Molecular Typing and Resistance Analysis of Travel-Associated *Salmonella enterica* Serotype Typhi

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Salmonella enterica serotype Typhi is a human pathogen causing 12 to 30% mortality and requiring antibiotic therapy to control the severity of the infection. Typhoid fever in United States is often associated with foreign travel to areas of endemicity. Increasing resistance to multiple drugs, including quinolones, is associated with decreased susceptibility to ciprofloxacin (DCS). We investigated 31 clinical strains isolated in Florida from 2007 to 2010, associated with travel to six countries, to examine the clonal distribution of the organism and apparent nalidixic acid (NAL) resistance. The strains were isolated from blood or stool of patients aged 2 to 68 years. The isolates were subtyped by ribotyping and pulsed-field gel electrophoresis. Susceptibilities to 15 antimicrobials were determined, and the isolates were screened for integrons and gyrase A gene mutations. Both typing techniques effectively segregated the strains. Identical clones were associated with different countries, while diverse types coexisted in the same geographic location. Fifty-one percent of the strains were resistant to at least one antimicrobial, and five were resistant to three or more drugs (multidrug resistant [MDR]). All 12 isolates from the Indian subcontinent were resistant to at least one drug, and 83% of those were resistant to NAL. Three of the MDR strains harbored a 750-bp integron containing the *dfr7* gene. Ninety-three percent of the resistant strains showed a DCS profile. All the NAL-resistant strains contained point mutations in the quinolone resistance-determining region of *gyrA*. This study affirms the global clonal distribution, concomitant genetic heterogeneity, and increased NAL resistance of *S. enterica* serovar Typhi.

Salmonella enterica serotype Typhi causes typhoid fever in humans. The symptoms include high fever, abdominal pain, chills, and dizziness. In severe cases, the disease is life-threatening, with a mortality rate of 12 to 30%; therefore, administration of antibiotics is important. Asymptomatic carriers act as reservoirs and may spread this organism to other people by the fecal-oral route. Typhoid fever is a global public health problem since it is endemic in developing countries and is often associated with foreign travel to underdeveloped countries. According to the Centers for Disease Control and Prevention (CDC), approximately 400 cases per year are reported in the United States. Southern Asia is considered a very high risk area for foreign travelers, followed by the Caribbean region, Central and South America, and Africa (4). Two kinds of vaccines have been effective in preventing the infection: the live attenuated Ty21a and the parenteral Vi vaccine (8).

Studies examining the conserved aspect of the *S. Typhi* genome suggest that the organism has evolved recently (approximately 50,000 years ago) and is younger than other *Salmonella enterica* serotypes and than *Escherichia coli* (15). Multilocus sequence typing (MLST) with a seven-housekeeping-gene scheme has illustrated that *S. Typhi* is genetically homologous (15). Therefore, it is considered to be clonal in distribution across the world, with a few clones circulating globally (16, 39). Methods studying variation based on genomic rearrangements have been more discriminatory than MLST and have been very useful for understanding the epidemiological aspects of this disease. For epidemiological studies, methods including pulsed-field gel electrophoresis (PFGE) (5, 13, 16, 38), ribotyping (25), and variable number tandem repeat typing (21) have successfully segregated closely related strains. More recently, single nucleotide polymorphism (SNP) typing (27, 30, 33) has proven valuable in both phylogenetic and epidemiological studies.

Antimicrobial resistance to multiple drugs is a growing prob-

lem in *S. Typhi*. Plasmid-associated integrons are frequently implicated in multidrug resistance (MDR) phenotypes (2, 24, 28, 29, 37). Increasing multidrug resistance to the first line of drugs, including chloramphenicol, trimethoprim, ampicillin, streptomycin, and tetracycline, has been observed by several groups (18, 23, 28, 35, 37). Therefore, administration of fluoroquinolones has become an important treatment option. However, there has been a global increase in nalidixic acid (NAL)-resistant strains causing decreased ciprofloxacin susceptibility (DCS) (1, 7, 22). This DCS may lead to longer fever clearance times and frequent treatment failures (6, 7, 40). Quinolone resistance in *Salmonella* spp. is often due to mutations in the DNA gyrase (*gyrA* and *gyrB*) or topoisomerase (*parC* and *parE*) genes (7, 9, 10) or to decreased permeability to the agents or to overexpression of efflux pumps (34). More recently, *qnr* genes, the products of which inhibit quinolone action by binding to *gyrA* and *gyrB* subunits, have been reported (11, 26). Nucleotide changes in the quinolone resistance-determining region (QRDR) of *gyrA* in *Salmonella* are more common than mutations in *gyrB* or the topoisomerase genes (6, 7, 12, 33, 36). In *S. Typhi*, nucleotide substitutions at Ser-83, Asp-87, Glu-133, Asp-76, Phe-72, Leu-55, and Gln-106 of the *gyrA* gene have been

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previously reported, with mutation at codon 83 being the most common (6, 9, 36).

