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Trimethoprim resistance gene in *Shigella dysenteriae* 1 isolates obtained from widely scattered locations of Asia

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SUMMARY

Trimethoprim-resistance genes of *Shigella dysenteriae* 1 strains, isolated from a different location of six different countries of Asia over a 5-year period were characterized by using three different dihydrofolate reductase (DHFR) gene probes. The trimethoprim-resistant (TMP^R) strains hybridized only with the type I DHFR gene probe by colony hybridization. None of the strains hybridized with types II and III DHFR gene probes. Southern blot experiments using plasmid DNA extracted from these resistant strains indicated that the type I DHFR genes were either on a 20 MDa plasmid or might be located on the chromosome. None of the other plasmids present in *S. dyysenteriae* 1 strains hybridized with the probe. This indicates that the TMP resistance in these *S. dysenteriae* 1 strains are mediated by type I DHFR enzyme, and there may be transposition of this type I DHFR gene occurs between the 20 MDa plasmid and the chromosome in this serotype of shigella.

INTRODUCTION

Trimethoprim-sulfamethoxazole is regarded as the treatment of choice for shigellosis [1, 2]. Over the last decade, *Shigella flexneri*, the major cause of endemic shigellosis in Bangladesh, has shown little increase in resistance to this drug combination [3]. *S. dysenteriae* 1 causes the most virulent type of bacillary dysentery both in terms of the severity of disease and in its ability to cause epidemics. In 1982 a new epidemic of *S. dysenteriae* 1 resistant to trimethoprim-fulfamethoxazole, began to occur in Bangladesh [3], India [4], and Sri Lanka [5]. In contrast to the slow emergence of trimethoprim-resistance in *S. flexneri*, by 1984, *S. dysenteriae* 1 outbreak strains were uniformly resistant to trimethoprim [3]. The rapid emergence of trimethoprim resistance among *S. dysenteriae* 1 was apparent in unrelated epidemics occurring in Africa [6]. Trimethoprim-resistance is due to modified DHFR enzymes the genes for which may be plasmid or chromosomal [7]. Trimethoprim resistance in many bacterial species has been reported from many parts of the world and varies from species to species and from country to country. In many bacteria, it has been shown that high level

trimethoprim resistance is mediated by plasmid-encoded dihydrofolate reductases (DHFR) [8–11]. Recently, additional DHFRs have also been described [12–15]. Analysis of drug-resistance patterns of *S. dysenteriae* 1 isolates obtained from widely scattered geographical locations indicated that 281 of 343 strains were resistant to trimethoprim-sulfamethoxazole in combination with other drugs [16]. Two major drug resistance patterns were observed. The purpose of this study was to characterize the trimethoprim resistance genes present in *S. dysenteriae* 1 strains, isolated from widely scattered locations of Asia using three kinds of specific trimethoprim-resistance DHFR gene probes.

MATERIALS AND METHODS

Bacterial strains

Twenty-two representative trimethoprim-resistant strains of *S. dysenteriae* 1 obtained from a previous study [16], with two major drug resistance patterns, were selected for this study. These were resistance to chloramphenicol, streptomycin, tetracycline and trimethoprim-sulfamethoxazole in 182/281 strains, and resistance to ampicillin, chloramphenicol, streptomycin, tetracycline and trimethoprim-sulfamethoxazole in 79/281 strains. They were collected from six different places throughout Asia and six trimethoprim-sensitive strains from Asia and USA were also included (Table 1). The recipient strain used in conjugation experiments was a nalidixic acid-resistant *Escherichia coli* K-12 14R525 ($Lac^+F^-Nx^R$), kindly supplied by Dr. B. Rowe, Central Public Health Laboratory, Colindale, London, UK. Plasmids containing cloned genes coding for type I DHFR (pFE 872) type II DHFR (pFE 700) and type III DHFR (pFE 1242) have been described earlier [10, 17, 18]. Strains P195 (type I), P237 (type II), P638 (type III) and *E. coli* K-12 Xac were used as controls in DNA hybridization experiments [10]. These organisms were kindly provided by M. Fling, Wellcome Research Laboratories, Triangle Park, North Carolina.

