

Analysis of Plasmid and Chromosomal DNA of Multidrug-Resistant *Salmonella enterica* Serovar Typhi from Asia

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Molecular analysis of chromosomal DNA from 193 multidrug-resistant (MDR) *Salmonella enterica* serovar Typhi isolates from 1990 to 1995 from Pakistan, Kuwait, Malaysia, Bangladesh, and India produced a total of five major different pulsed-field gel electrophoresis (PFGE) patterns. Even within a particular country MDR *S. enterica* serovar Typhi DNA was found to be in different PFGE groups. Similar self-transferable 98-MDa plasmids belonging to either incompatibility group incHI1 or incHI1/FIIA were implicated in the MDR phenotype in *S. enterica* serovar Typhi isolates from all the locations except Quetta, Pakistan, where the majority were of incFIIA. A total of five different PFGE genotypes with six different plasmids, based on incompatibility and restriction endonuclease analysis groups, were found among these MDR *S. enterica* serovar Typhi isolates.

It is estimated that each year there are over 20 million cases of typhoid fever, which result in 700,000 deaths worldwide (25). In developed countries, the incidence of cases and death has been greatly decreased by a combination of improved sanitation and hygiene, vaccines, and effective antimicrobial chemotherapy. The first two are difficult if not impossible to implement in many developing countries, and, unfortunately, the effectiveness of antimicrobial chemotherapy is also being eroded by the emergence of antibiotic resistance (5, 16, 27). In developing countries, the antibiotics most readily available for treatment of typhoid are chloramphenicol, ampicillin, and cotrimoxazole. Plasmid-encoded chloramphenicol resistance emerged first in the early 1970s (2), followed by large epidemics in Central America (20). Although slightly less effective than chloramphenicol, ampicillin was used both for therapy and for elimination of the carrier state (22). Again, plasmid-encoded resistance soon developed (1, 20). Finally, cotrimoxazole was introduced in 1980, and plasmid-encoded resistance to trimethoprim and sulfonamides was observed shortly afterwards (7). The first cases of typhoid due to *Salmonella enterica* serovar Typhi carrying plasmid-encoded resistance to chloramphenicol, ampicillin, and cotrimoxazole were reported from southeast Asia (14). Cases in the area increased exponentially, and it is possible to define an epidemic zone for multidrug-resistant (MDR) *S. enterica* serovar Typhi which encompasses China, southeast Asia, and the Indian subcontinent (16). There is also a potential epidemic zone in the Middle East (Kuwait, Oman, and Saudi Arabia) due to importation of MDR *S. enterica* serovar Typhi by migrant workers from the epidemic zone. Interestingly MDR *S. enterica* serovar Typhi is rarely, if at all, reported from Africa or South and Central America, where some of the early epidemics of chloramphenicol-resistant typhoid were first reported (2, 20). We investigated the antimicrobial susceptibility and the genetic basis of resistance

of MDR *S. enterica* serovar Typhi isolates from various countries in Asia and the Middle East. Pulsed-field gel electrophoresis (PFGE) was used to study the genotypic relationship among the isolates.

MATERIALS AND METHODS

Bacterial isolates. A total of 193 MDR *S. enterica* serovar Typhi isolates obtained from 1990 to 1995 were studied; 149 isolates were from patients from Rawalpindi and Quetta, Pakistan, 30 were from Bangladesh, 6 were from Kuwait, 6 were from Malaysia, and 2 were from India. The identity of *S. enterica* serovar Typhi isolates was confirmed using biochemical tests on Analytab Products strips (bioMérieux, Basingstoke, United Kingdom), and they were serotyped using agglutinating antisera (Murex Diagnostics, Dartford, United Kingdom). Isolates were stored at -70°C on Protect beads (Technical Service Consultants Ltd., Heywood, United Kingdom) until analyzed.

Antimicrobial susceptibility testing. *S. enterica* serovar Typhi isolates were tested for susceptibility to antimicrobials by a controlled disk diffusion technique according to the guidelines provided by the National Committee for Clinical Laboratory Standards (18) on diagnostic sensitivity testing (DST) agar (Oxoid Ltd., Basingstoke, United Kingdom) plates containing 5% lysed horse blood. The antibiotic discs (all from Oxoid) contained ampicillin (10 μg), tetracycline (30 μg), cotrimoxazole (1.25 μg), chloramphenicol (30 μg), streptomycin (30 μg), gentamicin (10 μg), amoxicillin (20 μg)-clavulanic acid (10 μg), ofloxacin (10 μg), ciprofloxacin (10 μg), and nalidixic acid (10 μg). MICs of these antibiotics were determined using the E-test strips (AB BIODISK, Solna, Sweden) following the manufacturer's instructions. Adjusted inocula of bacteria (ca. 10^6 CFU/ml [ca. 0.5 MacFarland standard]) were used on DST agar plates. *Escherichia coli* ATCC 25922 (the MICs for which are known) was used as a control for potency of antibiotics.

