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Molecular characterization of trimethoprim resistance in *Shigella sonnei* in Sicily

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SUMMARY

During the 3-year period 1985–7, all strains of *Shigella sonnei* isolated in Catania, Sicily, showed a high level of resistance to trimethoprim (Tp) which was invariably associated with resistance to other antibiotics.

Plasmid analysis showed 18 different electropherotypes: 35 of 37 strains harboured a plasmid of 70 Megadaltons (MDa), and 29 of 37 strains a plasmid of 130 MDa.

Restriction endonuclease fingerprinting of purified 70 MDa plasmid DNA from different strains demonstrated that these plasmids were similar but not identical.

In some strains with transferable Tp resistance, DNA hybridization analysis demonstrated the presence of genes coding for the production of dihydrofolate reductase (DHFR) type V. In contrast, there was no detectable hybridization with DNA probes specific for genes coding for DHFR types I, II and IV. This is the first report of the DHFR type V gene outside Sri Lanka.

INTRODUCTION

Trimethoprim, Tp, was introduced into general clinical use in 1969. It is a synthetic drug which has been shown to inhibit bacterial dihydrofolate reductase enzymes (DHFR), being far less active against the equivalent enzymes in mammalian cells. Unfortunately the intensive use of Tp in both human and veterinary medicine has provided the appropriate selective pressure for the emergence and spread of Tp-resistant bacterial strains in many countries [1].

Transferable resistance to Tp has been shown to be mediated by plasmids coding for the production of additional DHFR enzymes, less susceptible than chromosomal enzymes to inhibition by Tp. The genes responsible have been reported to have the ability to integrate into the bacterial chromosome [1]. So far, 11 genes coding for the production of different DHFR enzymes have been found. Two of these enzymes, DHFR types I and II, confer high levels of Tp resistance

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Table 1. *Characteristics of the trimethoprim-resistant dihydrofolate reductases*

Enzyme	Heat sensitivity	Plasmid source	Reference	Tp MIC for the host strains μg/ml	Molecular mass (KDa)
DHFR I a	Labile	R 483 (Tn7)	[24]	1000	35·2 (2 × 17·6)
DHFR I b	Labile	pUK153 (Tn4132)	[30]	1000	24·5
DHFR II a	Stable	R67	[31]	1000	33·6 (4 × 8·4)
DHFR II b	Stable	R388	[31]	1000	33·2 (4 × 8·3)
DHFR II c	Stable	R751 (Tn402)	[31]	1000	34 (4 × 8·5)
DHFR III	—	pAZ1	[32]	64	16·9
DHFR IV	—	pUK1123	[33]	Variable depending on the testing medium	46·7
DHFR V	Stable	pLM020	[21]	1000	5·0
DHFR VI	Labile	pUK672	[3]	1000	10·0
DHFR VII	—	pUN835	[4]	1000	11·5
DHFR SI	Stable	pSK1, PGO1	[34]	1000	19·7

(MIC > 1000 μg/ml) and have been reported to be widely distributed in the United States and Europe. Other DHFR enzymes which also confer high levels of resistance include: type V, common in Sri Lanka [2], type VI, found in South Africa [3], and type VII, identified in the United Kingdom [4] (Table 1).

Tp used in combination with sulphamethoxazole is considered particularly efficacious in the treatment of invasive diarrhoea caused by ampicillin-resistant *Shigellae* sp. [5], especially in those areas where the majority of strains exhibit multiple resistance to a range of therapeutic antibiotics. A study of strains belonging to A, B and C subgroups of *Shigellae* sp. isolated in England and Wales in the period 1974–83 revealed a marked increase in antibiotic resistance, and in particular Tp resistance during the period of study [6]. In Spain, 96·9% of strains of *S. sonnei* isolated in 1982 were Tp-resistant, whereas in 1979 there was a 34·4% frequency [7]. A more recent report [8] indicates that 89–97% of *S. sonnei* isolated in Spain from 1981 to 1987 were Tp-resistant. In Bulgaria, where the first Tp-resistant strain of *S. sonnei* was isolated in 1980, the rapid spread of the DHFR type I gene was such that by 1987, 43% of all strains were resistant [9]. In Canada, Tp-resistant *S. sonnei* were first isolated in 1977–8 [10]. Out of 65 strains of *S. sonnei* isolated in Korea in the period 1980–1, 63 (96·9%) were Tp-resistant [11]. In India and Bangladesh, where resistance to Tp alone or in combination with sulphonamides was rarely encountered in shigella control until 1981, a dramatic increase in the resistance of *S. dysenteriae* to Tp was observed in the period 1982–3 which was attributed to the increasing use of the drug in those two countries [12].

