. Int J Antimicrob Agents. 2003;22:622–5.
9. 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.
10. Piddock LJ. Fluoroquinolone resistance in *Salmonella enterica* serovars isolated from humans and food animals. FEMS Microbiol Rev. 2002;26:3–16.
11. Eaves DJ, Randall L, Gray DT, Buckley A, Woodward MJ, White AP, et al. Prevalence of mutations within the quinolone resistance-determining region of *gyrA, gyrB, parC*, and *parE* and association with antibiotic resistance in quinolone-resistant *Salmonella enterica*. Antimicrob Agents Chemother. 2004;48:4012–5.
12. Piddock LJ, Ricci V, McLaren I, Griggs DJ. Role of mutation in the *gyrA* and *parC* genes of nalidixic-acid-resistant salmonella serotypes isolated from animals in the United Kingdom. J Antimicrob Chemother. 1998;41:635–41.
13. Wang M, Saum DF, Jacoby GA, Hooper DC. Emerging plasmid-mediated quinolone resistance associated with the *qnr* gene in *Klebsiella pneumoniae* clinical isolates in the United States. Antimicrob Agents Chemother. 2004;48:1295–9.
14. Selander RK, Beltran P, Smith NH, Helmuth R, Rubin FA, Kopecko DJ, et al. Evolutionary genetic relationships of clones of *Salmonella* serovars that cause human typhoid and other enteric fevers. Infect Immun. 1990;58:2262–75.
15. Chandel DS, Nisar N, Thong KL, Pang T, Chaudhry R. Role of molecular typing in an outbreak of *Salmonella paratyphi* A. Trop Gastroenterol. 2000;21:121–3.

Address for correspondence: Belgode Narasimha Harish, Department of Microbiology, JIPMER, Pondicherry 605006, India; fax: 91-413-2272067; email: drbnharish@yahoo.com

www.cdc.gov/eid

To receive tables of contents of new issues send an email to listserve@cdc.gov with subscribe eid-toc in the body of your message.