Beta-lactamase study. Preparations of protein extracts from *S. enterica* serovar Typhi isolates were made using the method of Corkill et al. (6) and were analyzed by isoelectric focusing on sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels (Pharmacia, St. Albans, United Kingdom). Ampholytes with pIs in the range of 3.5 to 9.5 were used to generate the pH gradient, and proteins of known pIs were included as controls, as were extracts from bacteria expressing TEM-1 and TEM-2.

PFGE of macrorestricted genomic DNA. Chromosomal DNA from *S. enterica* serovar Typhi isolates was prepared in agarose plugs as described by Thong et al. (26). DNA in agarose plugs was digested using 25 U of *Xba*I or 20 U of *Spe*I (Life Technologies, Paisley, United Kingdom). PFGE of agarose plug inserts was then performed on a CHEF-DR II system (Bio-Rad Laboratories, Richmond, Calif.) on a horizontal 1% agarose gel for 22 h at 120 V, with a pulse time of 1 to 40 s at 14°C . A lambda DNA digest consisting of a ladder (ca. 22 fragments) of increasing size from 48 kb to approximately 1,000 kb was included as a DNA size standard. The gel was stained with ethidium bromide and photographed on a UV transilluminator (UVP Inc., San Gabriel, Calif.). The restriction endonuclease (RE) digest patterns were compared, and their similarities were scored by the

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TABLE 1. Patterns of antibiotic resistance transfer from MDR *S. enterica* serovar Typhi isolates using ampicillin and chloramphenicol for selection of transconjugant phenotypes

Isolate origin (no.)	Phenotype of donor ^a	No. of successful transfers	Phenotype transferred to <i>E. coli</i> K-12 ^b
Rawalpindi (25)	Cm Ap Te Tm Su Sm	25	Cm Ap Te Tm Su Sm
Quetta (122)	Cm Ap Te Tm Su Sm	72	Cm Ap Te Tm Su Sm (62) Cm Te Tm Sm (5) Cm Ap Te Tm Sm (5)
Bangladesh (30)	Cm Ap Te Tm Su Sm	10	Cm Tm Ap
Kuwait (8)	Cm Ap Te Tm Su Sm	6	Cm Te Ap
Malaysia (6)	Cm Ap Te Tm Su Sm	6	Cm Te Ap
India (2)	Cm Ap Te Tm Su Sm	2	Cm Ap Te Tm

^a Ap, ampicillin; Cm, chloramphenicol; Tm, trimethoprim; Su, sulfonamide; Te, tetracycline; Sm, streptomycin.

^b Numbers in parentheses are numbers of isolates with successful transfers.

method of Tenover et al. (24). By these criteria isolates that gave PFGE banding patterns that were indistinguishable were assumed to be from a single outbreak strain. Isolates that gave banding patterns showing differences in fewer than four bands were assumed to be closely related, as they may represent isolates differing by a single genetic event. In addition, isolates with differences in four to six bands may also be part of an outbreak. However, isolates showing a difference in more than seven bands of their banding patterns may represent more than three genetic events, in which case they were considered epidemiologically unrelated.

Conjugation of plasmids and incompatibility grouping. Conjugation experiments were carried out in broth using the method of Walia et al. (28) with *E. coli* K-12 (Nal^r Lac⁺) as the recipient. All *S. enterica* serovar Typhi isolates were susceptible to nalidixic acid. Transconjugants were selected on MacConkey agar (Oxoid) supplemented with nalidixic acid (32 mg/l each) and ampicillin or chloramphenicol (32 mg/l each). Plasmid DNA was then extracted from the transconjugants using an alkaline lysis method (3). Plasmids were electrophoresed on horizontal 0.8% agarose gels and stained with 0.05% ethidium bromide (Sigma, Poole, United Kingdom). DNA bands were then visualized using a UV transilluminator (UVP).

Incompatibility grouping was performed on plasmids extracted from *E. coli* K-12 transconjugants using DNA-DNA hybridization (15) on nitrocellulose membranes (Sigma). A total of eight rep probes (incFII, incFI, incB/O, incHI1, incK, incN, incP, and incW) labeled with biotinylated UTP by nick translation were employed as described previously (13). Probe-target hybridization was detected using a streptavidin-alkaline phosphatase conjugate and nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate for visualization (Sigma).