A clonal expansion of NAL-resistant strains with or without the MDR phenotype has been observed by SNP analysis. These NAL-resistant strains belong to a single haplotype, H-58, and are circulating globally, especially in Southeast Asia (14, 17, 33).

In this study, we investigated a set of 31 travel-associated clinical *S. Typhi* isolates that were collected in Florida from 2007 to 2010. Using molecular typing techniques, we examined these isolates for the presence of multiple clones with global distribution as noted in other studies as well as for geographical associations among the strains. We also wanted to determine if the reported increase in NAL-resistant isolates could be found in a small set of global strains. This study will contribute to an understanding of the types of travel-associated clones circulating globally. Information obtained by the molecular characterization and resistance profiling of this set of strains will enhance efforts to control and treat this important global pathogen.

MATERIALS AND METHODS

Bacterial strains and patient history. Thirty-one clinical *S. Typhi* strains isolated in Florida in 2007, 2008, and 2010 were examined in this study (Table 1). Twenty-eight strains were provided by the Florida Department of Health, Bureau of Laboratories, Jacksonville, FL, and three were provided by the Florida Department of Health, Bureau of Laboratories, Tampa, FL. The isolates were biochemically characterized with API 20E panels (bioMérieux, Hazelwood, MO) and screened for the somatic (O) serogroup using *Salmonella* antiserum (Becton, Dickinson, Sparks, MD) or CDC antisera. All the isolates were serotyped and confirmed as *S. Typhi* by the Florida Department of Health, Bureau of Laboratories, Jacksonville, FL. About 97% (29/30) of the isolates associated with a known travel history involved international travel. The majority of cases were related to travel in southern Asia or in Haiti (Table 1). Two cases associated with Haiti were attributed to a known outbreak. The age range of patients was 2 to 68 years, with a median age of 17 years. Twenty of the 31 isolates were from male patients, and 10 were from female patients; information was unavailable for one isolate. The source of isolation was either blood or stool, and there were no reported deaths. The common symptoms recorded were fever, chills, headache, and anorexia, and the majority of patients were hospitalized (74%). Three of 31 cases were food handlers, and one was a health care worker (Table 1). Either the patients were either not vaccinated, or their vaccination status was unknown.

Ribotyping. Ribotyping was performed with an automated RiboPrinter (DuPont Qualicon, Wilmington, DE) using the manufacturer's instructions and reagents. Bacterial cells were suspended in sample buffer, heat treated (80°C for 15 min), and lysed with two lysing agents. The samples were then analyzed on the RiboPrinter. Restriction digestion with the enzyme *EcoRI*, followed by electrophoretic size separation of the DNA fragments, transfer of the fragments to a membrane, hybridization with a ribosomal DNA (rDNA) probe, and treatment with chemiluminescent agents were automatically carried out in the RiboPrinter. Images in tagged-image file format were exported for manual analysis as described below.

PFGE. Pulsed-field gel electrophoresis (PFGE) was performed according to a CDC standardized laboratory protocol as previously described (31) with modifications. *Salmonella* colonies were suspended in 1 ml of cell suspension buffer (100 mM Tris–100 mM EDTA, pH 8.0). Cell concentration was measured on a spectrophotometer (model DU 640; Beckman Instruments, Inc., Fullerton, CA) at a wavelength of 610 nm and adjusted to obtain an absorbance of 1 to 1.4. A total of 200 μ l of the cell suspension was mixed well with 10 μ l of proteinase K (20 mg/ml; Sigma, St. Louis, MO) and 200 μ l of molten 1% SeaKem agarose (Cambrex Bio Science, Rockland, ME) containing 1% sodium dodecyl sulfate (Sigma). The cell-agarose mixture was pipetted into disposable plug molds (Bio-

TABLE 1 Strain reference numbers correlated to travel history, year of isolation, and epidemiological data of patient source