The epidemiological surveillance of shigella isolated in Sicily from 1975 revealed that until 1981, 99% were multiply antibiotic-resistant but Tp sensitive. In that year, two strains of *S. sonnei* were isolated, each harbouring a Tp resistance plasmid. One of these plasmids belonged to the I1 incompatibility group, whilst the other was compatible with reference plasmids representative of 11 different *Inc* groups (G. Giammanco, unpublished observations).

From 1985 to the present day, *S. sonnei* strains resistant to cotrimoxazole have increased in frequency in Sicily. We report the molecular characterization of Tp resistance in such strains.

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MATERIALS AND METHODS

Bacterial strains

During the period 1985–7, 37 strains of *S. sonnei* were isolated in Catania (Sicily) from the faeces of hospitalized patients affected with acute enteritis; 4 strains were isolated from one epidemic episode and 33 from apparently unrelated cases of diarrhoea.

Biotype was determined by the Szturm-Rubinstein method [13], on the basis of rhamnose and xylose fermentation.

Antibiotic sensitivity was determined by the Bauer–Kirby method [14]. Ampicillin (Ap), 25 μg; chloramphenicol (Cm), 30 μg, kanamycin (Km), 30 μg; tetracycline (Tc), 30 μg; nalidixic acid, 30 μg; rifampicin, 30 μg; streptomycin (Sm), 10 μg; spectinomycin (Sp), 50 μg; cotrimoxazole (Cot), 25 μg were tested. For those strains resistant to cotrimoxazole, Tp and sulphamethoxazole (Su) were tested separately, and the Tp resistance level (MIC) was determined by the method of Ericsson and Sherris [15].

Conjugation experiments were performed using *Escherichia coli* K12 J53 Rif^+ strain as recipient. Mixtures of donors and recipients were incubated at 37 °C for 18 h, after which one loopful (100 μl) of bacteria was streaked on appropriate selective plates. In instances in which mating frequency was very low or undetectable, the conjugation experiments were repeated with mixtures of cells cultivated on Millipore filters [17].

Plasmid incompatibility group determination

R plasmids were classified into incompatibility groups as described by Chabbert and colleagues [18], using standard plasmids of the following incompatibility groups (Inc): B, C, FI, FII, H, I1, I2, M, N, P, W.

Plasmid profile and endonuclease restriction analyis

Plasmid DNA was extracted by the method of Kado and Liu [19]. For endonuclease restriction analysis, plasmid DNA was prepared and purified as described by Kraft and co-workers [20]. Restriction enzymes *Hin*d III, *Hin*d II and *Cla* I were used as recommended by the manufacturer (Bethesda Research Laboratories Ltd, U.S.A.). Samples were then electrophoresed on a 0·8% horizontal agarose gel in Tris acetate buffer, at 80V for 2 h, together with lambda DNA digested with *Hin*d III, as a fragment size marker.

DNA probes

Standard methods and conditions for ligation of DNA fragments to appropriate plasmid vectors and for the preparation of frozen competent cells of *E. coli* JM103 by the calcium chloride procedure for transformation have been described [21, 22]. Plasmid DNA from strains containing plasmids coding for different types of DHFR enzymes, Col E1 Tn7 for DHFR type I, R388 and R751 for DHFR II (National Collection of Type Cultures), pUK1148 for DHFR type IV and pLK09 for DHFR type V (kindly supplied by K. J. Towner), were prepared according to the method of Holmes and Quigley [23].