Restriction endonuclease analysis (REA). Plasmid DNA was extracted from MDR *S. enterica* serovar Typhi transconjugants as described previously (4) and subsequently was digested using *Hind*III and *Eco*RI (Life Technologies) according to the manufacturer's instructions. Restriction fragments were resolved by agarose gel electrophoresis. *Hind*III digests of lambda DNA were used as size standards.

RESULTS

Antimicrobial susceptibility. A total of 193 MDR *S. enterica* serovar Typhi isolates were examined. All the isolates were uniformly resistant to the five antimicrobial agents commonly available in developing countries, namely, ampicillin, co-trimoxazole, chloramphenicol, tetracycline, and streptomycin. However, they were all sensitive to nalidixic acid, cefuroxime, cefotaxime, ciprofloxacin, and ofloxacin. MICs of ampicillin, chloramphenicol, streptomycin, and tetracycline were all >256 mg/l, and the MICs of co-trimoxazole were 16 to 32 mg/l. MICs of nalidixic acid ranged between 2 and 4 mg/l, and amoxicillin-clavulanic acid MICs ranged between 0.25 and 10 mg/l. Resistance in each case was associated with self-transferable 98-MDa plasmids.

Beta-lactamases. On isoelectric focusing all the MDR *S. enterica* serovar Typhi isolates produced beta-lactamases with a pI of 5.4 that cofocused with the control TEM-1 beta-lactamase. As these isolates did not show resistance to the extended-spectrum beta-lactams, no further analysis of their beta-lactamases was carried out.

Conjugation of resistance plasmids and incompatibility grouping. As shown in Table 1 121 of the 193 (62.7%) MDR *S. enterica* serovar Typhi isolates from the various countries

transferred resistance to three or more antimicrobials to *E. coli* K-12. For all the MDR *S. enterica* serovar Typhi isolates resistance was carried on 98-MDa plasmids. Apart from *S. enterica* serovar Typhi isolates from Quetta, Pakistan, which transferred three different resistance phenotypes, all the isolates transferred the same resistance phenotype in each case. The 98-MDa resistance plasmids fell into five different incompatibility groups (Table 2). Apart from resistance plasmids from Pakistan, which were in three different incompatibility groups, the plasmids were either of incHI1 or incHI1 cross-reacting with incFIIA (incHI1/FIIA). The distribution of the other plasmid incompatibility groups is shown in Table 2. Interestingly, the predominance of incFIIA plasmids among the *S. enterica* serovar Typhi isolates from Pakistan contrasts with the general association between incHI1 plasmids and *S. enterica* serovar Typhi worldwide.

REA of resistance plasmids. RE digestion of the 98-MDa plasmids from MDR *S. enterica* serovar Typhi with *Eco*RI or *Hind*III produced 7 to 12 fragments ranging in size from 2 to 10 kb (Table 3). Two isolates from Quetta, Pakistan, had distinct unique restriction digestion patterns. In order to improve resolution, the plasmid DNA was digested with *Hind*III and *Eco*RI together. There was better discrimination between strains following digestion with the two enzymes combined than with the individual enzymes. As expected more DNA fragments (15 to 18) were produced with the combined restriction enzyme digestion than with digestion by the individual enzymes, and their sizes varied from less than 2,000 to 10,000 bp (Table 3). With the combination of *Hind*III and *Eco*RI, REA patterns for plasmids from four of the five different countries (the exception being Pakistan [Rawalpindi and Quetta]) were similar (Table 3). REA of plasmids from Quetta using *Hind*III and *Eco*RI, either alone or in combination, gave two distinct patterns; these plasmids were clearly different

TABLE 2. Incompatibility groups of the transferable 98-MDa resistance plasmids from 121 *E. coli* transconjugants

Source	No. of isolates with incompatibility group ^a :					
	FIIA	FIIA	P	B/O	HI1	HI1/FIIA ^b
Rawalpindi						25
Quetta	69		2	1		
Bangladesh					1	9
Kuwait					5	1
India					2	
Malaysia		1				5

^a The prefix inc is omitted throughout.

^b HI1/FIIA, plasmids hybridized with both the rep incHI1 and incFIIA probes.