Antimicrobial susceptibility tests

Antimicrobial drug susceptibility tests were performed by a disk diffusion method [19] using commercially prepared disks (BBL Microbiology Systems, Cockeysville, MD, USA) with concentrations as follows: ampicillin (Ap) (30 μg), chloramphenicol (Cm) (30 μg), streptomycin (Sm), tetracycline (Tc) (30 μg), trimethoprim-sulfamethoxazole (TMP-SMZ) (1·25 and 23·75 μg) and nalidixic acid (Nx) (30 μg). Minimum inhibitory concentrations (MIC) to TMP were determined by the agar dilution method [20, 21] using Mueller Hinton agar (Difco Laboratories, Detroit, Michigan, USA).

Plasmid analysis and conjugation tests

Plasmid DNA was extracted by the method of Birnboim and Doly [22] and separated by vertical agarose gel (0·7%) electrophoresis techniques [23]. Marker plasmids used as standards for molecular size estimations in megadaltons (MDa) of plasmid DNA were pDK-9 (140 and 105 MDa), R_1 (62 MDa), RP_4 (36 MDa) and Sa (23 MDa). Conjugation experiments were performed with 22 TMP^R strains of *S. dysenteriae* 1 to determine whether the TMP resistance could be self-transferred

Table 1. Shigella dysenteriae *1 strains resistant to trimethoprim*

Strains	Place of isolation	Year of isolation	Resistance patterns	Colony hybridization with type I gene probe (types II, III negative)	Position in Fig. 2
Bangladesh					
26732	Dhaka	1984	Ap Cm Sm Tc TMP-SMZ	+	1
33891	Dhaka	1984	Ap Cm Sm Tc TMP-SMZ	+	2
20331	Dhaka	1984	Ap Cm Sm Tc TMP-SMZ	+	3
31790	Dhaka	1984	Ap Cm Sm Tc TMP-SMZ	+	4
31431	Dhaka	1984	Cm Sm Tc TMP-SMZ	+	9
26406	Dhaka	1984	Cm Sm Tc TMP-SMZ	+	10
24623	Dhaka	1984	Cm Sm Tc TMP-SMZ	+	11
Saudi Arabia					
17247	Dammam	1985	Ap Cm Sm Tc TMP-SMZ	+	12
4215	Riyadh	1985	Cm Sm Tc TMP-SMZ	+	14
612	Riyadh	1985	Cm Sm Tc TMP-SMZ	+	15
Nepal					
46	Kathmandu	1985	Ap Cm Sm Tc TMP-SMZ	+	16
791	Kathmandu	1984	Cm Sm Tc TMP-SMZ	+	17
896	Kathmandu	1985	Ap Cm Sm Tc	−	5
679	Kathmandu	1984	Ap Cm Sm Tc	−	6
831	Kathmandu	1984	Ap Cm Sm Tc	−	7
India					
104	Varanasi	1980	Ap Cm Sm Tc TMP-SMZ	+	18
225	Varanasi	1985	Ap Cm Sm Tc TMP-SMZ	+	19
D 16	Calcutta	1984	Cm Sm Tc TMP-SMZ	+	20
C 152	Calcutta	1984	Cm Sm Tc TMP-SMZ	+	22
B 1	Delhi	1984	Cm Sm Tc TMP-SMZ	+	23
B 6	Delhi	1984	Cm Sm Tc TMP-SMZ	+	24
F 36	Bombay	1985	Cm Sm Tc TMP-SMZ	+	25
F 127	Bombay	1985	Cm Sm Tc TMP-SMZ	+	26
Burma					
1	Rangoon	1984	Cm Sm Tc TMP-SMZ	+	27
2	Rangoon	1984	Cm Sm Tc TMP-SMZ	+	28
USA					
3381	CDC	1972	Cm Sm	−	8
3073	CDC	1980	Cm Sm Tc	−	13
1007	CDC	1974	Cm Sm Tc	−	21
E. coli K-12 exconjugants					
14R525 (26732)			Ap Cm Sm Tc	−	30
14R525 (33891)			Ap Sm TMP	=	29

to the *E. coli* K-12 recipient strain [24]. Antibiotic-resistant *E. coli* exconjugants were selected by spreading the mixture of cultures from the top of the nitrocellulose membrane onto a TSA plate containing NxTMP, NxCm, NxTc, and NxAp. Concentrations of antibiotics were as follows. Nx (30 μg/ml), TMP (200 μg/ml), Cm (30 μg/ml, Tc (30 μg/ml) and Ap (10 μg/ml).