Samples of plasmid DNA extracted from test strains and controls were transferred to nylon filters (Nytran 13, Schleicher & Schuell) using a vacuum gene

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apparatus (LKB). To bind the DNA, filters were baked for 2 h at 80 °C. To determine the type of DHFR responsible for Tp resistance, different probes were used. The probe for DHFR type I was a 499 base-pairs (bp) *Hpa* I fragment from plasmid pFE872, derived originally from R483 and kindly supplied by K. J. Towner [24, 25]. To prepare the probe for DHFR type II, a *Bam*H 1 – *Eco*R 1 digest of plasmid R388 was ligated into a pUC19 vector which had been digested with *Bam*H 1 – *Eco* R1. Competent cells of *E. coli* JM103 were then used as recipients for the recombinant clones. Clones resistant to Tp were selected on Ap–Tp plates, and plasmid DNA was extracted as described above. The resultant clone, containing the 1·77 kb *Bam*H 1 – *Eco*R 1 DHFR type II fragment was then digested with *Eco*R 1 – *Hin*c II to obtain a 710 bp fragment, which was initially used as a probe for DHFR type II. For a more precise determination of the occurrence of DHFR type II the 710 bp *Eco*R 1 – *Hin*c II fragment was recovered from a 1% agarose gel and purified using a Gene-Clean Kit (Stratech Scientific Ltd). This fragment was digested with *Sau*3A 1 to obtain a 275 bp *Eco*R 1 – *Sau*3A 1 fragment. This fragment was then used as a probe for DHFR type II [2, 26]. The probe for DHFR type IV was a 1700 bp *Cla* I fragment from plasmid pUK1148 [27]. For DHFR type V a 500 bp *Hin*c II – *Hin*c II fragment from plasmid pLK09 was used, as described by Towner and colleagues [27].

Preparation of labelled probes

DNA fragments prepared as described above were recovered from horizontal 1% agarose gels in Tris acetate buffer and purified by using a Gene Clean Kit. These fragments were then labelled by random primed incorporation of digoxygenin dUTP (Boehringer, Mannheim), using the conditions recommended by the manufacturers.

Hybridization conditions

Filters were prehybridized for 2 h at 68 °C in hybridization solution containing 5 × SSC buffer (1 × SSC buffer contains 0·15 M sodium chloride plus 0·015 M sodium citrate); 0·1% *N*-lauroyl-sarcosine, Na salt; 0·02% sodium dodecyl sulphate; 1% blocking reagent (Boehringer, Mannheim). Hybridization was then performed for 18 h at 68 °C in hybridization solution containing freshly denatured DNA probe. Detection of a positive hybridization result was by means of a nonradioactive DNA labelling and detection kit (Boehringer, Mannheim), using the post-hybridization washes and procedures recommended by the manufacturers.

RESULTS

Antibiotic resistance

During the 3-year period 1985–7, 37 Tp-resistant *S. sonnei* were isolated in Catania, Sicily. All were classified as biotype a. All strains showed a high level of resistance to Tp (MIC > 1000 μg/ml) associated with resistance to Su and Sm, and in 34 of the 37 strains, with resistance to Sp. Furthermore, 17 strains were resistant to Ap, 6 to Tc and in one strain these resistances were combined with resistance to Cm and Km. In 34 strains, streptomycin resistance was mediated by adenylation and in the remaining three strains, by phosphorylation (Table 2).

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Plasmid profile analysis

Plasmid profile analysis demonstrated 18 different electropherotypes. The number of plasmids per strain ranged from 5 to 7; their molecular weights ranged from approximately 1·3 MDa to 130 MDa. Thirty-five strains harboured a plasmid of approximately 70 MDa and 29 a plasmid of 130 MDa. Five strains carried additional plasmids: one of 50 MDa, two of 32 MDa and two of 15 MDa. Furthermore, plasmids with molecular weights ranging from 9·5 MDa to 1·3 MDa were identified in 21 strains.