TABLE 3. REA patterns of 98-MDa plasmids and PFGE analysis of *Xba*I (5'-TCTAGA-3')-digested chromosomal DNA

Isolate origin (no.)	No. of REA fragments from digestion with:			REA pattern(s) of plasmids	PFGE pattern(s) ^a
	<i>Hind</i> III	<i>Eco</i> RI	<i>Hind</i> III + <i>Eco</i> RI		
Rawalpindi (25)	9–10	9–10	15–18	I	A1 (18), A2 (3), B1 (4)
Quetta (122)	8–9	11–12	16–17	III and IV	A1 (92), B1 (12), B2 (10), E1 (2), E2 (6)
Kuwait (8)	7–8	8–9	15–18	II	C3 (8)
Bangladesh (30)	7–8	8–9	15–18	II	C1 (30)
Malaysia (6)	7–8	8–9	15–18	II	D1 (1), D2 (2), E1 (3)
India (2)	7–8	8–9	15–18	II	C2 (2)

^a The PFGE patterns were arbitrarily designated groups A to E. The pattern for each group differs by more than seven bands from those of the other groups. The subgroup patterns (e.g., C1, C2, C3) differed from each other by fewer than seven bands. Numbers in parentheses are numbers of isolates in the subgroup.

from the plasmids from Rawalpindi, Pakistan, and from the other four countries.

PFGE. *Xba*I-digested DNA from *S. enterica* serovar Typhi isolates from the five different countries produced a total of five different patterns consisting of 13 to 22 fragments ranging in size from ca. 23 to 730 kb. Figure 1 shows the PFGE banding patterns of representative MDR *S. enterica* serovar Typhi isolates from various countries. The 30 MDR *S. enterica* serovar Typhi isolates from Bangladesh all produced the same PFGE fragment pattern (designated subgroup C1 in Table 3). This pattern was closely related to the PFGE patterns for the isolates from India (C2) and Kuwait (C3). The Malaysian isolates produced one pattern (D) that was distinct from all the other isolates and one (E) that was also found in eight isolates from Quetta, Pakistan. The MDR *S. enterica* serovar Typhi isolates from Rawalpindi, Pakistan, fell into two groups (A and B), and the majority of the isolates from Quetta, Pakistan, fell into these two groups, which were not detected among isolates from any of the other regions. As with the *Xba*I-digested DNA, the *Spe*I-digested DNA fragment pattern analysis resulted in similar types and a similar distribution of digestion patterns of *S. enterica* serovar Typhi isolates from the five countries and hence did not offer further discrimination among the *S. enterica* serovar Typhi isolates. However, *Spe*I digestion pro-

duced more fragments (18 to 24), ranging in size from ca. 20 to 750 kb.

DISCUSSION

In this study we have examined a total of 193 MDR isolates of *S. enterica* serovar Typhi from five different countries in Asia and the Middle East. Each was included because of resistance to chloramphenicol, ampicillin, and co-trimoxazole, and this resistance phenotype is predominant in many parts of the continent (9, 16, 19, 23, 27). The MICs of ampicillin, chloramphenicol, and co-trimoxazole among our MDR *S. enterica* serovar Typhi isolates are similar to those reported from India and Vietnam (23, 27). In each case ampicillin resistance was mediated by a β -lactamase with a pI of 5.4 that cofocused with TEM-1. This confirms and extends findings from Vellore, India, which demonstrated that all the MDR *S. enterica* serovar Typhi isolates examined expressed TEM-1 (23). All our MDR *S. enterica* serovar Typhi isolates were sensitive to cefuroxime and cefotaxime and, unlike those from Vietnam and Tajikistan (17, 27), all were sensitive to nalidixic acid, ciprofloxacin, and ofloxacin. This is of particular importance since the fluoroquinolones ciprofloxacin and ofloxacin are particularly valuable in the treatment of typhoid fever whether due to MDR *S. enterica* serovar Typhi or not (3, 5, 12).

In each case antimicrobial resistance was transferable to *E. coli* K-12 on ca. 98-MDa plasmids. In previous studies the large (90- to 110-MDa) self-transmissible plasmids from MDR *S. enterica* serovar Typhi have invariably been of incompatibility group IncHI1 (1, 10, 23). Although all of the tested plasmids from the MDR *S. enterica* serovar Typhi isolates from Bangladesh, Kuwait, India, and Rawalpindi, Pakistan, fell into this or a cross-reacting incHI1/FIIA group, none of the plasmids from the Quetta isolates did so. The majority of the Quetta plasmids were of incFIIA but two were of incP and one was of IncB/O, a distribution which has not been reported previously. In addition one of five Malaysian plasmids was of incFIIA. RE digestion of the plasmids revealed a total of five different patterns. The plasmids from the MDR *S. enterica* serovar Typhi isolates from Kuwait, Bangladesh, Malaysia, and India all produced the same pattern regardless of their incompatibility groups and antibiotic resistances transferred. The isolates from Rawalpindi all produced the same plasmid RE pattern, which was different from all the others. Finally the isolates from Quetta carried plasmids that fell into two different RE patterns that were also unique. Those of incFIIA produced one pattern, and the rest produced the other pattern. It is thus clear that the MDR phenotype in the isolates from our study is encoded on a variety of different plasmids, as determined by incompatibility group and REA. However, most of the diversity arises in the isolates from Pakistan.