Reference no. ^a	Travel-associated country	Year of isolation	Age range (yr)	Hospitalization
CBD 1265	Lebanon	2007	26	Yes
CBD 1266	Haiti	2007	28	UN ^c
CBD 1267	FL	2007	4	UN
CBD 1346 ^{\$}	Pakistan	2008	48	UN
CBD 1347*	India	2007	20	UN
CBD 1348	Peru	2008	6	Yes
CBD 1349	India	2008	24	Yes
CBD 1350	NA ^b	NA	NA	NA
CBD 1351 [†]	Haiti	2008	16	No
CBD 1352 [†]	Haiti	2008	2	Yes
CBD 1353	Bangladesh	2008	10	Yes
CBD 1354	Haiti	2008	12	Yes
CBD 1355	Haiti	2008	13	Yes
CBD 1356	Haiti	2008	18	Yes
CBD 1357	Haiti	2008	41	Yes
CBD 1358 [‡]	Haiti	2008	31	NA
CBD 1359	Haiti	2008	68	Yes
DOH 10269*	Haiti	2010	35	No
DOH 10341 [‡]	India	2010	34	Yes
DOH 10355	India	2010	31	Yes
DOH 10511	India	2010	6	Yes
DOH 10621	India, UAE	2010	42	Yes
DOH 10630	Haiti	2010	6	Yes
DOH 10636	Haiti	2010	3	Yes
DOH 10676	India	2010	7	Yes
DOH 10766*	Haiti	2010	30	Yes
DOH 10854	India	2010	33	Yes
DOH 101170	Haiti	2010	14	Yes
DOH 101174	Haiti	2010	5	No
DOH 101093 [‡]	India	2010	14	Yes
DOH 101094 [‡]	India	2010	14	Yes

^a Information about the source of the isolate is indicated as follows: *, from a food handlers; \$, from a health care worker; ‡, from a person visiting Florida from the respective country; †, part of an outbreak.

^b NA, not available.

^c UN, unknown.

Rad, Hercules, CA) and allowed to solidify. Cells suspended in plugs were then lysed in 5 ml of cell lysis buffer (50 mM Tris–50 mM EDTA, pH 8.0, 1% sarcosyl) with 25 μ l of proteinase K (20 mg/ml) for 2 h at 54°C in a shaking water bath. The plugs were then washed twice with prewarmed water and four times with prewarmed Tris-EDTA buffer (10 mM Tris–1 mM EDTA, pH 8.0) at 10-min intervals in a shaking water bath at 50°C. Plug slices 2 mm in width were digested each with 50 U of *XbaI* (Promega, Madison, WI) for 2 h at 37°C. Digested plug slices were adhered to a gel comb (Bio-Rad) and embedded in 1% SeaKem agarose that was electrophoresed on a CHEF (contour-clamped homogenous electric field) Mapper (Bio-Rad) for 18 h with an initial switch time of 2.16 s and a final switch time of 63.8 s. *XbaI*-digested *Salmonella enterica* serotype Braenderup H9812 (CDC strain) plug slices were used as molecular weight standards. Bands below 54 kb were not considered for analysis to exclude plasmid DNA. The gel was stained in ethidium bromide and visualized on a GelDoc (Bio-Rad).

Profile analysis. Ribotyping and PFGE images were analyzed by BioNumerics software (version 5.1) using the Dice coefficient. The phylogenetic relationships among isolates based on both ribotyping and PFGE were studied by dendrograms constructed with the unweighted pair group method with arithmetic averages (UPGMA) with 1% position tolerance. Two isolates with a similarity of $\geq 90\%$ were defined as clones as previously described (20, 32). Strains with less than 90% similarity were

each assigned a distinct pulso- or ribotype number. Each clonally related strain was assigned an alphabetic subtype designation. Strains above 95.3% and 99.99% similarity for PFGE and ribotyping, respectively, were considered identical. These thresholds were established by cluster analysis of at least two molecular weight standards from each PFGE gel or riboprint, followed by calculation of the percent similarity among each respective set of standards.

Antibiotic susceptibility testing. Antibiotic resistance profiles were determined by the Sensititre system, which is based on the classic broth macrodilution method (Trek Diagnostics, Cleveland, OH) using the manufacturer's instructions and reagents. One to two isolated colonies from freshly streaked plates were suspended in 5 ml of demineralized water to obtain a 0.5 McFarland density. Ten microliters of the bacterial water suspension was added to 11 ml of Mueller-Hinton broth (MHB). A 50- μ l aliquot of the MHB cell suspension was dispensed into each well of a 96-well panel consisting of 15 antimicrobials. The panels were incubated at 35°C for 18 h and then read by the autoreader. The 15 antimicrobials tested included amikacin (AMK), amoxicillin/clavulanic Acid (AMC), ampicillin (AMP), cefoxitin (FOX), ceftiofur (FUR), ceftriaxone (CRO), chloramphenicol (CHL), ciprofloxacin (CIP), gentamicin (GEN), kanamycin (KAN), nalidixic acid (NAL), streptomycin (STR), sulfisoxazole (FIS), tetracycline (TET), and trimethoprim/sulfamethoxazole (SXT). *E. coli* strain ATCC 25922 was used for quality control. Results were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (6a) where available. Breakpoints for ceftiofur and streptomycin were defined as MICs of ≥ 8 and ≥ 64 μ g/ml, respectively, as described elsewhere (11).

