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Molecular Typing of *Salmonella typhi* Strains from Dhaka (Bangladesh) and Development of DNA Probes Identifying Plasmid-Encoded Multidrug-Resistant Isolates

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Seventy-eight *Salmonella typhi* strains isolated in 1994 and 1995 from patients living in Dhaka, Bangladesh, were subjected to phage typing, ribotyping, IS*200* fingerprinting, and PCR fingerprinting. The collection displayed a high degree of genetic homogeneity, because restricted numbers of phage types and DNA fingerprints were observed. A significant number of the *S. typhi* strains (67%) were demonstrated to be multiple drug resistant (MDR). The vast majority of the MDR strains were resistant to chloramphenicol, ampicillin, trimethoprim, streptomycin, sulfamethoxazole, and tetracycline (R type CATmSSuT), a resistance phenotype that has also frequently been observed in India. Only two strains displayed a distinct MDR phenotype, R type ATmSSuT. Pulsed-field gel electrophoresis demonstrated the presence of large plasmids exclusively in the MDR strains of both R types. The plasmids present in the *S. typhi* strains of R type CATmSSuT could be conjugated to *Escherichia coli* and resulted in the complete transfer of the MDR phenotype. PCR fingerprinting allowed discrimination of MDR and susceptible strains. The DNA fragments enabling discrimination of MDR and susceptible *S. typhi* strains by PCR were useful genetic markers for identifying MDR encoded by large plasmids of the H1 incompatibility group.

Typhoid fever, caused by *Salmonella typhi*, yearly affects more than 12.5 million people worldwide (3). The vast majority of cases occur in Southeast Asia, Africa, and South America, although both the case fatality rate and the spectrum of clinical complications of typhoid fever vary considerably between different areas where the disease is endemic (9).

The alarming ability of the microbe to acquire persistent, high-level resistance to the clinically most relevant antibiotics (chloramphenicol, ampicillin, and trimethoprim-sulfamethoxazole) is a major problem (12, 18). Of particular concern are those strains that have acquired multiple drug resistance (MDR) against two or more therapeutic agents. Since 1989, MDR *S. typhi* strains have become prevalent in most of the areas where the disease is endemic (21, 27). MDR in *S. typhi* strains is often encoded by plasmids. Of particular interest are the plasmids encoding resistance to chloramphenicol, ampicillin, trimethoprim, sulfonamides, and tetracyclines (R type CATmSSuT). These large plasmids (approximately 180 kb) belong to the H1 incompatibility group and originate from Southeast Asia, in particular, India, Bangladesh, and Pakistan (1, 18–20, 25, 28).

One of the main approaches for combatting typhoid fever today is the control of the dissemination of the causative agent. To this end, investigation of the epidemiology of *S. typhi* is of major importance. The classical method of distinguishing different clinical isolates is bacteriophage typing (for a review, see reference 7). Recently, molecular techniques have been applied to epidemiological investigations of *S. typhi* infections. Restriction fragment length polymorphism analysis of chromosomal DNA (11, 14, 17, 23) and ribotyping (6, 24) have led to a significant increase in discriminatory power. By these techniques, various distinct fingerprint types can be distinguished, even within phage types (2, 15). Recently, the mobile genetic element IS*200*, initially described by Lam and Roth (10), has also been applied for DNA fingerprinting. The number of insertion sequence (IS) copies in *S. typhi* varies from 10 to 25 (4), suggesting its usefulness in epidemiological investigations. The discriminatory power of IS*200* fingerprinting was found to be poor among strains isolated in areas such as India and Pakistan, where a restricted number of phage types occur (26, 27).

In the study described here, we examined 78 *S. typhi* strains isolated in Dhaka, Bangladesh, in 1994 and 1995 by phage typing, ribotyping, IS*200* fingerprinting, and PCR fingerprinting. PCR was also used to develop MDR-specific probes. Evidence for the clonal spread of genetically strongly related *S. typhi* isolates is given, and the significance of plasmid-encoded MDR is discussed.

MATERIALS AND METHODS

Bacterial strains. Seventy-eight *S. typhi* strains were isolated in 1994 and 1995 from 60 pediatric patients living in Dhaka city. The strains were isolated from the blood of patients who were suffering from clinically diagnosed enteric fever. Eighteen of these patients were readmitted to the health center 16 to 41 days after primary diagnosis and treatment, and *S. typhi* infection or reinfection was confirmed by culture. Both primary isolates and relapse isolates (paired *S. typhi* isolates) were used in the study. Ten additional *S. typhi* strains, provided by the National Institute of Public Health and Environmental Protection (Bilthoven,

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The Netherlands), were also included in the study. This group consisted of clinical isolates from different geographic areas (see Table 1).

Drug susceptibility testing by the agar disk diffusion assay. The disk diffusion method was performed on Iso-Sensitest agar with Oxoid disks (Oxoid, Hampshire, United Kingdom) according to the instructions of the manufacturer. The disks of ampicillin, ampicillin-clavulanic acid, chloramphenicol, tetracycline, ciprofloxacin, trimethoprim, sulfamethoxazole, and streptomycin were placed on plates that were inoculated with bacteria to obtain semiconfluent growth. Inocula were prepared by suspending colonies from blood agar plates incubated at 37°C for 18 h to achieve a density equivalent to that of a McFarland 0.5 standard. The photometrically adjusted suspensions were swabbed onto the surfaces of the Iso-Sensitest agar plates; this was followed by the application of up to four disks with antimicrobial agents per plate. The plates were incubated for 18 to 24 h at 37°C. Zones of inhibition were then recorded. Zone diameter interpretive standards and equivalent MIC breakpoints for gram-negative organisms were used as recommended by the National Committee for Clinical Laboratory Standards (16).