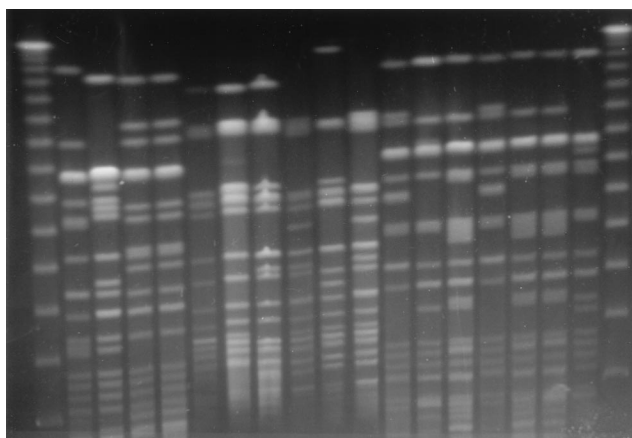


FIG. 1. *Xba*I RE fragment patterns of representative *S. enterica* serovar Typhi isolates from various countries. Lanes 1 and 19 (numbering is from left to right), 48.5-kb ladder molecular size standard; lane 2, B1 from Bangladesh in PFGE group C1; lane 3, I1 from India in PFGE group C2; lanes 4 and 5, K1 and K2, respectively, from Kuwait in PFGE group C3; lanes 6, 7, 8, and 9, M1, M2, M3, and M4 from Malaysia in PFGE groups D1, D2, D2, and E1, respectively; lanes 10, 11, 12, 13, and 14, Q1, Q2, Q3, Q4, and Q5 from Quetta, Pakistan, in PFGE groups E2, E1, B1, B2, and A1, respectively; lanes 15, 16, 17, and 18, R1, R2, R3, and R4 from Rawalpindi, Pakistan, in PFGE groups B1, A1, A1, and A2, respectively.

PFGE of macrorestricted chromosomal DNA from the MDR *S. enterica* serovar Typhi isolates produced five major patterns which differed from each other in more than seven bands. This was apparent when either *Xba*I or *Spe*I was used. Although until recently it has been suggested that *S. enterica* serovar Typhi represents a single clone with little interspecies divergence (21), molecular analyses such as that by PFGE are demonstrating much greater genetic heterogeneity among *S. enterica* serovar Typhi isolates (9, 23, 25, 26). It is possible that some of the apparent genomic diversity could result from homologous recombinations between *rnm* operons, as has been observed in epidemiologically linked outbreaks in Spain (8). It is unlikely that such events could account for all the diversity detected in the present study and by others, especially in those isolates that are not epidemiologically linked (8, 19, 21, 22). Previously PFGE analysis of *S. enterica* serovar Typhi isolates from Vellore, India, has demonstrated that although antibiotic-sensitive isolates showed great genomic variability, the MDR *S. enterica* serovar Typhi constituted a single genotype (23). Analysis of MDR *S. enterica* serovar Typhi isolates from visitors to Canada (10) and from Bangladesh (11) also demonstrated single genotypes. In the present study two different PFGE patterns were detected in both Pakistan and Malaysia. In contrast the isolates from India, Bangladesh, and Kuwait produced identical patterns. Thus a total of five different genotypes of MDR *S. enterica* serovar Typhi were detected. Similarly, Hampton et al. (9) were able to detect different genotypes among MDR *S. enterica* serovar Typhi isolates originating from Pakistan, India, Bangladesh, and Tajikistan. In conclusion, our study has demonstrated that there are at least five different genotypes of MDR *S. enterica* serovar Typhi in circulation in Asia. In addition it appears that there are at least six different plasmids encoding the MDR phenotype based on a combination of REA and incompatibility grouping. Clearly the MDR *S. enterica* serovar Typhi isolates that are circulating in Asia are not derived from a single clone of *S. enterica* serovar Typhi that has acquired one particular resistance plasmid.

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