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Usefulness of ribotyping in a molecular epidemiology study of shigellosis

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SUMMARY

Ribotyping performed with six restriction endonucleases was used to study the molecular epidemiology of shigellosis in Asturias, Spain. The series included *Shigella sonnei* from 34 sporadic cases, 3 outbreaks and 3 reference strains, and *S. flexneri* from 13 sporadic cases and 1 reference strain. The *S. sonnei* strains were grouped into 5 ribotypes with *Sal* I, 4 with *Hind* III and *Pvu* II, 3 with *Bgl* II and *Eco*R I and 2 with *Hinc* II (Discriminatory Index (DI) between 0·54 and 0·14); the *S. flexneri* into 5 ribotypes with *Sal* I, Hinc II and *Hind* III, and 4 with the other enzymes (DI = 0·71–0·63). The combination of results for 2 or more enzymes facilitated an additional discrimination, the highest values in *S. sonnei* were for the 6 enzymes (16 types, DI = 0·91) and in *S. flexneri* for some combinations of 3 or more enzymes (7 types, DI = 0·81). Ribotypes with the 6 enzymes defined 16 clonal lines in *S. sonnei* and 7 in *S. flexneri*, which showed a different degree of genetic heterogeneity, and all the lines of each species falling into a different cluster. No line appeared as clearly endemic in the bowels of Asturian people.

INTRODUCTION

Molecular epidemiological studies of pathogenic bacteria are designed to differentiate species into types, lineages or clonal lines in order to know which are prevalent as well as their maintenance, dispersion and evolution throughout space and time, and in order to associate different clinical cases with outbreaks, cases with reservoirs and transmission vehicles. The differentiation can be made using different molecular typing methods, including ribotyping [1] which has been previously used by us in salmonella outbreak diagnosis [2, 3] and appears to be a useful genetic marker in studies on circulation [4–6] and evolution [7] of shigellas. The ribotyping procedure identifies and compares restriction fragments of the chromosomal 'rDNA region', which includes DNA carrying RNA ribosomal genes – grouped as operons – and DNA flanking regions, after hybridization with rRNA or rDNA probes. For optimum discrimination in ribotypes it is important to use the restriction endonucleases (REs) yielding the most accurate and discriminatory rDNA banding patterns within each species.

In this paper, a retrospective molecular epidemiological study of shigellosis was carried out using ribotyping performed with 6 REs. The results, from a series of 50 clinical isolates of *S. sonnei* and 13 isolates of *S. flexneri*, representing those causing shigellosis in Asturias between 1982 and 1994, and four reference strains, have been used in the attempt to address a number of questions including: (i) to ascertain the REs yielding the most accurate and differentiating DNA-fragments and highest discrimination power for ribotyping of both species; (ii) to determine whether results from different combinations of REs increase

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the discriminatory power of the method; (iii) to define within each species clonal lines, in terms of combined ribotypes (CRT) with the 6 REs, and to study the genetic relationship among the different lines, (iv) to assign isolates from 3 epidemic water-borne outbreaks, registered between 1982 and 1984, to clonal lines and survey the maintenance of organisms of these lines. We also attempt to ascertain the presence of the endemic and the sporadic clonal lines causing shigellosis in Asturias over the last decade; (v) to compare the ribotypes found in the series under study with the ribotypes of reference strains and ribotypes previously described in series from other countries in order to have additional information about the dispersion and evolution of shigellas in different geographic areas.