Preparation of the trimethoprim-resistance gene probes

Plasmid DNA from *E. coli* C 600 (pFE 872), (pFE 700) and (pFE 1242) was isolated by the method described by So and colleagues [25]. DNA from pFE 872 and pFE 1242 was digested with *Eco*R I-Hind III and from pFE 700 with *Eco*R I according to the manufacturer's instructions (BRL, Gaithersburg, MD, USA). Digested fragments were separated by polyacrylamide gel electrophoresis. The fragments used as DHFR gene probes were a 1680 bp fragment of pFE 872 (type I), 800 bp of pFE 700 (type II) and 885 bp of pFE 1242 (type III). The fragments were removed from the gel by electroelution, extracted with phenol, precipitated with ethanol, and finally labelled with [^{32}P]deoxyribonucleotide triphosphates (New England Nuclear Corp., Boston, MA, USA) by nick translation [26].

DNA–DNA hybridization

Twenty-two TMP^{R} and six TMP^{S} *S. dysenteriae* 1 strains were examined by colony hybridization with the three type specific DHFR gene probes [27]. Hybridizations were performed under stringent conditions at 42 °C in 50% formamide [28]. Filters were washed in 2 × SSC.0·2 % SDS at room temperature, then at 65 °C, air dried and exposed to X-Omat AR X-ray film at −70 °C for 24 h. The X-ray films were developed according to the manufacturer's instructions (Eastman Kodak Company, Rochester, NY, USA). Plasmids from isolates that hybridized with the type I DHFR gene probe by colony hybridization were electrophoresed on agarose gel (0·7 %) and transferred to nitrocellulose paper by the Southern [29] technique and examined with the three gene probes.

RESULTS

Drug susceptibility and plasmid analysis of 28 strains of *S. dysenteriae* 1 showed that 14 strains were resistant to Cm Sm Tc TMP-SMZ (i.e. Ap^{S} and TMP^{R}) and 8 strains showed resistance pattern of Ap Cm Sm Tc TMP-SMZ (i.e. Ap^{R} and TMP^{R}). All 14 strains in the first group harboured 140, 6, 4 and 2 MDa plasmids (Fig. 1, lane B) and 7 of the 8 strains in the latter group harboured 140, 20, 6, 4 and 2 MDa plasmids (Fig. 1, lane C); the remaining strain contained a 65 MDa plasmid instead of the 20 MDa (Fig. 1, lane E).

Conjugation experiments showed that none of Ap^{S} TMP^{R} strains transferred TMP resistance to the *E. coli* K-12 recipient. However, 7 of the 8 Ap^{R} TMP^{R} *S. dysenteriae* 1 strains transferred the 20 MDa plasmid coding for both Ap and TMP resistance to *E. coli* K-12 (Fig. 1, lane D). The remaining strain transferred the 65 MDa plasmid which codes for only Ap resistance but not TMP resistance (Fig. 1, lane F). None of the other plasmids (i.e. 140, 6, 4 and 2 MDa) or resistance markers (i.e. Tc and Cm) were transferred during conjugation.

Colonies of all the 22 TMP^{R} *S. dysenteriae* 1 strains, with or without the 20 MDa plasmid, hybridized with only the type I TMP-resistant DHFR gene probe (Table 1, Fig. 2). None of these isolates hybridized either with type II or type III DHFR gene probes. All six TMP^{S} strains failed to hybridize with any of the DHFR probes. A TMP^{R} colony of an *E. coli* K-12 exconjugant, containing the 20 MDa plasmid, also hybridized only with the type I DHFR probe.