Resistance transfer

When conjugation experiments were performed, transfer of the 130 MDa plasmid was never observed; in contrast, the 70 MDa plasmid was transferable in 29 strains together with most of the antibiotic resistances and with one or more of the plasmids with molecular weights lower than 9·5 MDa. Tp resistance was transferable in only six strains, alone in three strains and together with resistances to Ap Sm Su Cm Km Tc in the remaining three strains (Table 2). Plasmids coding for Tp resistance were compatible with plasmids of all the incompatibility groups tested and therefore could not be assigned to definitive groups. In contrast, those plasmids coding for resistance to Ap, Sm and Su were of 70 MDa and belonged to *Inc* B (Table 2).

Restriction endonuclease analysis

Restriction endonuclease analysis (using *Hin*d III, *Hin*d II and *Cla* I) of the DNA of the 70 MDa plasmids from different strains demonstrated a considerable degree of homogeneity. For example, the *Cla* I endonuclease patterns of plasmids coding for Ap Sm Su Tp and Ap Sm Su, showed that the number of fragments varied between 10 and 12, with 9–10 fragments in common. Similarly, the number of fragments for plasmids encoding for Tp and Sm Su Tp varied between 6 and 9, with 3–9 fragments in common (Fig. 1).

Hybridization analysis

The six strains with transferable Tp resistance and two strains which did not transfer this resistance were chosen for further analysis by DNA–DNA hybridization.

When the probe for DHFR type I was used, no hybridization was detected with any of the plasmids tested. When the 710 bp DHFR type II probe was used there was no detectable hybridization to control plasmids containing DHFR types I or IV. However, some degree of cross-hybridization to the plasmids containing the DHFR type V gene was observed. When the smaller 275 bp fragment was used, no hybridization was observed either with control plasmids carrying known DHFR types different from type II, or with any of the plasmids tested.

When the probe for DHFR type IV was used, no hybridization was detected with any of the plasmids except pUK1148, that known to contain the control gene for DHFR IV. This result provided additional evidence of the high specificity of the DHFR type IV probe.

When the 500 bp *Hinc* II – *Hinc* II fragment from pLK09 was used as a probe

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Table 2. *Phenotype and R-plasmid characteristics of* Shigella sonnei *strains isolated from 1985 to 1987*

Bacterial strain characteristics					Characteristics of R-plasmids transferred to *E. coli* K12 J53 Rif^r		
No. of strains	Biotype	Resistance pattern	Plasmid pattern	Enzyme inactivating SM	R determinants	Inc. group	MW (MDa)
1	a	Ap Sm Su Tp	70	Adenylase	Ap Sm Su	B	70
13	a	Ap Sm Su Tp	70, 130	Adenylase	Ap Sm Su	B	70
1	a	Ap Sm Su Tp	50, 70, 130	Adenylase	Ap Sm Su	B	70
1	a	Ap Sm Su Tp	70	Adenylase	Ap Sm Su	B	70
1	a	Ap Sm Su Tp	70, 130	Adenylase	Ap Sm Su Tp	Compatible	70
					Ap Sm Su	B	70
1	a	Ap Sm Su Tp Cm Km Tc	32, 70, 130	Adenylase	Ap Sm Su Tp Cm Km Tc Cm Km Tc Tp		70
					Ap Sm Su Cm Km Tc	B	70
2	a	Tc Sm Su Tp	70	Adenylase	NT		
1	a	Tc Sm Su Tp	70, 130	Adenylase	NT		
2	a	Tc Sm Su Tp	130	Adenylase	NT		
1	a	Tc Sm Su Tp	70	Adenylase	NT		
1	a	Sm Su Tp	70, 130	Phosphorylase	Sm Su Tp	Compatible	70
					Tp	Compatible	70
1	a	Sm Su Tp	15, 70, 130	Phosphorylase	Sm Su Tp	Compatible	70
					Tp	Compatible	70
1	a	Sm Su Tp	15, 70	Phosphorylase	Sm Su Tp	Compatible	70
					Sm Su	B	70
					Tp	Compatible	70
7*	a	Sm Su Tp	70, 130	Adenylase	Sm Su	B	70
1	a	Sm Su Tp	32, 70, 130	Adenylase	Sm Su Tp	Compatible	70
					Sm Su		32, 70
2	a	Sm Su Tp		Adenylase	NT		

* Four from a single epidemic episode in one family.