Phage typing. All *S. typhi* strains were analyzed by phage typing by the method described by Guinee and Van Leeuwen (7). This method can discriminate more than 100 different phage types. The phages and bacterial strains used for phage propagation were supplied by B. Rowe, National Institute of Public Health, London, United Kingdom.

DNA fingerprint analyses. The bacterial cultures were grown in Luria-Bertani (LB) broth (22) to an optical density of 0.6 at 560 nm. The cells were harvested by centrifugation at 5,000 × *g* for 10 min. Genomic DNA was extracted by the cetyltrimethylammonium bromide (CTAB) method (31). The restriction enzymes *Pst*I and *Eco*RI were used to digest the CTAB-purified chromosomal DNA samples (22). The restriction fragments were separated electrophoretically on a 0.8% agarose gel, denatured, and transferred onto a nylon membrane (Hybond N+; Amersham International plc, Amersham, United Kingdom) by vacuum blotting (VacuGene; Pharmacia LKB Biotechnology AB, Uppsala, Sweden). Southern blot hybridization and detection were carried out by the chemiluminescence gene detection system according to the guidelines of the manufacturer (Amersham International plc). A 529-bp DNA probe consisting of part of the gene encoding 16S rRNA (rDNA probe) was generated by PCR with purified *S. typhi* chromosomal DNA (8). On the basis of the IS*200* sequence (10), a 400-bp IS*200* DNA probe was generated by PCR with *S. typhi* chromosomal target DNA and the primers IS*200*-F2 (5′-TATCTTCGCCGTGTTCTTAC-3′) and IS*200*-R2 (5′-CAAAAGTTCAACAAGAAATC-3′). The PCR conditions used to amplify the 16S rDNA probe and the IS*200* DNA were as described previously (8).

Random amplification of polymorphic DNA was performed in a PCR buffer system containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 2.5 mM $MgCl_2$, 0.01% gelatin, 0.1% Triton X-100, 0.2 mM (each) the respective deoxynucleotide triphosphates, 50 pmol of primer, and 0.2 U of *Taq* polymerase (Supertaq; HT Biotechnology, Cambridge, United Kingdom), to which CTAB-purified DNA was added (50 ng per amplification mixture). The PCR mixtures were overlaid with 100 μl of mineral oil. Cycling was performed in a Biomed PCR thermocycler (Model 60; Biomed, Theres, Germany) and consisted of the following steps: predenaturation at 94°C for 4 min and 35 cycles of 94°C for 1 min, 25°C for 1 min, and 74°C for 2 min. The primers used to discriminate the *S. typhi* strains were ERIC1 (5′-ATGTAAGCTCCTGGGGATTCAC-3′), ERIC2 (5′-AAGTAAGT GACTGGGGTGAGCG-3′), and RAPD1 (5′-GGTTGGGTGAGAATTGCA CG-3′) (29, 30, 32). Primer RAPD1 and the primer combination ERIC1-ERIC2 were applied in single PCR assays, respectively. The amplification products were separated by electrophoresis in 1.5% agarose gels (5 mm thick; Hispanagar; Sphaero Q, Leiden, The Netherlands). Gels were run in 0.5× Tris-borate-EDTA buffer (TBE) at a constant current of 100 mA for 2 h. Prior to electrophoresis, samples were mixed with a 5× concentrated layer mixture consisting of 50% glycerol in water and 0.8 mg of bromophenol blue per ml. Thirty-five microliters of the amplified material was loaded onto the gel, and a molecular weight marker was run in parallel with the PCR fingerprint samples. Gels were stained after electrophoresis by the addition of 10 μl of ethidium bromide (10 mg/ml) to a total volume of 300 ml of 0.5× TBE (22). Gels were photographed with a Polaroid MP4 Landcamera and Polaroid 57 high-speed film; the exposure time was 0.125 to 0.25 s (diaphragm F5.6). The banding patterns visualized in this manner were indexed by letter codes.

All PCR-amplified DNA probes were purified from the agarose gel by Geneclean extraction as recommended by the manufacturer (Bio 101 Inc., La Jolla, Calif.).

Pulsed-field gel electrophoresis. *S. typhi* isolates and *Escherichia coli* conjugant strains were grown in LB broth (22) to an optical density of 0.5 at 560 nm. The cells were spun down, washed twice in phosphate-buffered saline (PBS), and resuspended in PBS in volumes of the material concentrated 20 times. The bacterial suspensions were embedded in agarose plugs by mixing with equal volumes of a 2% agarose solution (FMC BioProducts, Rockland, Maine); 200-μl plugs were prepared. The plugs were incubated overnight with lysozyme (10 mg/ml; Sigma Chemicals, Zwijndrecht, The Netherlands) in 5 mM EDTA and were subsequently washed four times for 1 h each time with PBS. The plugs were then incubated overnight with proteinase K (1 mg/ml; Merck Chemicals, Amsterdam, The Netherlands) in 250 mM EDTA containing 1% sarcosyl, 0.04% desoxycholate, and 0.1% Brij 58. To inactivate the proteinase K, the plugs were washed for 1 h with 10 mM Tris-HCl–1 mM EDTA (pH 8.0) containing 1 mM phenylmethylsulfonyl fluoride. The plugs were finally washed three times for 1 h each time with PBS. The DNA present in the agarose plugs was analyzed on a 1% agarose gel by pulsed-field gel electrophoresis (CHEF DRIII; Bio-Rad Laboratories Inc., Veenendaal, The Netherlands) at 14°C at 6 V/cm in 0.5× TBE (22) by using pulse times of 2 to 17 s at an angle of 120°. Both the agarose gel and the electrophoresis buffer contained 0.1 μg of ethidium bromide per ml.