DNA extraction. DNA was extracted on a MagNA Pure LC instrument using DNA Isolation Kit III (Roche Applied Sciences, Indianapolis, IN) following the manufacturer's protocol. DNA was diluted in molecular-grade water (Fisher Scientific, Pittsburg, PA) to 1:20 for PCR for integron analysis (150 to 200 μ g/ml) and to 1:10 for pyrosequencing PCR (300 to 400 μ g/ml).

Integron screening and sequencing. The isolates were screened for class 1 integrons by PCR as described previously (19) with forward primer intF (GGCATCCAAGCAGCAAG) and reverse primer intR (AAGCAGACTTGACCTGA) (IDT DNA, Coralville, IA). All PCR reagents were from TaKaRa (Clontech Laboratories, Mountain View, CA). A 50- μ l reaction volume was set up with 4 μ l of 10 $\times$ PCR buffer, 0.8 μ l of 2.5 mM deoxynucleotide triphosphate mix, 11.2 pM each of forward and reverse primer, 6 μ l of magnesium chloride (25 mM/ μ l; Thermo Fisher), 35.6 μ l of molecular biology-grade water, 0.4 μ l of *Taq* polymerase, and 2 μ l of DNA template. The reaction was carried out for 35 cycles with initial denaturing at 94°C for 5 min, followed by denaturation (at 94°C for 30 s), annealing (at 55°C for 30 s), and extension (at 72°C for 2 min 30 s), with a final extension for 5 min at 72°C. The amplified product was purified (Wizard PCR Prep DNA Purification System; Promega) and sequenced on a CEQ 8000 sequencer (Beckman Coulter Fullerton, CA) following the manufacturer's protocol. The results were analyzed with Lasergene software, version 5.6 (DNASTar, Inc., Madison, WI) and compared to the National Center for Biotechnology Information (NCBI) database.

SNPs in the gyrase A gene (*gyrA*) at the QRDR. Single nucleotide polymorphisms (SNPs) at Ser-83 and Asp-87 of the *gyrA* gene quinolone resistance-determining region (QRDR) were determined by pyrosequencing technology (Qiagen, Valencia, CA). All the reagents, tools, and Pyromark PCR kits were obtained from the manufacturer. Based on *S. Typhi* *gyrA* gene sequence (accession number [AM283474](#)) and using the PSQ pyrosequencing assay design software (Qiagen), primers were designed to amplify a 127-bp region (273 to 400) in the *gyrA* QRDR to include the triplet base codons TCC (Ser-83) and GAC (Asp-87). The following primers were used: *gyrA*F1, TAAAAAATCTGCCGTGTC GTTG (forward); *gyrA*R1, CTGACCATCCACCAGCATGTAAC (reverse); and *gyrA*S1, CGGTAAATACCATCCC (sequencing primer). The reverse primer was purified by high-performance liquid chromatography (HPLC) and biotinylated (IDT DNA). PCR was performed with an initial

denaturation at 95°C for 15 min, followed by 45 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, with a final extension at 72°C for 10 min. The PCR product was analyzed by agarose gel electrophoresis before SNP analysis. The biotinylated PCR product was captured with Sepharose beads, and the sample was cleaned and washed with a vacuum prep tool before being analyzed on the Q96 ID pyrosequencer, according to the manufacturer's instructions. Each sample was examined twice for reproducibility.

RESULTS

Thirty-one travel-related *S. Typhi* strains isolated by the Florida Department of Health were analyzed by molecular typing and antibiotic resistance profiling. Factors contributing to MDR and NAL resistance were investigated.