Ap, ampicillin; Sm, streptomycin; Su, sulphonamides; Tc, tetracycline; Cm, chloramphenicol; Km, kanamycin; Tp, trimethoprim; NT, not transferring.

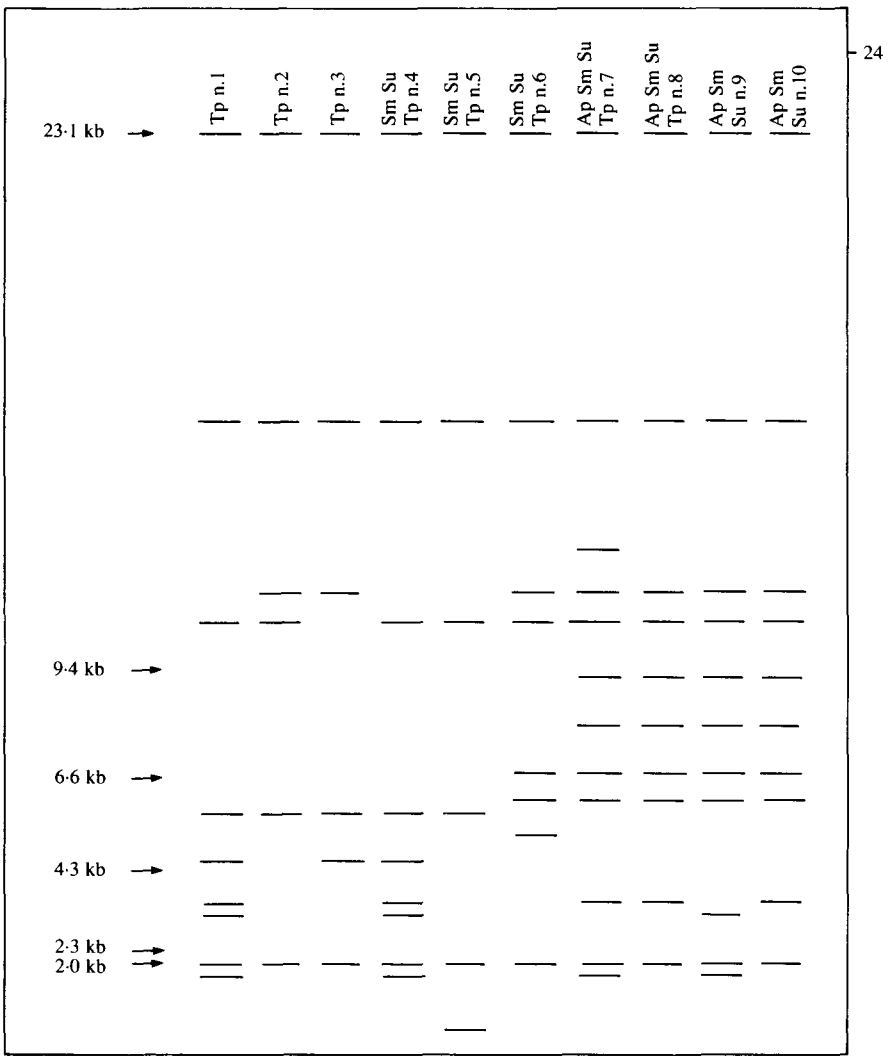

Fig. 1. *Cla* I restriction patterns of *S. sonnei* 70 MDa R plasmids transferred into *E. coli* K12.

for DHFR type V, high specificity was observed and hybridization was detected with three *S. sonnei* strains and with their corresponding Tp-resistant transconjugants.