Conjugational transfer of drug resistance. MDR isolates were used to transfer resistance to a plasmid-free strain of *E. coli* K-12 (strain WG1 F^- lac^+ Nal^r) and an F factor-containing *E. coli* strain (strain WG2). All mating procedures were carried out at 28 and 37°C for 18 h before plating onto appropriate selective medium containing 50 μg of ampicillin per ml or 40 μg of tetracycline per ml. Nalidixic acid at a final concentration of 80 μg/ml was used for counterselection. Transfer frequencies were expressed in terms of the ratio of the number of drug-resistant conjugants to the number of viable cells of the recipient strain after incubation of the mating mixture for 18 h at 37°C. The antibiograms of the conjugants were determined by the agar disk diffusion assay as described above.

RESULTS

Antibiotic resistance patterns of the *S. typhi* strains isolated in Dhaka. The antibiograms of all 78 isolates were determined by the disk diffusion assay with a variety of antibiotics. Twenty-six strains were susceptible to all antibiotics tested. Fifty strains appeared to be resistant to chloramphenicol, ampicillin, trimethoprim, streptomycin, sulfamethoxazole, and tetracycline (R type CATmSSuT). Two strains were resistant to all of the antibiotics mentioned above except chloramphenicol (R type ATmSSuT). Of the 36 paired isolates, 28 were resistant to multiple antibiotics. The primary and secondary isolates from 14 patients displayed the R type CATmSSuT. Both primary and secondary isolates from only one patient were susceptible. The primary and secondary isolates from three patients displayed different antibiograms. The R type ATmSSuT for the primary isolate from one patient was succeeded by R type CATmSSuT for the secondary isolate. Furthermore, the drug-susceptible primary isolate from the second patient was succeeded by an MDR isolate of R type CATmSSuT, whereas an MDR isolate (R type CATmSSuT) from the third patient was followed by a susceptible isolate.

Analysis of *S. typhi* strains of different geographic origins by various typing methods. To investigate the discriminatory power of the fingerprint techniques used in the present study, we analyzed 10 *S. typhi* strains of different geographic origins by phage typing, ribotyping, IS*200* DNA fingerprinting, and PCR fingerprinting. The results are summarized in Table 1. Eight distinct phage types were observed. *Pst*I ribotyping generated eight distinct fingerprints (Fig. 1A), whereas only a single *Eco*RI ribotype was observed. IS*200* DNA fingerprinting generated two distinct DNA types (Fig. 1B). By PCR fingerprinting, the primer combination ERIC1-ERIC2 did not generate DNA polymorphism (Fig. 2). However, primer RAPD1 was able to generate polymorphism, because four distinct types were observed (Fig. 3A). We also investigated the resistance profiles of the *S. typhi* strains a to j. Strain i from Pakistan was resistant to chloramphenicol, trimethoprim, streptomycin, and tetracycline but was susceptible to ampicillin and sulfamethoxazole (R type CTmST). Strain j was resistant to ampicillin, trimethoprim, streptomycin, sulfamethoxazole, and tetracycline but was susceptible to chloramphenicol (R type ATmSSuT). Furthermore, strains a to h were susceptible to all antibiotics tested (Table 1).

Phenotypic and genotypic analysis of the *S. typhi* strains isolated in Dhaka. (i) Phage typing. All 78 *S. typhi* strains were analyzed by phage typing. The results are summarized in Table 2. Phage type E1a was predominantly present, because 56 strains (72%) had this type. Type E1a was observed in 85% of the MDR strains, whereas only 46% of the susceptible strains

TABLE 1. Phenotypic and genotypic polymorphisms among *S. typhi* strains of different geographic origins[a]

Strain code	Geographic origin	Phenotyping		Genotyping				
		Resistance profile	Phage type	Ribotype		IS*200* type	PCR fingerprint type	
				*Eco*RI	*Pst*I		ERIC1-ERIC2	RAPD1
a	The Netherlands	Susceptible	D1	A	C	A	A	B
b	Turkey	Susceptible	C5	A	D	A	A	C
c	Morocco	Susceptible	C1	A	E	A	A	C
d	Turkey	Susceptible	A	A	F	C	A	D
e	Unknown	Susceptible	M1	A	G	A	A	D
f	Pakistan	Susceptible	D2	A	B	A	A	B
g	Morocco	Susceptible	E4	A	B	A	A	B
h	Morocco	Susceptible	E1a	A	H	A	A	B
i	Pakistan	CTmST	E1a	A	I	A	A	A
j	Ghana	ATmSSuT	A	A	H	A	A	B

[a] The letter codes correspond to the codes used in Table 2.

had this phage type. This observation indicates elevated clustering among the MDR strains, possibly as a result of the recent establishment of the MDR strains. Furthermore, three strains of phage type A and two strains of phage type F1 were identified. Seventeen strains were nontypeable. Among the paired isolates from the 18 patients who were readmitted to the health center, the primary and relapse isolates displayed phage type E1a, whereas the paired isolates from four patients were nontypeable (Table 2).