MATERIALS AND METHODS

Bacterial strains

This study included 34 *S. sonnei* and 13 *S. flexneri* strains causing sporadic episodes of shigellosis in the Principality of Asturias. Of these, 26 were *S. sonnei* and 10 *S. flexneri* representing strains isolated over the period 1982–7; the other 8 *S. sonnei* and 3 *S. flexneri* were representative of 11 episodes microbiologically diagnosed and attended in 3 hospitals situated in different cities (Oviedo, Avilés and Luarca) over the last 2 years, 1993–4. Sixteen other isolates of *S. sonnei* associated with 3 outbreaks registered in Asturias in 3 different localities were also tested; these consisted of 4 isolates from each of the 2 strains causing the Oviedo outbreak occurring in 1982 [8]; 4 from the Luanco outbreak in 1982 and the other 4 from the Blimea outbreak in 1984 [9]. In addition *S. sonnei* American Type Culture Collection (ATCC) 25931 and 11060 (= Colección Española de Cultivos Tipo, CECT 457), CECT 542; and *S. flexneri* CECT 585 (NCTC 1) were also tested (Table 1).

Ribotyping

Chromosomal DNA was obtained and purified as described by Deener and Boychuk [10] and samples of 2 μg were digested with the endonuclease under study for 6 h according to the manufacturer's instructions (Boehringer-Mannheim, Germany). DNAs from the gels with clearly digested DNA and visible fragments were blotted onto hybridization membranes (Hybond-N Nylon, 0·45 Micron from Amersham, U.K.) using a vacuum transfer apparatus.

The plasmid pKK3535 which carried the *rrn*B ribosomal RNA operon from *Escherichia coli* cloned in the *Bam*H I site of pBR322 [11] was used as the source of probe DNA. The probe, a 7·5 Kb *Bam*H I fragment was collected as in [12]. *Hin*d III and/or *Pst* I fragments of bacteriophage lambda DNA were also run in each gel as molecular size markers. Both lambda DNA and the probe were separately labelled with 11-d-UDP-digoxigenin using the non-radioactive DNA Labelling and Detection Kit (Boehringer-Mannheim, Germany); the membranes were hybridized with the probe for 18 h at 68 °C according to the manufacturer's instructions. In order to discard the bands corresponding to possible partial digestions, DNAs from selected isolates were tested at different cleave time periods (1, 2, 4, 6 h and overnight). For the shorter periods of time, the DNA digestions were frequently incomplete (at the top of the agarose gels there appeared one undefined band of DNA, or DNA smear along the track), in these cases in the hybridization banding patterns there frequently appeared a strong band at the top and/or additional bands along the track, which possibly corresponded to partial digestions. These bands were not considered in the definition of types. The patterns of bands containing rRNA gene sequences were designated ribotypes.

To test the reproducibility (relative position and intensity of the DNA hybridized fragments) of the method, DNA from isolates representing each one of the different rDNA restriction patterns were tested several times under the same conditions (5 U of RE per 1 μg of DNA, and 6 h of cleave time).

The polymorphic restriction sites (PRSs) were deduced by the presence or absence of bands in the overall ribotypes from each RE and species as described by Karaolis and colleagues [7].

Strains showing identical ribotypes with the six REs (combined ribotype) were assigned to the same clonal line.

Statistical methods

The discrimination index (DI), i.e. the probability that two unrelated strains sampled from the population would be placed into different typing groups, was calculated by Simpson's index of diversity [13].

The phylogenetics analysis using data from the ribotypes generated with the 6 REs (clonal lines) in

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Table 1. *Discrimination of* S. sonnei *and* S. flexneri *strains in ribotypes*