None of the probes used hybridized with plasmid DNA extracted from the two strains which did not transfer Tp resistance and which were chosen for further study.

DISCUSSION

All Tp-resistant *S. sonnei* strains isolated from 1985 to 1987 were multiresistant, and plasmid analysis showed 18 different electropherotypes. The almost ubiquitous presence of transferable 70 MDa plasmids first suggested that an

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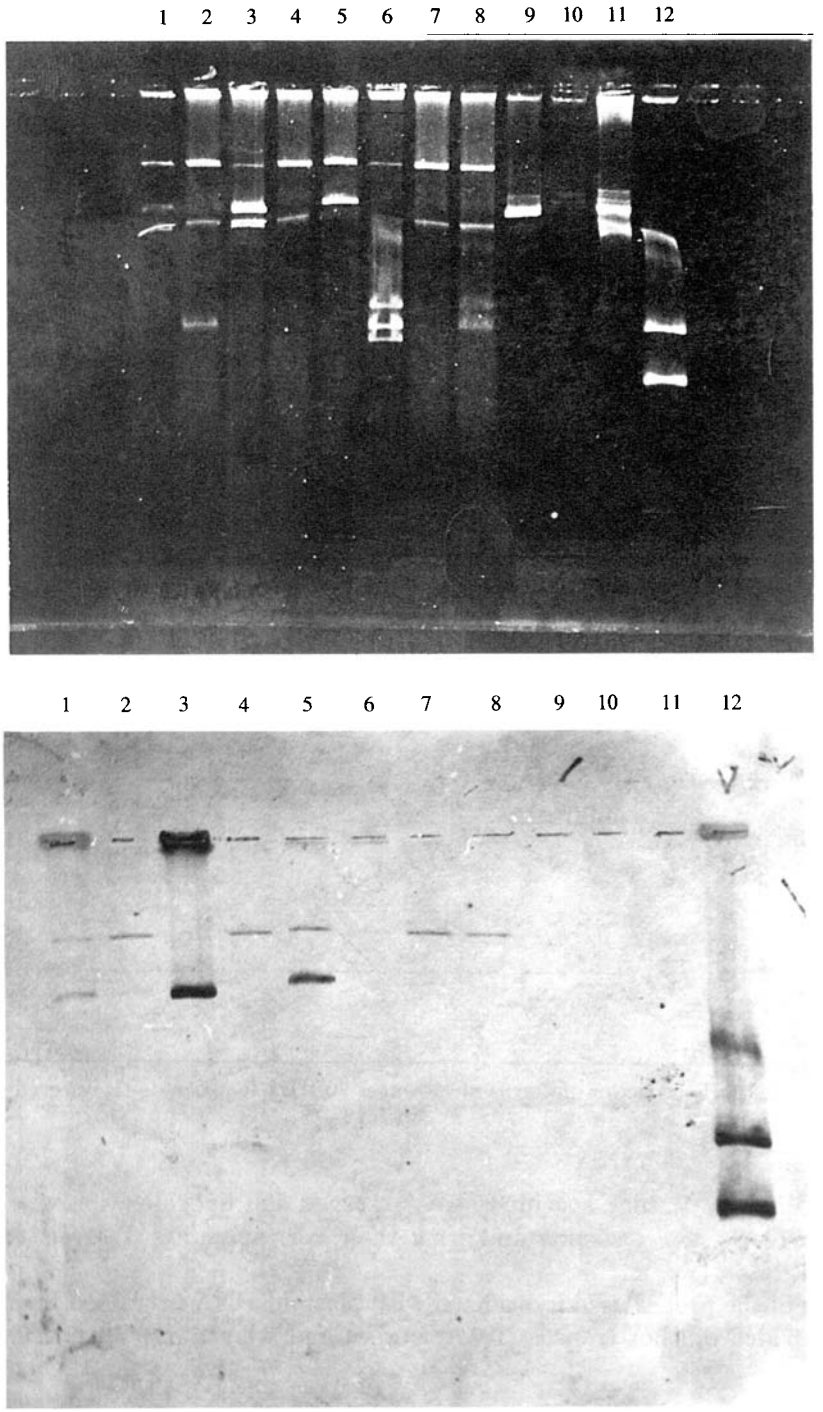

Fig. 2. For legend see opposite.