(ii) Genotyping. Several fingerprinting methodologies were performed to investigate the genetic relatedness of the 36 paired *S. typhi* strains. The results are summarized in Table 2. Ribotype analysis with *Eco*RI-digested DNA did not distinguish these isolates, since an identical banding pattern, type A, was always observed. The *Eco*RI ribotype was identical to that invariably observed in the group of isolates of different geographic origins. Ribotyping was also performed with *Pst*I-digested DNA. By this approach, two related but distinct ribotypes were observed (Fig. 1A). The A type was predominantly present among the MDR isolates, whereas the majority of the susceptible strains belonged to the B type. In one patient, the *Pst*I ribotype from the primary MDR isolate (R type CAT mSSuT) differed from that of the susceptible relapse isolate.

The 36 paired strains were further analyzed by IS*200* DNA fingerprinting. Among all strains tested, 13 copies of the insertion element were observed, assuming that all restriction fragments highlighted by Southern blot hybridization analysis contained a single IS*200* copy. Identical to *Pst*I ribotyping, two related but distinct IS types were observed (Fig. 1B). The IS fingerprint types differed only in the position of a single IS*200* copy. Moreover, IS*200* type A was invariably linked to *Pst*I ribotype A, and IS type B was exclusively observed among strains of *Pst*I ribotype B.

We also analyzed the strains by PCR fingerprinting using primer pair ERIC1-ERIC2. The ERIC1-ERIC2 amplification data once more revealed the genetic homogeneity of the strains from Dhaka. Clonality could be deduced for all strains tested, because this primer combination did not enable discrimination between any of the strains tested (Fig. 2). The banding patterns, comprising 11 major DNA fragments, were identical for all strains. In contrast, two distinct fingerprint types were observed when using primer RAPD1 (Fig. 3A). Interestingly, the divergence of the strains into two fingerprint types (types A and B) correlated entirely with the presence or absence of drug resistance, respectively (Table 2). Consistent with these findings, strain i from Pakistan displayed the resistance fingerprint type A with primer RAPD1. However, MDR strain j displayed type B, which invariably correlated with the drug-susceptible phenotype of the Dhaka strains (Table 1).

To investigate the possible correlation between the genotypic differences observed by PCR fingerprinting with primer RAPD1 and the amplification of extrachromosomal DNA, which is present exclusively in the MDR strains, we isolated four MDR-specific amplicons (amplicons A to D) (Fig. 3B). For this purpose, we used *S. typhi* 1 (lanes 1 of Fig. 1 to 3). DNAs from the 36 paired isolates were hybridized with probe A. As shown in Fig. 1C, DNAs from the MDR strains of both R types CATmSSuT and ATmSSuT invariably hybridized with probe A. Additionally, DNAs from the non-Bangladesh isolates hybridized with probe A. DNA from strain i from Pakistan (R type CTmST) also hybridized with probe A, whereas DNA from strain j from Ghana, sharing the R type ATmSSuT with two strains from Dhaka, did not hybridize with the probe.

MDR characteristics of the *S. typhi* isolates. The antibiograms of the MDR isolates suggest a clustering of antibiotic resistance markers. Conjugation experiments with six randomly picked MDR *S. typhi* isolates of R type CATmSSuT, the two strains of R type ATmSSuT, and the two resistant isolates from Pakistan (R type CTmST) and Ghana (R type ATmSSuT) were performed. Among the MDR strains from Bangladesh, only the resistance phenotype CATmSSuT was transferable to the plasmid-free strain *E. coli* WG1, whereas the resistance phenotype ATmSSuT could not be transferred. The frequency at which the CATmSSuT transfer occurred was 5×10^{-2} at a temperature of 28°C. At 37°C this frequency decreased to 10^{-6}. Conjugational transfer of the resistance to the F factor-containing strain *E. coli* WG2 was not possible. Conjugation experiments of the MDR *S. typhi* strains from Pakistan and Ghana demonstrated that both R types CTmST (strain i; Pakistan) and ATmSSuT (strain j; Ghana) were both transferable to *E. coli* WG1. Identical to the Dhaka *S. typhi* strains of R type CATmSSuT, the frequency of MDR transfer to *E. coli* WG1 shifted from 5×10^{-2} at 28°C to 1×10^{-6} at 37°C. Furthermore, conjugational transfer of the resistance trait to the F factor-containing strain *E. coli* WG2 was not possible.