Ribotyping. Ribotyping with *EcoRI* generated 10 types (R1 to R10) and eight subtypes (R1a, R1b, R6a, R6b, R6c, R6d, R6e, and R6f) at approximately 26 to 100% similarity (Fig. 1). Three distinct clusters, A, B, and C, were observed at about 55% similarity. The majority of the strains ($n = 22$) clustered at 84% or greater relatedness (Fig. 1, cluster B). Fourteen strains in cluster B, isolated from 2007 to 2008 and linked to five different countries, Haiti, India, Pakistan, Bangladesh, and Florida, had identical ribotypes (R6d). Eight of these 14 strains were susceptible to all antimicrobials tested, and 4 had a common MDR profile of AMP-CHL-STR-SXT. Two other strains, DOH 10766 (intermediate resistance to CIP) linked to Haiti (isolated in 2010) and the NAL-CIP-resistant strain DOH 10854 associated with travel to India (isolated in 2010) also had identical ribotypes (Fig. 1, R1a, cluster A). One strain (DOH 101094) was an outlier and did not group with cluster A, B, or C. Since two isolates with a similarity of $\geq 90\%$ were defined as clones and as ribotypes, the method reinforced the clonal nature as well as the heterogeneous distribution of *S. Typhi*. For example, a subcluster of cluster B (Fig. 1), including 18 strains linked to Haiti, Pakistan, India, Peru, and Bangladesh, was identical or highly clonal ($\geq 98\%$). Conversely, the 15 strains associated with Haiti were distributed in at least five different ribotypes and were grouped along with strains linked to India, Pakistan, Peru, and Bangladesh. Similarly, the 10 strains associated with India belonged to five ribotypes and demonstrated similarity with strains linked to other countries, including Haiti, Bangladesh, Pakistan, and Peru.

PFGE. Macrorestriction digestion with *XbaI* of the DNA from the 31 strains followed by pulsed-field gel electrophoresis (PFGE) generated 22 (P1 to P22) distinct PFGE types (pulsotypes) and four subtypes (P7a, P7b, P10a, and P10b). Two major clusters were linked at 55% similarity (Fig. 2). Cluster 1 included 11 strains isolated in 2007, 2008, and 2010 associated with travel to Haiti, Lebanon, Bangladesh, and India. The majority of the strains ($n = 20$) were grouped in cluster 2, which can be further divided into two subclusters. Twelve isolates (from 2008 and 2010) linked to Peru, Haiti, and India were grouped at about 72% relatedness in subcluster 2a (Fig. 2). Subcluster 2b grouped eight isolates (2007, 2008, and 2010) linked to Florida, Pakistan, and India at approximately 76% similarity. Five pairs of identical strains with pulsotypes P7a, P12, P14, P17, and P20 were associated with travel to the same country, were isolated in the same year, and demonstrated identical resistance profiles. A sixth identical pair of strains (CBD 1267 and CBD 1346), with the same resistance profile of AMP-CHL-NAL-STR-SXT, was associated with different travel histories (Florida and Pakistan) and was isolated in different years (2007 and 2008). Though strains CBD 1348 and DOH 10269 were

FIG 1 Ribotyping patterns, year of isolation, travel history and antibiotic resistance profiles of *S. Typhi* isolates. Band comparison was performed by using the Dice coefficient with 1% position tolerance (tol). H, minimum height; S, minimum surface; S, susceptible to all 15 drugs tested; NA, data not available; AMP, ampicillin; AMC, amoxicillin-clavulanic acid; CHL, chloramphenicol; SXT, trimethoprim/sulfamethoxazole; NAL, nalidixic acid; STR, streptomycin; CIP, ciprofloxacin.

related to travel in Peru (2008) and Haiti (2010), respectively, and had different resistance profiles, they were clonal (~91% similar) (Fig. 2). Though not clonal by the criteria applied, strains DOH 10621 and DOH 10766, associated with India-United Arab Emirates (UAE) and Haiti, respectively, were about 85% similar by PFGE. Ribotyping called all five MDR strains identical. In contrast, PFGE effectively segregated all five of the MDR strains. Four MDR strains belonged to subcluster 2b (Fig. 2) and were approximately ≥80% similar. The fifth strain was placed in a different subcluster, 2a (Fig. 2), and was ~65% similar to 2b. Only two MDR isolates (CBD 1267 and CBD 1346) were clonal by PFGE. Three of four MDR strains with known travel histories are associated with the Indian subcontinent; the fourth (CBD 1267) is from Florida. Overall, the 12 strains from the Indian subcontinent were distributed in 9 pulsotypes, and the 15 Haiti strains were distributed in 11 pulsotypes.

Antibiotic resistance. All of the isolates were susceptible to amikacin, cefoxitin, ceftiofur, ceftriaxone, gentamicin, kanamycin, sulfisoxazole, and tetracycline. Overall, 16 strains (~51%) were resistant to one or more drugs, including two strains that were intermediately resistant to CHL and CIP (Table 2). Five strains (~16%) were MDR to three or more antimicrobials. All five MDR strains were resistant to CHL, STR, and SXT. Addition-

ally, four of them were resistant to ampicillin, and one was also resistant to amoxicillin-clavulanic acid. Two of the MDR strains were also resistant to NAL (MIC > 32 µg/ml) (Table 2), and four were intermediately resistant to CIP, with a MIC of 0.12 or 0.25 µg/ml (Table 2). All 12 isolates linked to the Indian subcontinent were resistant to at least one antimicrobial. Only one of the 15 isolates associated with Haiti was resistant to any drug tested. Twelve of the 16 resistant isolates (75%) were resistant to NAL, with a MIC of 32 µg/ml or greater. None of the 14 strains isolated in 2010 were resistant to any drugs other than NAL or CIP. Overall, 12 out of 31 strains (38.7%) were NAL resistant. All strains resistant to one or more drugs had a ciprofloxacin MIC of 0.12 or 0.25 µg/ml, with the exception of one (CBD 1347, with a MIC of 0.03 µg/ml). It was interesting that all 15 pan-sensitive strains had a ciprofloxacin MIC of ≤0.015 µg/ml.