Strains	Ribotypes (no isolates)					
	Sal I	*Pvu* II	*Hinc* II	*Hind* III	*Eco*R I	*Bgl* II
S. sonnei						
Sporadic episodes	S1 (18)	P1 (25)	H1 (14)	Hd1 (13)	E1 (23)	B1 (26)
1982–7 ($n = 26$)	S2 (8)	P2 (1)	H2 (12)	Hd2 (8)	E2 (2)	
				Hd3 (5)	E3 (1)	
1993–4 ($n = 8$)	S1 (4)	P1 (5)	H1 (4)	Hd1 (4)	E1 (5)	B1 (5)
	S2 (2)	P3 (2)	H2 (4)	Hd2 (2)	E2 (2)	B2 (1)
	S3 (1)	P4 (1)		Hd3 (1)	E3 (1)	B3 (2)
	S4 (1)			Hd4 (1)		
Oviedo outbreak, 1982						
O-I strain ($n = 4$)	S1 (4)	P1 (4)	H2 (4)	Hd1 (4)	E1 (4)	B1 (4)
O-II strain ($n = 4$)	S1 (4)	P1 (4)	H1 (4)	Hd3 (4)	E1 (4)	B1 (4)
Blimea outbreak, 1982 ($n = 4$)	S2 (4)	P1 (4)	H2 (4)	Hd1 (4)	E1 (4)	B1 (4)
Luanco outbreak, 1984 ($n = 4$)	S1 (4)	P1 (4)	H1 (4)	Hd2 (4)	E1 (4)	B1 (4)
ATCC 25931	S2	P1	H2	Hd2	E1	B1
ATCC 11060 (CECT 457)	S5	P1	H1	Hd1	E1	B1
CECT 542	S1	P1	H1	Hd1	E1	B1
PRS*	7	11	1	5	6	3
DI†	0·52	0·19	0·51	0·54	0·27	0·14
S. flexneri						
Sporadic episodes	S6 (7)	P5 (8)	H3 (7)	Hd5 (7)	E4 (6)	B4 (8)
1984–7 ($n = 10$)	S7 (2)	P7 (2)	H4 (2)	Hd6 (2)	E5 (2)	B5 (2)
	S8 (1)		H5 (1)	Hd7 (1)	E6 (2)	
1993–4 ($n = 3$)	S8 (1)	P6 (1)	H4 (1)	Hd6 (1)	E5 (2)	B5 (1)
	S9 (1)	P7 (1)	H6 (1)	Hd8 (1)	E7 (1)	B6 (1)
	S10 (1)	P8 (1)	H7 (1)	Hd9 (1)		B7 (1)
CECT 585	S7	P6	H4	Hd6	E5	B5
PRSs*	8	7	17	14	4	4
DI†	0·7	0·65	0·7	0·7	0·71	0·63

* PRSs, polymorphic restriction sites in the totality of ribotypes with each RE.
† DI, discrimination index with each RE.

both species was carried out as described in [14]. Clustering analysis between clonal lines was achieved by using Jaccard's distance coefficient (D) and unweighted pair group method with arithmetic averages [15] in a Basic programme and the corresponding dendrogram was drawn.

RESULTS

In the first step, the 50 clinical isolates and the 3 reference strains of *S. sonnei* were tested by ribotyping using *Sal* I, *Pvu* II and *Hinc* II since these have been described as the REs providing the greatest strain discrimination in this species [4–7]. Results with *Sal* I showed that the clinical isolates could be discriminated into four ribotypes, labelled S1 to S4 (Fig. 1). Each of the three reference strains showed a different ribotype; CECT 542 was ascribed to S1, ATCC 25931 to S2 and ATCC 11060 to a fifth ribotype, S5. The 5 *Sal* I ribotypes showed 14 or 15 fragments of very different sizes (between 40 and 0·5 Kb) and so were well distributed along the gel, 11 fragments were common to all types. The analysis of the uncommon fragments enabled the detection of 7 PRSs within the overall ribotypes.

Pvu II and *Hinc* II yielded 4 and 2 ribotypes, labelled P1 to P4 and H1 to H2, respectively; P1 and H1 include both pathogenic and reference strains. The 4 *Pvu* I ribotypes included between 17 and 19 fragments (between 40 and 1·8 Kb), 13 of these common, and a total of 11 PRSs. The H1 and H2 ribotypes showed 19 and 18 fragments, respectively, all in the region lower than 4·6 Kb and differing only in the presence of an additional fragment of about 2·9 Kb in H1 (Fig. 1).