The group of strains used in the conjugation experiments were also analyzed by pulsed-field gel electrophoresis. Figure 4A demonstrates the presence of extrachromosomal DNAs with large molecular sizes in the MDR strains from Dhaka (R types CATmSSuT and ATmSSuT), whereas no such plasmids were observed in the two susceptible strains. The sizes of the plasmids from strains of R types CATmSSuT and ATmSSuT differed significantly, being approximately 220 and 290 kb, respectively. The MDR strain from Pakistan (R type CTmST)

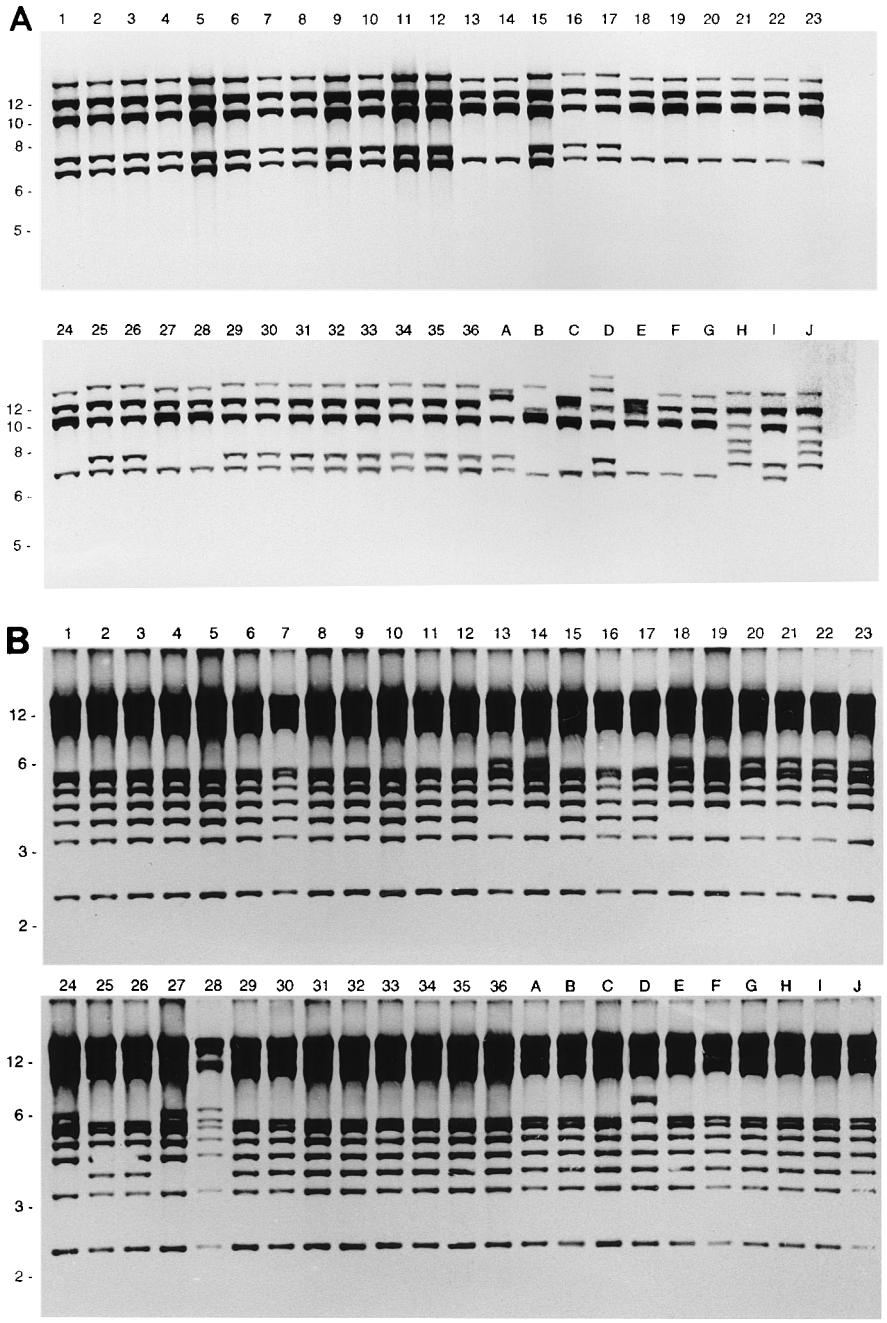

FIG. 1. *Pst*I ribotyping (A), IS*200* DNA fingerprinting (B), and hybridization analysis with the randomly amplified DNA fragment A (C) of 36 paired *S. typhi* strains isolated from 18 patients in Dhaka (lanes 1 to 36, respectively) and 10 isolates from geographically distinct areas (lanes A to J, respectively). The paired isolates are grouped (patient 1, lanes 1 and 2, patient 2, lanes 3 and 4, etc.). Even lane numbers refer to the relapse strains. Lanes A to J refer to strains a to j in Table 1. The numbers on the left indicate the sizes of standard DNA fragments (in kilobase pairs).

harbored extrachromosomal DNA of 210 kb, whereas in the MDR strain from Ghana (R type ATmSSuT), no large extrachromosomal DNA (>90 kb) was present. However, a 50-kb extrachromosomal DNA band was observed in the latter strain when the pulsed-field gel electrophoresis time was reduced from 18 to 12 h (data not shown).

Southern blot analysis of the pulsed-field gel demonstrated the exclusive hybridization of probe A with the large extrachromosomal DNA (Fig. 4B). Identical hybridization patterns were obtained with probes B, C, and D (data not shown).

We also analyzed the *E. coli* conjugants by pulsed-field gel electrophoresis. Acquisition of the different MDR phenotypes by *E. coli* invariably coincided with the presence of extrachromosomal *S. typhi* DNA in the conjugants (data not shown).

