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rRNA gene restriction patterns and biotypes of *Shigella sonnei*

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SUMMARY

Shigella sonnei is a major agent of diarrhoeal disease in developed as well as in developing countries. Several phenotypic methods to define strain differences have been applied to this species of *Shigella* including, more recently, analysis of extrachromosomal and chromosomal DNA.

In this study, 432 endemic and epidemic strains isolated between 1975 and 1991 in Italy, France and Switzerland were submitted to rRNA gene restriction pattern analysis, after digestion of whole-cell DNA by *Hin*c II, and to concomitant biotyping.

Thirteen ribotypes, H1 to H13, and five biotypes, a, d, e, f, g, were detected. Ninety-five percent of the sporadic strains were assigned to ribotypes H1 to H4, which could be subtyped, except for H4, in different biotypes. Strains from each of seven different outbreaks had indistinguishable ribotype–biotype patterns. In contrast, 65 strains, isolated in Sicily in 1980 over an extended period of apparently epidemic increase of isolations and which had previously been considered to be a single bacterial clone on the basis of resistance pattern and phage type, were found to belong to two different and scarcely related ribotypes.

Ribotyping and biochemical subtyping appear to be a useful epidemiological tool in studies on the circulation and distribution of strains of *S. sonnei*.

INTRODUCTION

Bacteria of the genus *Shigella* are common enteric pathogens in developing countries, where strains of the different species are responsible for epidemic and endemic cases of diarrhoeal diseases. In developed countries shigellosis is mainly caused by *S. sonnei* [1].

Unlike the other *Shigella* species, *S. sonnei* contains only one serovar. Because serological typing cannot be used, several procedures have been developed in order to differentiate strains of *S. sonnei* for epidemiological purposes. To this end colicine typing, phage typing, drug resistance pattern and plasmid profile analysis [2–6] have been applied. However, all these methods have some disadvantages or limitations. In particular, colicine and phage typing are time-consuming and difficult to standardize; and not all strains are able to produce colicines or to

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maintain their plasmid determinants. Finally, instability of extrachromosomal DNA can also invalidate the results of drug resistance pattern analysis and plasmid fingerprinting.

In recent years a method for the genetic characterization of bacterial strains has been offered by the restriction fragment analysis of the chromosomal genes coding for ribosomal RNA (rRNA), that has proved to be a reliable epidemiological tool for molecular typing of several bacterial pathogens [7–11].

We have used the rDNA fingerprinting technique for typing of a large number of endemic and epidemic isolates of *S. sonnei*. In addition these strains have been submitted to a traditional biochemical typing procedure [12].

MATERIAL AND METHODS

Bacterial strains

Four hundred and thirty-two isolates of *S. sonnei* from epidemic and sporadic cases were studied: 249 sporadic strains were isolated from different geographical areas of northern (Lombardy, Trentino, Venetia), central (Tuscany) and southern Italy (Sicily, Calabria, Apulia) during the years 1975–91; 41 were isolated in France between 1978 and 1991 and 9 in Switzerland between 1976 and 1988. Epidemic strains consisted of 68 isolates from 7 different outbreaks occurring in Italy and in France (Table 2). Finally, 65 strains were isolated in Sicily during 1980, when an unusually high frequency of isolations had been recorded.

Biochemical typing

Standard methods for the fermentation of rhamnose and xylose and hydrolysis of ortho-nitrophenyl-β-D-galactopyranoside (ONPG) were used for biochemical typing [13]. The following biotypes were recognized: 'a' (ONPG +, rhamnose +, xylose −), 'd' (ONPG −, rhamnose +, xylose +), 'e' (ONPG +, rhamnose +, xylose +), 'f' (ONPG −, rhamnose −, xylose −), 'g' (ONPG +, rhamnose −, xylose −).