Integron screening and sequencing. All 31 isolates were screened for integrons by PCR; three (9.6%) carried a 750-bp integron. The three integron-positive strains had a common resistance profile of AMP-CHL-STR-SXT (Table 2). Sequencing of the integron revealed the presence of the dihydrofolate reductase VII gene (*dhfr*) conferring resistance to trimethoprim. Our sequence showed 99% similarity to the *S. Typhi* class 1 integron *dhfr7* gene (GenBank accession number AY245101).

FIG 2 Macrorestriction analysis of *S. Typhi* strains by PFGE with the XbaI enzyme. The information to the right of the panel includes, in order, strain number, patient travel history, year of isolation, and resistance profile. Band comparison was performed by using the Dice coefficient with 1% position tolerance (tol). H, minimum height; S, minimum surface; NA, data not available; S, susceptible to all 15 drugs tested; AMP, ampicillin; AMC, amoxicillin-clavulanic acid; CHL, chloramphenicol; SXT, trimethoprim/sulfamethoxazole; NAL, nalidixic acid; STR, streptomycin; CIP, ciprofloxacin.

SNPs in the gyrase A gene (*gyrA*) at the QRDR. The NAL-resistant isolates, along with NAL-susceptible controls, were analyzed for the presence of single nucleotide polymorphisms (SNPs) in the *gyrA* gene by pyrosequencing (Table 2). All 12 NAL-resistant strains had nucleotide changes at either Ser-83 or Asp-87 of the *gyrA* quinolone resistance-determining region (QRDR). Eleven of the 12 strains had a point mutation in the Ser-83 (TCC) region, and one strain had the mutation at Asp-87 (GAC). Seven strains (58%) had a C-to-T nucleotide change in the second base of Ser-83, changing the amino acid to phenylalanine (TTC; Phe-83) and four strains (33%) had a C-to-A substitution causing the amino acid change to tyrosine (TAC; Tyr-83). Only one isolate had a mutation with a nucleotide change of G to A at region Asp-87, resulting in the amino acid change to asparagine (AAC; Asn-87). No mutations in either of the two regions screened were detected in the six NAL-susceptible control strains (two controls shown in Table 2).

DISCUSSION

We investigated 31 *S. Typhi* strains that were associated with travel to six different countries. Ribotyping with EcoRI and PFGE with XbaI segregated the isolates into 10 and 22 profiles, respectively. Sixteen strains were resistant or intermediately resistant to at least one antimicrobial, and five showed MDR profiles. Twelve of the

resistant strains were NAL resistant, all of which had a nucleotide alteration at either Ser-83 or Asp-87.

PFGE was more discriminatory than ribotyping, as observed previously (13, 17). Strains from diverse countries, isolated in different years, and with different resistance profiles were identical by ribotyping (Fig. 1), whereas only the isolates sharing geographical linkage, year of isolation, and resistance profiles were identical by XbaI PFGE, with one exception (Fig. 2). This demonstrates that PFGE clusters strains into epidemiologically meaningful groups compared to ribotyping, as reported previously (13). One exception to this was the identical pulsotypes of strains CBD 1267 and CBD 1346 (Fig. 2). While the two strains had identical ribotypes and PFGE and antibiotic resistance profiles, they were linked to Florida (isolated in 2007) and Pakistan (2008), respectively. An apparent association between the two isolates was not supported by the epidemiological data. Strain CBD 1267 was not associated with international travel and was isolated in a different year than CBD 1346, suggesting that either the patient was not truthful about his/her travel history or that he/she came in close contact with an asymptomatic carrier. However, we have no further information regarding an epidemiological investigation of the patient's exposure history.