DISCUSSION

In the present study, the close genetic relatedness of the *S. typhi* strains isolated in Dhaka in 1994 and 1995 is clearly demonstrated. First, phage type E1a was highly predominant among the 78 isolates, whereas 10 randomly selected strains

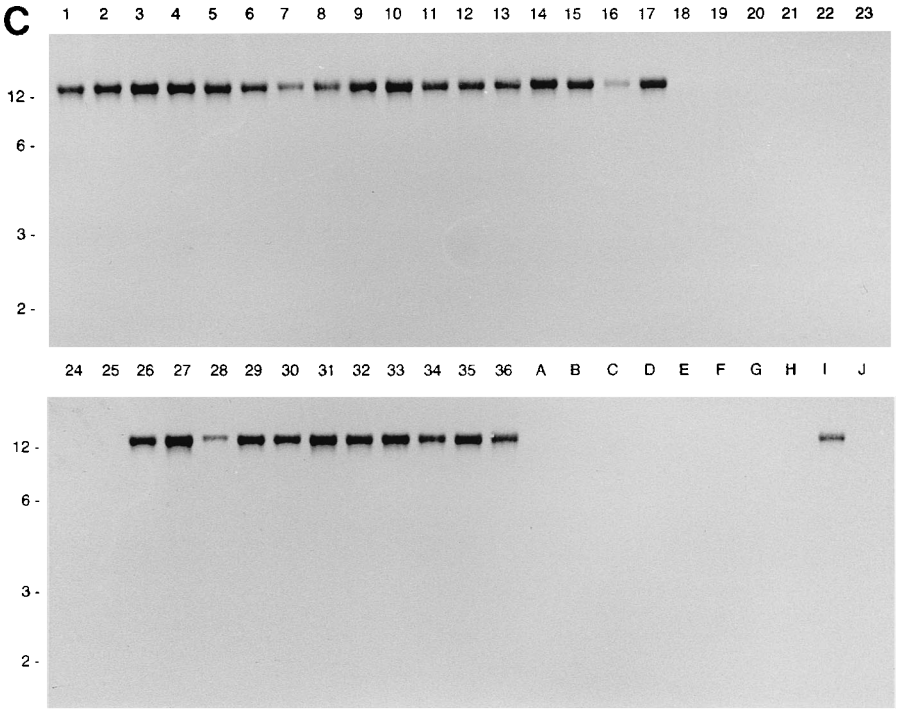

FIG. 1—*Continued.*

with distinct geographic origins displayed eight different phage types. Second, *Pst*I ribotyping revealed only two related fingerprint types. The 10 strains of different geographic origins displayed eight different ribotypes. Consistent with observations made by other investigators, there was no correlation between phage typing and ribotyping (2, 15). *Eco*RI ribotyping, IS*200* DNA fingerprinting, and PCR fingerprinting favored the assumption that genetic relatedness was strong among the strains, because these methods detected limited or even no genetic polymorphism among the *S. typhi* strains tested. However, since the discriminatory power of these genotyping assays was poor among the geographically nonrelated isolates, we conclude that the usefulness of the individual methods for molecular typing of *S. typhi* is questionable. The genotypic divergences of the *S. typhi* strains observed by *Pst*I ribotyping and IS*200* fingerprinting were identical. These data suggest the clonal spread of two strongly related families of *S. typhi* strains in Dhaka.

Detailed comparison of the IS*200* DNA fingerprints with those observed in isolates from India and Pakistan revealed the exclusive presence of patterns IS*200Sty1* and IS*200Sty3* (27). Threlfall and coworkers (27) have concluded that although IS*200* fingerprinting is not as discriminatory as phage typing for the primary subdivision of *S. typhi*, it may be a useful tool for differentiating drug-resistant and -susceptible isolates in India and Pakistan (27). However, our data demonstrate that there is no clear division between IS*200* patterns A and B and MDR *S. typhi*, although IS type A was more frequently observed in the MDR group of strains. Therefore, we conclude that the usefulness of IS*200* as a marker or as an additional marker for MDR screening of *S. typhi* strains is limited.

Among the strains from patients who were readmitted to the health center, *Pst*I ribotyping and IS*200* fingerprinting demonstrated that only in one patient did the relapse strain differ from the primary isolate. These data demonstrate that at least 1 of the 18 patients with relapses was reinfected with another strain. Since the overall genetic relatedness of the *S. typhi* isolates is very high, reinfection cannot be ruled out for the other patients. In addition, the resistance patterns of the paired isolates were identical to those of the primary isolates

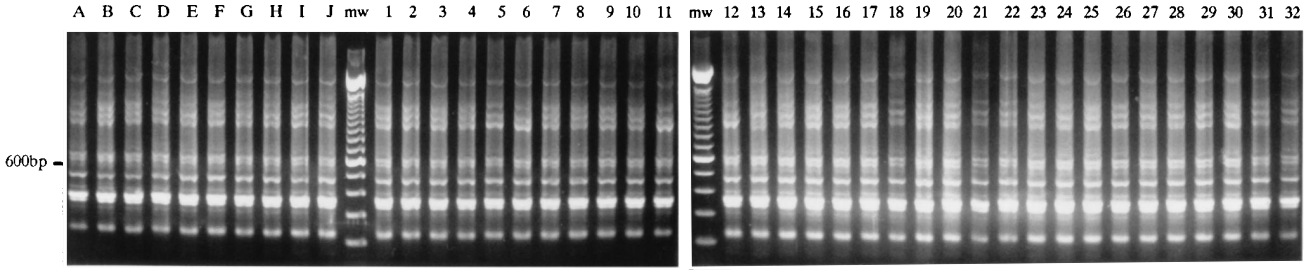

FIG. 2. PCR fingerprint analysis of 32 paired *S. typhi* strains isolated from 16 patients in Dhaka (lanes 1 to 32, respectively) and 10 isolates from geographically distinct areas (lanes A to J, respectively) with the primer pair ERIC1-ERIC2. Lane numbers correspond to those in Fig. 1. The numbers on the left indicate the sizes of standard DNA fragments (in kilobase pairs) (lanes mw).