Isolation of DNA

For whole-cell DNA preparation, bacterial cells from 5 ml overnight broth cultures were harvested, washed in saline and resuspended in 400 μl of 150 mM-NaCl, 10 mM-Tris-HCl, pH 8·0 and 10 mM-EDTA. RNAse (20 μl of 5 mg/ml), proteinase K (60 μl of 0·5 mg/ml) and sodium dodecyl sulphate (20 μl of a 10% (w/v) solution) were sequentially added. After an incubation of at least 30 min at 50 °C, the cell lysate was submitted to several extractions with phenol–chloroform–isoamylalcohol (25:24:1). DNA was precipitated with ethanol, pelleted by centrifugation, dried and resuspended in distilled water. Concentration and purity of DNA were evaluated by agarose gel electrophoresis.

Photobiotin labelling of rRNA

Commercially available of rRNA (Sigma) from *Escherichia coli* was labelled according to the technique described by Forster and colleagues [14], by mixing of equal volumes of the rRNA sample (1 μg/μl) and of a solution of photobiotin acetate (Sigma) (1 μg/μl) and irradiation with a 250 watt sunlamp.

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Phage lambda DNA was photobiotin labelled by the same procedure.

Southern blot analysis

Approximately 2 μg of bacterial DNA were digested with restriction endonucleases (Gibco–BRL Ltd) under conditions recommended by the manufacturer. DNA fragments were electrophoretically separated through 0·8% horizontal agarose gels [15]. Photobiotin labelled lambda DNA was also digested by *Hin*d III and used on each gel as a molecular weight marker.

DNA was transferred to nitrocellulose membranes by vacuum-assisted transfer [15].

Nitrocellulose filters were prehybridized for 4–6 h and then hybridized overnight at 42 °C in 50% formamide with the photobiotin labelled rRNA (250 ng/ml of hybridization solution).

The hybridization patterns were visualized by the 'BLUGENE' non-radioactive nucleic acid detection system (Gibco–BRL Ltd) as suggested by the supplier.

Statistical methods

*Hin*c II rDNA fragment patterns were visually screened for the total of the hybridized bands and presence or absence was coded as 1 and 0, respectively. The similarity coefficient between each pair of fingerprints was expressed as the fraction of zero and non-zero matches to the total number of bands used for the comparison. Clustering was carried out from a matrix of pairwise genetic distances by using the commercial program STATPRO (Penton Software, Inc.).

RESULTS

Preliminary tests were performed on whole-cell DNA from ten *S. sonnei* isolates by using the restriction endonucleases *Ava* I, *Bam*H I, *Bgl* I, *Eco*R I, *Hin*c II, *Sma* I and *Sph* I. Digestion by *Hin*c II provided the highest strain differentiation.

Among the 432 isolates of *S. sonnei*, 13 rDNA patterns were observed, and were assumed to be distinct ribotypes (Fig. 1). rDNA patterns H1 to H4 included the 94·7% of the strains under examination. In Fig. 2 the dendrogram of genetic relatedness among the 13 ribotypes is presented: two major clusters with a low degree of similarity are evident, the first containing ribotypes H1 and H3 and the latter ribotypes H2 and H4.

Table 1 shows the distribution of the 299 sporadic isolates into different ribotypes and biotypes. The highest percentage of isolates was grouped into ribotypes H1 (37·1%), H3 (28·4%), H4 (17·1%) and H2 (12·4%) and biotypes 'a' (51·4%) and 'g' (33·1%). Only a minority of isolates was referred to ribotypes H5 to H13 and biotypes 'd', 'e' and 'f'. A remarkable correlation was evident between ribotypes H1 and H2 and biotype 'g' as well as between ribotypes H3 and H4 and biotype 'a': indeed, 74·8 and 83·8%, respectively, of the strains of ribotypes H1 and H2 belonged to biotype 'g', whereas 92·9 and 100·0% of isolates of ribotypes H3 and H4, respectively, were included in biotype 'a'. The remaining strains belonging to ribotypes H5 to H13 appeared evenly distributed among biotypes 'a' and 'g'.