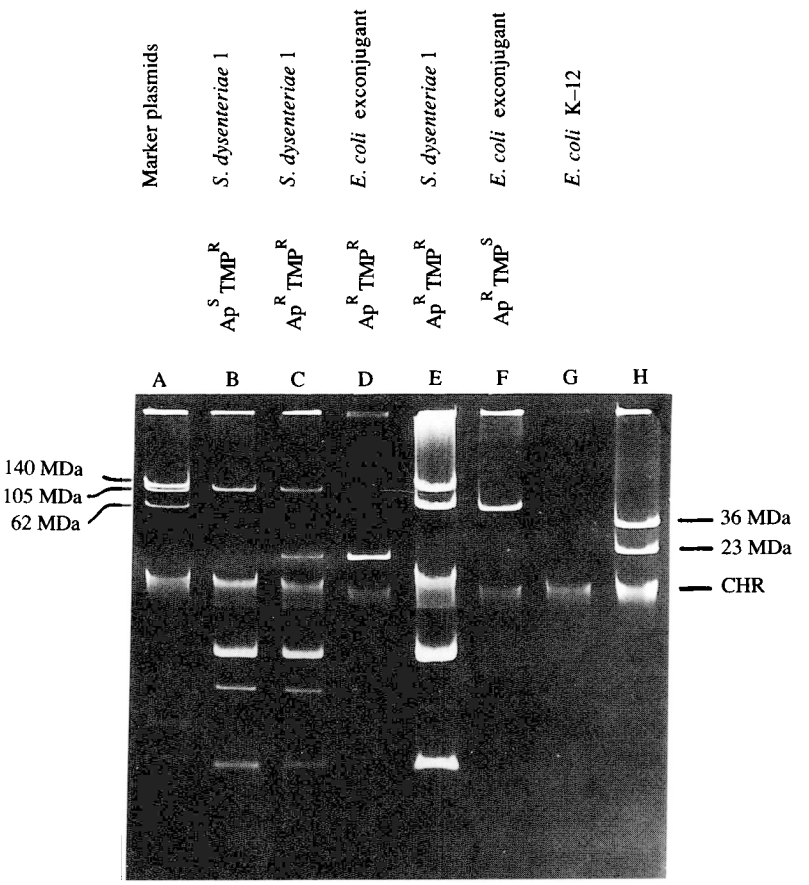

Fig. 1. Plasmid profiles of representative bacterial isolates. Lanes A–H, reference marker plasmids; lane B, Ap^S TMP^R *S. dysenteriae* 1 containing 140, 6, 4 and 2 MDa plasmids; lanes C and E, Ap^R TMP^R *S. dysenteriae* 1 containing 140, 20, 6, 4 and 2 MDa, and 140, 65, 6 and 2 MDa plasmids respectively; lane D, Ap^R TMP^R exconjugant with 20 MDa plasmid; lane F, Ap^R TMP^S exconjugant containing 65 MDa plasmid; and lane G, *E. coli* K-12 with no plasmid. CHR indicated chromosomal DNA.

DNA isolated from 23 TMP^R strains (22 *S. dysenteriae* 1 and one *E. coli* K-12 exconjugant) that hybridized with the type I DHFR DNA probe was examined by the Southern blot technique. The 20 MDa plasmid was present in seven *S. dysenteriae* 1 strains hybridized with the type 1 DHFR DNA probe (Table 2, Fig. 3). Similarly, the 20 MDa plasmid from the *E. coli* exconjugant also hybridized with this probe (Table 2, Fig. 4). The other plasmids (i.e. 140, 65, 6, 4 and 2 MDa) present in these strains did not contain genes encoding type I DHFR enzyme (Table 2, Figs. 3–4).

Isolates carrying the 20 MDa plasmid encoding for type I DHFR were examined for their level of resistance to TMP and compared with the level of TMP resistance in strains lacking the plasmid and presumably carrying DHFR genes on the chromosome. The MIC values for TMP determined in both types of TMP^R strains were similar ($\geqslant 1600$ μg/ml).