PFGE segregated some of the strains that were identical by ribotyping and vice versa (Fig. 1 and 2). Considerable genetic het-

TABLE 2 MICs of resistant strains to selected drugs, integron size and gene cassette, nucleotide changes in the *gyrA* gene, ribotype, and PFGE profile

Strain no. (travel association)	Ribotype	Pulsotype	MIC (μg/ml) ^b								Integron size (bp [gene]) ^c	Point mutation at <i>gyrA</i> (amino acid change) ^d	
			AMC	AMP	CHL	STR	SXT	NAL	CIP	Ser-83 (TCC)		Asp-87 (GAC)	
CBD 1267 (Florida)	R6d	P22	8	>32	>32	>64	>4	>32	0.25	750 (<i>dfr</i>)	TTC (Phe)	None	
CBD 1346 (Pakistan)	R6d	P22	2	>32	>32	>64	>4	>32	0.12	750 (<i>dfr</i>)	TTC (Phe)	None	
CBD 1347 ^a (India)	R6d	P21	≤1	≤1	16	>64	>4	16	0.03	None	None	None	
CBD 1348 (Peru)	R6f	P10a	≤1	≤1	8	≤32	0.25	>32	0.25	None	TTC (Phe)	None	
CBD 1349 (India)	R6d	P18	>32	16	>32	>64	>4	8	0.12	750 (<i>dfr</i>)	NA	NA	
CBD 1350 ^a	R6d	P11	8	>32	>32	>64	>4	4	0.12	None	None	None	
CBD 1353 (Bangladesh)	R6d	P3	≤1	≤1	4	≤32	≤0.12	>32	0.25	None	TTC (Phe)	None	
DOH 10341 (India)	R6b	P2	≤1	≤1	4	≤32	≤0.12	>32	0.25	None	TAC (Tyr)	None	
DOH 10355 (India)	R6c	P20	≤1	≤1	4	≤32	≤0.12	>32	0.25	None	TAC (Tyr)	None	
DOH 10511 (India)	R6c	P19	≤1	≤1	4	≤32	≤0.12	>32	0.25	None	TAC (Tyr)	None	
DOH 10621 (India, UAE)	R3	P15	≤1	≤1	4	≤32	≤0.12	>32	0.25	None	TTC (Phe)	None	
DOH 10676 (India)	R8	P20	≤1	≤1	4	≤32	≤0.12	>32	0.25	None	TAC (Tyr)	None	
DOH 10766 (Haiti)	R1a	P16	≤1	≤1	4	≤32	≤0.12	2	0.12	NA	NA	NA	
DOH 10854 (India)	R1a	P20	≤1	≤1	4	≤32	≤0.12	>32	0.12	None	None	AAC (Asn)	
DOH 101093 (India)	R1b	P14	≤1	≤1	4	≤32	≤0.25	>32	0.25	None	TTC (Phe)	None	
DOH 101094 (India)	R10	P14	≤1	≤1	4	≤32	≤0.12	>32	0.12	None	TTC (Phe)	None	

^a NAL-susceptible control for *gyrA* mutations.^b AMP, ampicillin; AMC, amoxicillin-clavulanic acid; CHL, chloramphenicol; SXT, trimethoprim/sulfamethoxazole; NAL, nalidixic acid; STR, streptomycin; CIP, ciprofloxacin. Boldface indicates resistance or intermediate resistance.^c *dfr*, dihydrofolate reductase VII; none, no integron or mutation detected.^d NA, not applicable.

erogeneity was revealed among the isolates since different clones coexisted within the same geographical location. In contrast, clones were distributed among multiple countries. This clonal nature of the *S. enterica* serovar Typhi was especially evident by ribotyping (Fig. 1, cluster B). Generally, only strains sharing the same travel history, year of isolation, and resistance patterns were identical by PFGE. However, PFGE also grouped certain strains with different travel histories at a very high similarity (Fig. 2). This pattern of global clonal distribution of strains is consistent with other studies employing SNP analysis, MLST, or PFGE, where diverse strains were identified in the same geography, and clones were distributed in various locations (3, 16, 21, 27).

MDR strains were discriminated well by PFGE. The 10 two-drug (NAL-CIP)-resistant isolates were distributed among all the clusters by both ribotyping and PFGE. By ribotyping these isolates showed similarity ranging from 26% to 100% with either the susceptible or the MDR strains (Fig. 1). Interestingly, none of these NAL-CIP-resistant strains had identical pulsotypes with either susceptible or MDR strains although they clustered at high similarity by PFGE. Le et al. have suggested that there was a clonal expansion of MDR *S. Typhi*, but the replacement of classical first-line antibiotics with fluoroquinolones resulted in loss of the MDR phenotype and an increase in NAL resistance in strains isolated in Vietnam (17). In a comparison study, these investigators have demonstrated that several different PFGE profiles were present in the H-58 haplotype. Our findings agree with the above concept of microevolution within *S. Typhi* clones based on our PFGE genetic relatedness data among MDR, NAL-CIP-resistant, and fully susceptible isolates. NAL-CIP resistance, although present in 10 of 12 strains associated with the Indian subcontinent, was not limited to that region since a NAL-CIP-resistant strain in this study was related to travel to Peru, and a CIP-resistant strain was from Haiti. However, the fact that insufficient numbers of strains were investigated from other regions needs to be considered. The recent