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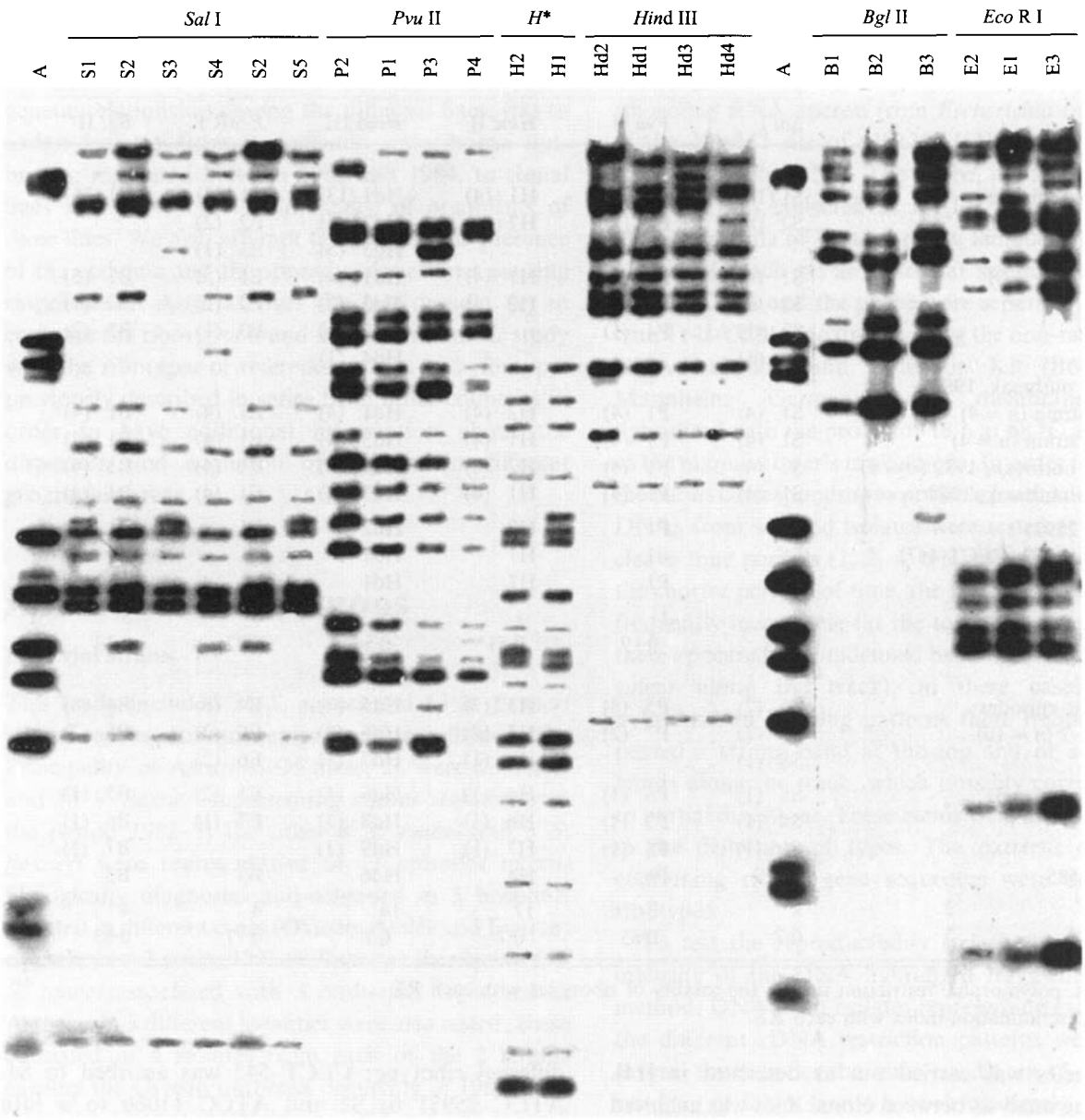

Fig. 1. Ribotypes from *S. sonnei*. H*, *Hinc* II: Lane A: lambda DNA cleaved with *Pst* I (sizes of fragments in Kb from top to bottom are 14·4, 11·6, 5·1, 4·6, 4·5, 2·8, 2·6, 2·5, 2·1, 1·9, 1·7, 1·1 and 0·8).