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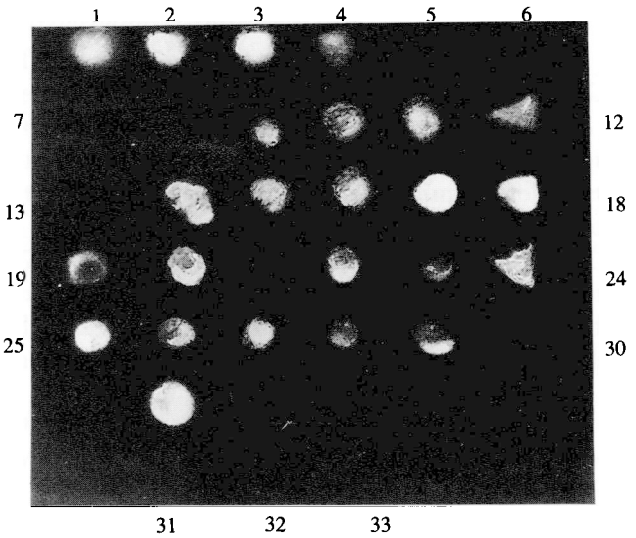

Fig. 2. Detection of trimethoprim-resistant gene in *S. dysenteriae* 1 strains by using ^{32}P-labelled type I DHFR gene probe. Total lysed DNA of 22 *S. dysenteriae* 1, and one *E. coli* K-12 exconjugant, resistant to TMP, hybridized with type I DHFR gene probe as indicated by the spots (see Table 1). Positive control for type I DHFR gene (P-872) is shown in position 31. Negative spots in positions 32 and 33 containing lysed total DNA of types II (P-700) and II (P-1242) respectively indicated the specificity of type I probe.

Table 2. *Plasmid profiles of TMP^R strains of* S. dysenteriae *1 and the plasmid-encoding type I DHFR genes*

Plasmid profiles (MDa)	No. of strains	Resistance patterns	DNA-hybridization with type I DHFR gene probe	
			No. of strains	Size of plasmid (MDa)
140, 6, 4, 2	14	Cm Sm Tc TMP-SMZ	0	—
140, 20, 6, 4, 2	7	Ap Cm Sm Tc TMP-SMZ	7	20
140, 65, 6, 2	1	Ap Cm Sm Tc TMP-SMZ	0	—

DISCUSSION

The colony hybridization of TMP^R *S. dysenteriae* 1 strains from various locations of Asia demonstrated that resistance to TMP was mediated only by type I DHFR enzyme in these isolates, irrespective of the drug resistance patterns or the geographic origin of the isolation. None of the TMP^R *S. dysenteriae* 1 strains contained the structural genes for type II or type III DHFR enzyme.

Results of DNA hybridization with a type I DNA DHFR probe demonstrated that the 20 MDa plasmid coded for TMP resistance in strains which contained the plasmid. The other plasmids were not thought to mediate TMP resistance. The involvement of the 20 MDa plasmid in TMP resistance was confirmed by the transfer of this plasmid to a recipient *E. coli* K-12 strain which became resistant to TMP, and by the observation that this 20 MDa plasmid in the exconjugant also