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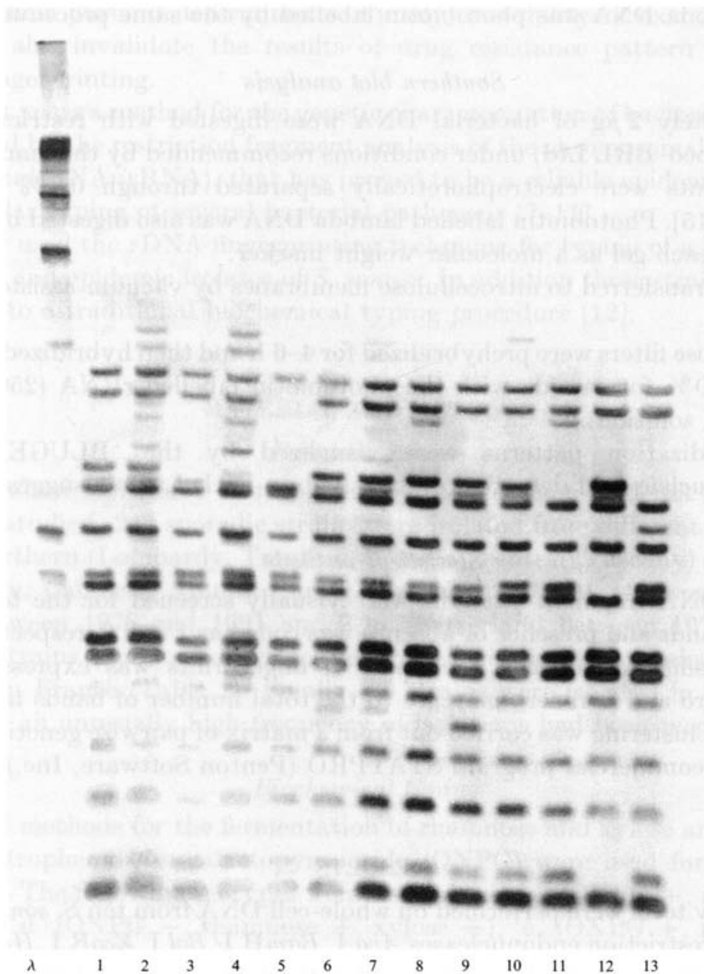

Fig. 1. rRNA gene restriction patterns of *Hinc* II digests of endemic and epidemic strains of *S. sonnei* (1–13). In the first lane are molecular size markers (phage lambda DNA digested with *Hind* III).

In Table 2 the results of ribotyping and biochemical typing of the epidemic isolates are reported. All isolates from the same outbreak exhibited identical rDNA and biochemical profiles. The ribotype H1 – biotype 'g' pattern characterized the strains from four Italian outbreaks, while the isolates from the French outbreak were of ribotype H4 – biotype 'a'. The strains recovered in Verona (Venetia) showed the pattern ribotype H2 – biotype 'f' and those from the outbreak which occurred in Livorno (Tuscany) exhibited the unusual rDNA profile H12 in association with biotype 'g'. This latter pattern was also detected in two sporadic strains from Prato (Tuscany) and from Switzerland.

The results of ribotyping of 65 Sicilian strains isolated in 1980, when an epidemic increase of infections by *S. sonnei* occurred, in comparison with the data of a previous study on the antibiotic resistance pattern and phage type of the same isolates [5], are reported in Table 3. According to their resistance patterns they

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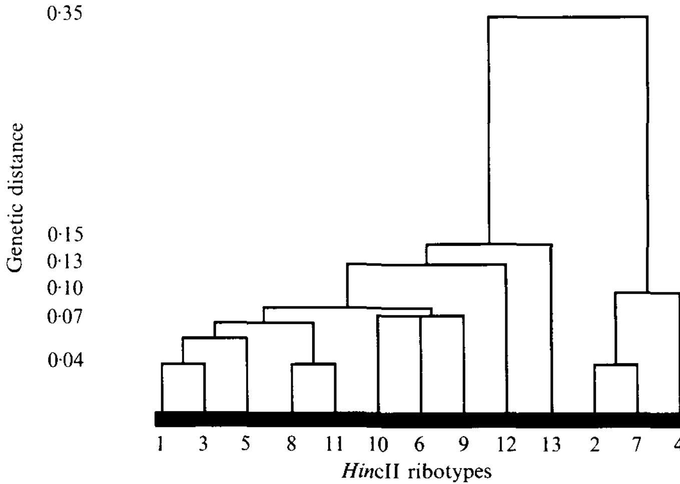

Fig. 2. Clustering of *Hinc* II rRNA gene restriction patterns of *S. sonnei*.