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epidemic plasmid was in circulation in *S. sonnei* in Sicily. However, molecular fingerprinting demonstrated that these plasmids, although closely related, were heterogenous and it could appear that multiple antibiotic resistance sequences were borne on structurally different plasmids. However, the degree of similarity between these plasmids does suggest that they may have evolved from a common progenitor.

The inability of the majority of strains to transfer resistance to Tp, despite the high level of resistance in these strains (MIC > 1000 μg/ml) could be explained by the integration of Tp resistance sequences into the bacterial chromosome, either by site-specific transposition [28] or by conventional mechanisms of recombination [29]. Of the six strains which showed transferable Tp resistance, three were able to transfer Tp alone. Hybridization experiments demonstrated that in these strains, Tp resistance was mediated by a gene coding for type V DHFR. This is the first report of DHFR type V outside Sri Lanka, where this Tp resistance mechanism has been found to dominate among different species of enterobacteria, including *Shigella* spp. [2]. In Sri Lanka, the occurrence of the DHFR type V gene on a transposon which also codes for sulphonamide resistance has been linked to selective pressure provided by the use of cotrimoxazole, the most widely distributed antimicrobial drug in that country. Although cotrimoxazole is the only antimicrobial drug used for the treatment of shigellosis in Sicily, plasmids in the three Sicilian strains which harboured the type V gene did not code for sulphonamide resistance.

None of the patients nor their families infected with *S. sonnei* with type V DHFR had travelled abroad. This finding could be explained by the introduction of Tp-resistant *S. sonnei* into Sicily as a consequence of a massive immigration of workers from Sri Lanka to Sicily which has taken place in the last few years. This hypothesis needs epidemiological confirmation.

The cross-hybridization of the 710 bp probe for DHFR type II gene and the plasmid containing the DHFR type V gene was presumably due to the similarity of the genetic areas external to the left of the resistance genes, as already observed by Sundstrom and colleagues [2]. None of the *S. sonnei* strains tested hybridized with the DHFR type I probe. This was quite unexpected because the DHFR type I gene, often associated with the Tn7 transposon, has been shown to have a world-wide distribution [2, 8, 9]. Furthermore, DHFR type I has been found in Tp-resistant Sicilian isolates of salmonella (A. Agodi, unpublished observations). Thus, *S. sonnei* could be considered a pocket of enterobacteria in which Tp resistance has evolved in a novel way, leading to the appearance of an unusual type of DHFR. Additional studies are necessary to investigate this hypothesis, and also to monitor the spread of the DHFR type V gene in Sicily.

Fig. 2. Plasmid DNA extracted by the method of Kado and Liu, electrophoresed on a 0·8% agarose gel (A), and corresponding vacuum blotted nylon filter (B), following hybridization with the biotin-labelled *Hinc* II–*Hinc* II probe for DHFR V. Lane 1, *S. sonnei* Sm Su Tp 1; 2, *E. coli* K12 J53 Rif^r Tp from *S. sonnei* 1; 3, *S. sonnei* Sm Su Tp 2; 4, *E. coli* K12 J53 Rif^r Tp from *S. sonnei* 2; 5, *E. coli* K12 J53 Rif^r Sm Su Tp from *S. sonnei* 2; 6, *S. sonnei* Sm Su Tp 3; 7, *E. coli* K12 J53 Rif^r Tp from *S. sonnei* 3; 8, *E. coli* K12 J53 Rif^r Sm Su Tp from *S. sonnei* 3; 9, Plasmid Col E1 Tn7 (DHFR I); 10, Plasmid R388 (DHFR II); 11, Plasmid pUK1148 (DHFR IV); 12, Plasmid plKO9 (DHFR V).