global increase in NAL-CIP-resistant strains is likely due to increased use of fluoroquinolones in the 1990s, leading to selection of resistant strains (33). The NAL-resistant H-58 haplotype has undergone a recent clonal expansion (14, 33). It is also likely that MDR strains belonging to the H-58 haplotype have recently lost their MDR plasmid, causing a decrease in the incidence of MDR strains with concomitant replacement by NAL single drug resistance, as shown in a previous study (17). A comparison of PFGE profiles and a complete SNP analysis will provide more information about the nature of the strains in our study and illustrate if they belong to the H-58 haplotype.

Three of the MDR strains had a 750-bp integron with the *dfr7* gene cassette conferring resistance to trimethoprim, as seen previously (18, 24, 37). Though resistant to SXT, two out of five MDR strains did not harbor integrons with the *dfr* gene (Table 2). While we did not screen the isolates for plasmids, a plasmid-integron association is highly possible, as noted previously (28, 29). The fact that the majority of the resistant isolates (~93%) had intermediate resistance to CIP regardless of whether they were resistant to NAL suggests that NAL resistance is not a good indicator of DCS, which is consistent with a previous study (7). In this study, the fact that strains with no resistance to any drug have demonstrated DCS is alarming because of the longer clearance times and treatment failures associated with DCS (7). We have examined a NAL-susceptible yet otherwise resistant strain (Table 2, CBD 1350) with DCS for the presence of point mutations in the *gyrA* gene. This strain had no mutations in the two regions investigated. The basis of the apparent DCS could be mutations either in other regions of *gyrA* which we have not examined or in other genes including *gyrB* or *parC*. Since this strain is resistant to other drugs, including AMP, CHL STR, and SXT, decreased membrane permeability or increased efflux pump action may have been involved in the DCS. This may also apply to other NAL-susceptible MDR isolates with intermediate resistance to CIP. The greater number

of SNPs encoding Phe-83 than Tyr-83 in the *gyrA* QRDR observed in this study is consistent with previous studies (10, 36).

Our molecular typing data shed light on the distribution patterns and the dominant *S. Typhi* types circulating throughout the globe. Twenty strains had the ribotype R6, making it the predominant type in this study. Haitian strains are equally diverse, similar to the Indian subcontinent strains, demonstrating that Haiti is an important reservoir of the organism. Various clones have been shown to be circulating and causing infections, implying that these types are maintained in the population. Since carriers are important reservoirs of this organism, screening and treatment of carriers in areas of endemicity will likely be very helpful in preventing spread of this disease, thus reducing the burden of disease.

Recent isolates (isolated in 2010) from our study are predominantly resistant to NAL-CIP and appear to have lost their MDR properties, as previously observed (17). Antibiotic recycling by reusing traditional first-line drugs (e.g., tetracycline, chloramphenicol, and trimethoprim) should be carefully considered. Clinicians in the United States need to be aware of the resistance of this organism to various antimicrobials, especially those associated with the Indian subcontinent, and consider azithromycin or ceftriaxone for empirical treatment (8). Continued monitoring of resistance patterns is essential for successful treatment of travel-related bacterial infections.

Approximately 51% of the cases in this study were in patients who were ≤ 18 years old, indicating that high-risk groups—especially children—need to be vaccinated and to take extra precautions regarding food and water that are consumed while traveling. The fact that the majority of the patients were unvaccinated also supports the need for expanded public awareness of vaccine availability and efficacy. Three patients in our study were food handlers, and one was a health care worker. Following foreign travel, given the nature of their work, these groups could represent heightened risk to public health. With the increased globalization of the food supply, and the large number of travelers to regions of endemicity, typhoid is not only a problem for developing countries but also for developed countries. An increased focus in regions of endemicity on improving sanitary conditions, the quality of food and water, identification and treatment of carriers, and surveillance for disease and an effective immunization program will all help reduce the incidence and burden of disease.

Our study represents 3 years of strain and epidemiological data associated with multiple countries of origin and elucidates typing and resistance patterns of travel-related *S. Typhi* in Florida. Strains isolated in the year 2009 were not available for study, which was a minor deficiency of our study. To our knowledge, this is the first report about the use of pyrosequencing technology to analyze the *gyrA* gene QRDR in *S. Typhi*. Our study is consistent with other studies and reaffirms the global clonal distribution and the increase in the NAL resistance of this very important organism.

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