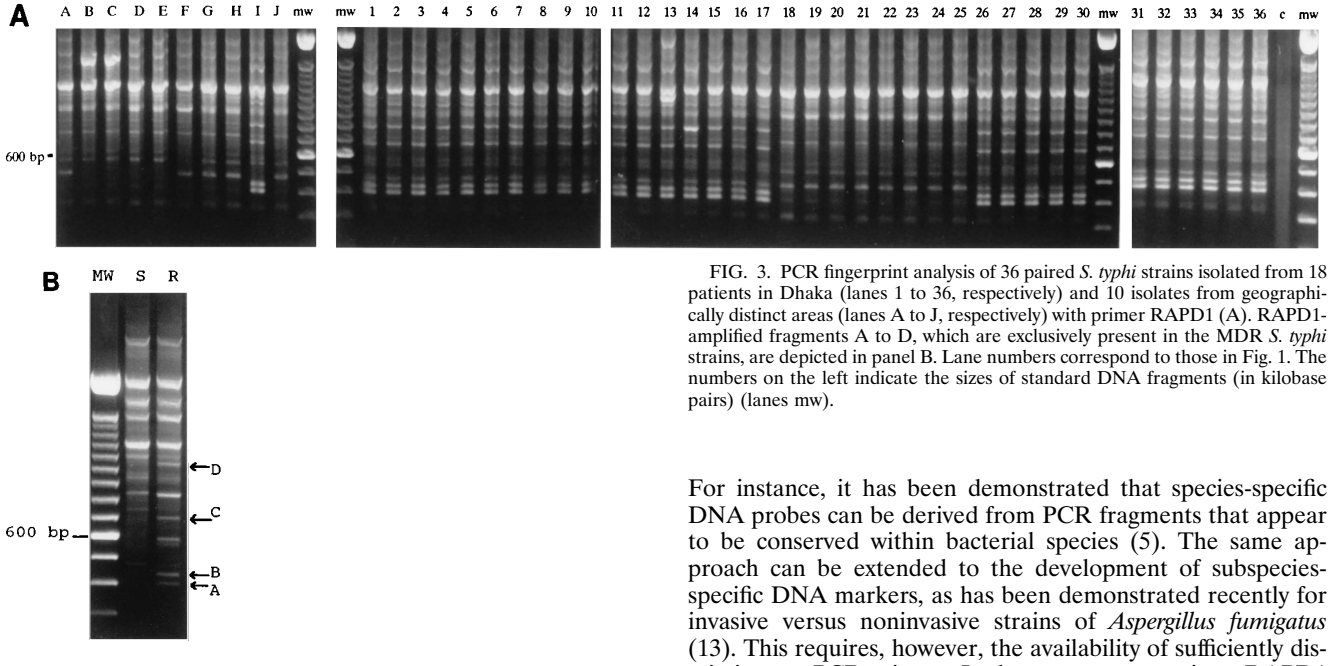

FIG. 3. PCR fingerprint analysis of 36 paired *S. typhi* strains isolated from 18 patients in Dhaka (lanes 1 to 36, respectively) and 10 isolates from geographically distinct areas (lanes A to J, respectively) with primer RAPD1 (A). RAPD1-amplified fragments A to D, which are exclusively present in the MDR *S. typhi* strains, are depicted in panel B. Lane numbers correspond to those in Fig. 1. The numbers on the left indicate the sizes of standard DNA fragments (in kilobase pairs) (lanes mw).

for 15 patients. The resistance profiles of the primary isolates from three patients differed from those of the relapse isolates. These data suggest that reinfection has occurred in at least three patients.

A significant number (67%) of the *S. typhi* strains were demonstrated to be resistant to multiple antibiotics. Among the MDR strains, the vast majority (96%) were resistant to chloramphenicol, ampicillin, trimethoprim, streptomycin, sulfamethoxazole, and tetracycline (R type CATmSSuT). Interestingly, the R type CATmSSuT is also frequently observed in isolates from India and Pakistan. This resistance phenotype is encoded by large plasmids belonging to the H1 incompatibility group (18–20, 25, 28). The R type CATmSSuT of isolates from Dhaka is most likely encoded by members of the same family of plasmids, since (i) plasmid DNA was demonstrated to be present in the MDR strains, (ii) conjugational transfer of MDR was linked with plasmid transfer, and (iii) the temperature- and F-factor-dependent conjugation characteristics of these MDR plasmids were similar to those of the H1 incompatibility group. The MDR phenotypes of two *S. typhi* strains of R type ATmSSuT were not transferable to *E. coli* strains, although they did contain extrachromosomal DNA of 290 kb. A significant difference in the sizes of these plasmids compared with the sizes of plasmids of R type CATmSSuT (220 kb) might explain the lack of conjugational transfer in our experiments. In contrast to these observations, the MDR phenotype of strain j from Ghana, which also displayed R type ATmSSuT, demonstrated conjugational transfer to *E. coli*. The MDR phenotype of strain j was located on a 50-kb plasmid. The MDR phenotype of strain i from Pakistan (R type CTmST) was also plasmid encoded, since temperature- and F-factor-dependent conjugational transfer of its 210-kb plasmid resulted in the acquisition of MDR by *E. coli*. The latter observation is consistent with the findings made by Threlfall and coworkers (28), who demonstrated that resistance among R type CTmST isolates in Pakistan was encoded by plasmids of the H1 incompatibility group (28).