Table 1. *Results of ribotyping and biochemical typing of 299 sporadic strains of* S. sonnei *isolated in Italy, France and Switzerland during the period 1975–91*

Ribotype	No. of strains	Biotype	No. of strains
H1	111	a	13
		d	5
		e	3
		f	7
		g	83
H2	37	a	4
		e	1
		f	1
		g	31
H3	85	a	79
		d	1
		e	1
		f	3
		g	1
H4	51	a	51
H5	3	a	3
H6	2	g	2
H7	3	g	3
H8	1	a	1
H9	1	g	1
H10	1	g	1
H11	1	a	1
H12	2	g	2
H13	1	a	1

could be divided into three groups. The largest group comprised 58 isolates characterized by the biotype 'a'-phage type 3 pattern and by the TcSmKmAp phenotype. Consequently these epidemic strains of *S. sonnei* were considered to be a single bacterial clone. rDNA analysis has now permitted the allocation of this group of strains to two ribotypes H3 and H4, which cluster very loosely (Fig. 1).

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Table 2. *Results of ribotyping and biochemical typing of 68 isolates of* S. sonnei *from seven outbreaks of shigellosis which occurred in Italy and in France*

Outbreak	No. of strains	Ribotype	Biotype
Massa 1969	3	H1	g
Livorno 1980	8	H12	g
Ragusa 1990	16	H1	g
Le Havre 1990	13	H4	a
Brindisi 1990	6	H1	g
Catania 1991	20	H1	g
Verona 1991	2	H2	f

Table 3. *Antibiotic resistance patterns, phage types and ribotypes of 65 strains of* S. sonnei *isolated in Sicily during 1980*

Antibiotic resistance pattern	No. of strains	Phage type	No. of strains	Ribotype	No. of strains
TcSmKmAp	58	3	57	H3	46
				H4	11
		7	1	H4	1
Various combinations*	5	3	3	H3	3
		NT†	2	H3	2
Sm	2	2	2	H3	2

* Various combinations of two or three resistances to Tc, Sm, Km, Ap.
† Untypable.

DISCUSSION

Molecular analysis of chromosomal DNA has been applied to typing of several bacterial species for taxonomic and epidemiological purposes. Recently adequate differentiation among independent clinical isolates of some *Shigella* serovars has been obtained by the application of pulsed field gel electrophoresis and genome hybridization with insertion sequences [16]. On the other hand, ribosomal DNA analysis has been proved useful for epidemiological characterization of sporadic and endemic isolates of *S. sonnei*: 9 distinct rDNA patterns were observed by the use of 4 restriction endonucleases among 100 isolates from sporadic cases and 45 strains from 4 different outbreaks of shigellosis occurred in the United States [17].

The sub division of the sporadic isolates of *S. sonnei* examined in this study into 13 different rDNA profiles, by using only the restriction enzyme *Hinc* II, suggests the existence of a remarkable degree of genomic heterogeneity within this serovar of *Shigella*.

Ribotyping appears a more reliable technique of strain differentiation than phenotypic methods; indeed the apparently epidemic strains isolated during 1980 in Sicily have been divided into two well-differentiated ribotypes, even though, on the basis of their resistance pattern and phage type, they had been previously included in a single bacterial clone.

There is no close correlation between biotype and rRNA gene restriction pattern; the most frequent ribotypes, except for H4, include almost all biotypes

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although in different proportions. Thus the association of these two typing methods should offer some advantages, as the analysis of the epidemic strains demonstrates: the isolates from the outbreak in Livorno belonged to the commonest biotype 'g', but were characterized by their unusual ribotype H12; in contrast, the two isolates from the outbreak in Verona that were assigned to the ribotype H2 exhibited the uncommon biotype 'f'.

Ribotyping appears more suitable than other techniques applied to the characterization of *S. sonnei* isolates for epidemiological purposes. The association of this procedure with biotyping can allow the classification of isolates of *S. sonnei* into ribotypes and biochemical subtypes in order to obtain useful and reliable results in epidemiological investigations.

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