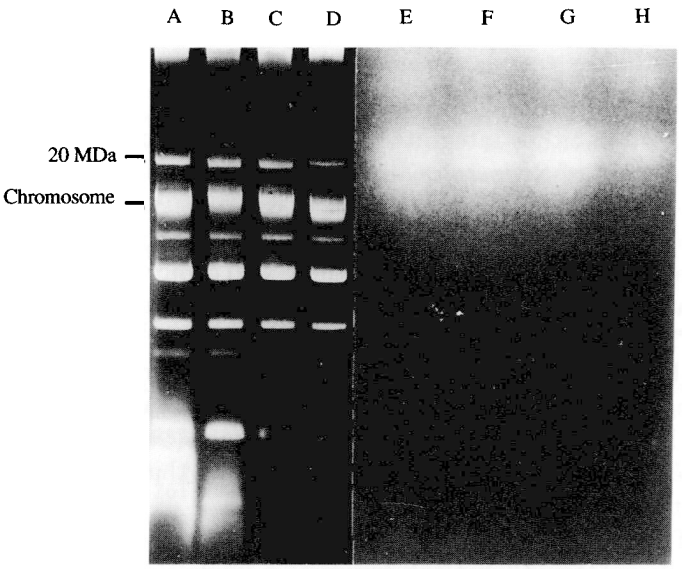

Fig. 3. Southern blot of DNA probe in representative strains of *S. dysenteriae* 1. Lanes A–D showed agarose gel electrophoresis of plasmid DNA isolated from TMP^R strains 26732, 17247, 46 and 104, respectively, and lanes E–H showed Southern blot of gel hybridized with type I DHFR gene probe showing hybridization with 20 MDa plasmid.

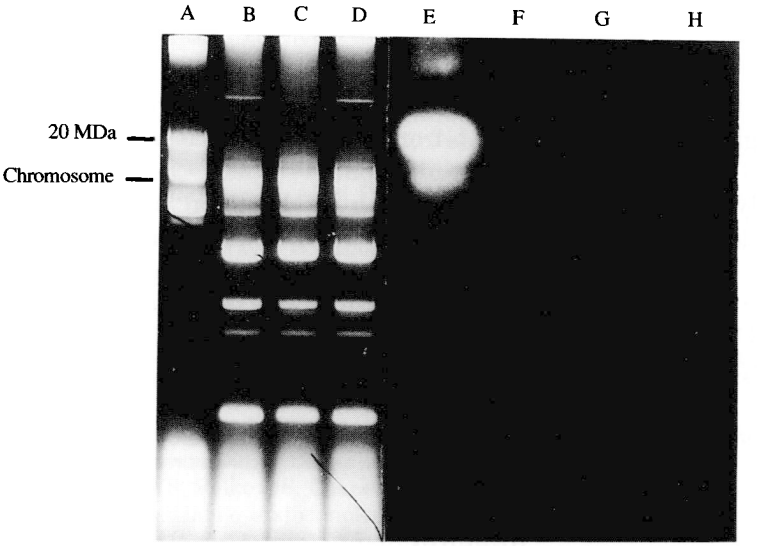

Fig. 4. Plasmid DNA isolated from TMP^R exconjugant 14R525 (33891) and TMP^R *S. dysenteriae* type 1 strains 31431, 4215 and 791 were respectively electrophoresed on 0·7 % agarose gel in lanes A–D. Lanes E–H showed corresponding Southern blot of the gel. Hybridization occurred only with the 20 MDa plasmid present in the exconjugant shown in lane E. *S. dysenteriae* 1 strains, without the 20 MDa plasmid showed no hybridization with the type I DHFR DNA probe (lanes F–H).

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hybridized with the type I DHFR DNA probe. The TMP^R gene in strains lacking the 20 MDa plasmid was presumed to be located in the chromosome. This theory is also supported by the fact that the genes coding for TMP resistance may be easily integrated into the chromosome with high efficiency [30, 31]. Thus, the TMP-resistance genes are either integrated into the chromosome or situated on 20 MDa plasmid in strain of *S. dysenteriae* 1.

Our work is supported by the results of Fling and colleagues [10] who found that 20% of their clinical isolates contained type I plasmid-encoded DHFR genes integrated into their chromosomes, and with those of Towner [32] who reported a significant increase in the isolation frequency of clinical strains with high level non-transferable TMP resistance.

It has been observed that high level trimethoprim resistance (MIC $\geqslant$ 2000 μg/ml) was mediated by the type I and type II DHFR enzymes in *E. coli* [8, 9]. Thus, the high MIC values for trimethoprim resistance ($\geqslant$1600 μg/ml) in our strains of *S. dysenteriae* 1 conforms with hybridization data that the type I DHFR enzyme-mediated the resistance to TMP.

The presence of uniform type I DHFR enzyme in *S. dysenteriae* 1 strains obtained from widely scattered locations, suggests a single clone origin. Analysis of plasmid profiles of *S. dysenteriae* 1 strains obtained from different geographical locations is also in agreement with the observation that there might be a global spread of a single strain of *S. dysenteriae* 1 [16].

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