Depending on primer choice and amplification parameters, PCR fingerprints can display variable degrees of resolution. For instance, it has been demonstrated that species-specific DNA probes can be derived from PCR fragments that appear to be conserved within bacterial species (5). The same approach can be extended to the development of subspecies-specific DNA markers, as has been demonstrated recently for invasive versus noninvasive strains of *Aspergillus fumigatus* (13). This requires, however, the availability of sufficiently discriminatory PCR primers. In the present case, primer RAPD1 displayed two distinct fingerprint types among the 36 paired *S. typhi* strains tested. Interestingly, this fingerprinting method

TABLE 2. Phenotypic and genotypic polymorphisms among MDR and susceptible *S. typhi* strains[a]

Test	Polymorphism among paired isolates (n = 36)			
	MDR (n = 28)[b]		Susceptible (n = 8)	
	Primary	Relapse	Primary	Relapse
Phage typing	E1a (11)[c,d]	E1a (11)	E1a (1)	E1a (1)
		E1a (1)	E1a (1)	
	E1a (1)			E1a (1)
	NT[e] (2)	NT (2)	NT (2)	NT (2)
Ribotyping				
*Eco*RI	A (14)[d]	A (14)	A (4)	A (4)
*Pst*I	A (11)	A (11)		
		A (1)	A (1)	
	A (1)			B (1)
	B (2)[d]	B (2)	B (3)	B (3)
IS*200* typing	A (11)	A (11)		
		A (1)	A (1)	
	A (1)			B (1)
	B (2)	B (2)	B (3)	B (3)
PCR fingerprinting				
ERIC1-ERIC2	A (14)[d]	A (14)	A (4)	A (4)
RAPD1	A (13)[d]	A (13)	B (4)	B (4)
		A (1)	B (1)	
	A (1)			B (1)

[a] Among 42 single isolates, 24 were MDR (20 type E1a and 4 nontypeable by phage typing) and 18 were susceptible (8 type E1a, 3 type A, 2 type F1, and 5 nontypeable by phage typing). The letter codes correspond to the codes used in Table 1.

[b] All MDR strains are of R-type CATmSSuT unless stated otherwise.

[c] The numbers of *S. typhi* strains representing a particular type are depicted in brackets.

[d] MDR group includes one strain of R-type ATmSSuT.

[e] NT, nontypeable.

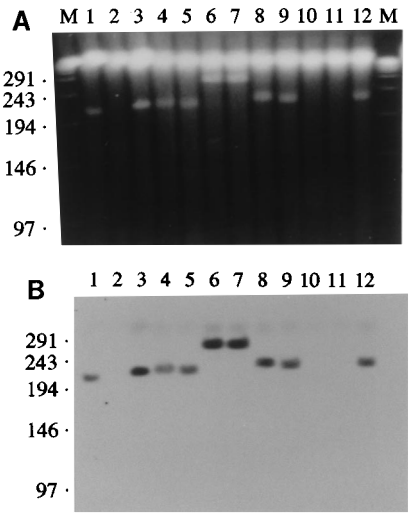

FIG. 4. Analysis of MDR and drug-susceptible *S. typhi* isolates by pulsed-field gel electrophoresis. The gel was stained with ethidium bromide (A) and was analyzed by Southern blot hybridization with the arbitrarily amplified DNA fragment A (B). Lane 1, strain i (Pakistan); lane 2, strain j (Ghana); lanes 3, 4, 5, 8, 9, and 12, strains of R type CATmSSuT; lanes 6 and 7, strains of R type ATmSSuT; lanes 10 and 11, drug-susceptible strains. The numbers on the left indicate the sizes of standard DNA fragments (in kilobase pairs).

discriminates between MDR and susceptible strains, because the amplified fragments A to D (Fig. 3B) were exclusively present in the MDR group. Consistently, the fragments hybridized only with DNAs from the MDR strains. Moreover, hybridization of these probes exclusively occurred with the large plasmid DNA present in the Dhaka MDR strains (R types CATmSSuT and ATmSSuT) and the MDR strain from Pakistan (R type CTmST). The MDR strain from Ghana (R type ATmSSuT) harbored a 50-kb plasmid which did not hybridize with the probes. These data suggest that the plasmid composition of the Ghanese strain differs significantly from those observed in isolates from Bangladesh and Pakistan. This might explain the lack of hybridization of this plasmid with probes A to D. This is the first example demonstrating the successful use of a random PCR method to develop probes that discriminate phenotypically different bacteria. We conclude that probes A to D recognize DNA sequences that are present in the large plasmids of the H1 incompatibility group. Since the hybridization of these probes is not influenced by the MDR phenotype, we assume that plasmid sequences other than resistance genes are recognized. Further characterization of the MDR probes by DNA sequencing is being undertaken.

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