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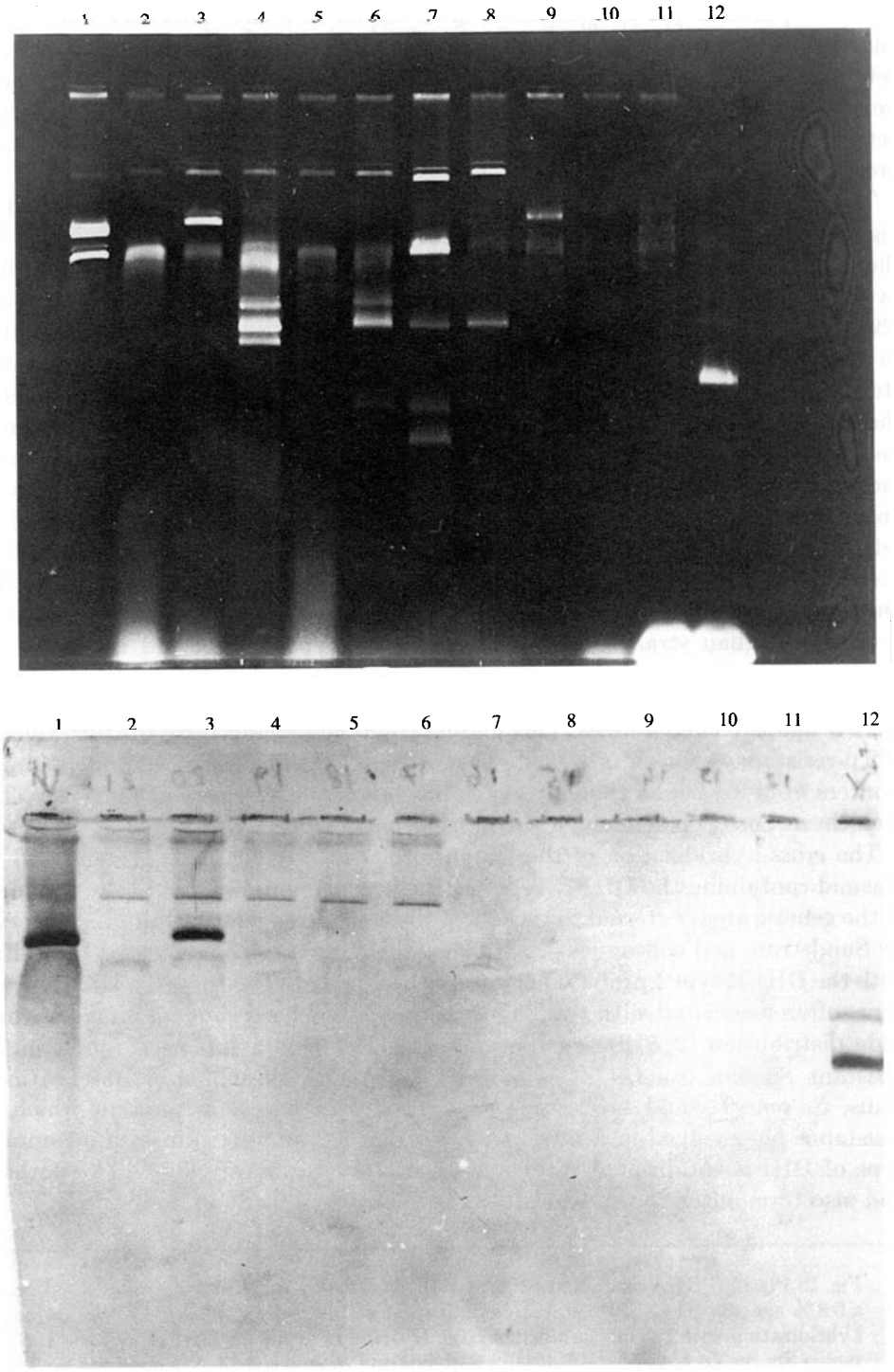

Fig. 3. For legend see opposite.

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