In order to determine if other REs yielded a higher number or more accurate ribotypes within *S. sonnei*, as well as compiling more data for a further study of genetic relationship, ribotyping was performed with three other REs: *Hin*d III, *Bgl* II and *Eco*R I. It was found that the organisms tested could be grouped into 4, 3 and 3 ribotypes, respectively; of these Hd1, Hd2, B1 and E1 included clinical isolates and reference strains. The 4 *Hin*d III ribotypes included 12 or 13 fragments of which 9 were common, and 5 RPSs; the 3 *Eco*R I ribotypes included 13 or 14 fragments, also distributed along the gel (between 40 and less than 1 Kb), 11 common and 6 PRSs. The 3 *Bgl* II ribotypes only included 8 or 9 fragments in the region between 40 and 4 Kb, 7 of these common and 3 PRSs. In Figure 1 it can be observed that the uncommon DNA fragments, used for the evaluation of the PRSs, were higher in number and more widely distributed in the *Sal* I and *Pvu* II ribotypes than in the others.

The distribution of organisms among ribotypes is shown in Table 1, where it can be observed that some ribotypes grouped several unrelated clinical isolates, whereas other ribotypes were represented by only one

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Table 2. *Clonal lines in* S. sonnei *and* S. flexneri

Line	Combined ribotype*	Presentation (No. strains)	Period
S. sonnei			
I	S1–P1–H1–Hd1–E1–B1	Sporadic (1) CECT 542	1986
II	S1–P1–H2–Hd1–E1–B1	O-I outbreak Sporadic (3)	1982–83
III	S1–P1–H1–Hd3–E1–B1	O-II outbreak Sporadic (5)	1982–83
IV	S1–P1–H1–Hd2–E1–B1	L. outbreak Sporadic (6)	1982–87
V	S1–P1–H1–Hd1–E2–B1	Sporadic (2)	1993
VI	S1–P1–H1–Hd1–E3–B1	Sporadic (1)	1985
VII	S1–P1–H1–Hd2–E2–B1	Sporadic (1)	1985
VIII	S1–P1–H2–Hd2–E2–B1	Sporadic (1)	1985
IX	S1–P1–H2–Hd2–E1–B1	Sporadic (2)	1993
X	S2–P1–H2–Hd1–E1–B1	B. outbreak Sporadic (7)	1982–87
XI	S2–P2–H2–Hd1–E1–B1	Sporadic (1)	1986
XII	S2–P1–H2–Hd2–E1–B1	ATCC 25931	
XIII	S2–P3–H2–Hd1–E1–B3	Sporadic (2)	1994
XIV	S3–P1–H1–Hd3–E1–B2–	Sporadic (1)	1993
XV	S4–P1–H1–Hd1–E1–B1	CECT 457	
XVI	S5–P4–H1–Hd4–E3–B1	Sporadic (1)	1994
S. flexneri			
XVII	S6–P5–H3–Hd5–E4–B4	Sporadic (6)	1984–86
XXVIII	S6–P5–H3–Hd6–E6–B4	Sporadic (1)	1984
XIX	S7–P6–H4–Hd6–E5–B5	Sporadic (1)	1987
XX	S7–P7–H4–Hd6–E5–B5	Sporadic (1) CECT 587	1994
XXI	S8–P5–H5–Hd7–E6–B4	Sporadic (2)	1987
XXII	S9–P7–H6–Hd8–E5–B6	Sporadic (1)	1993
XXIII	S10–P8–H7–Hd9–E7–B7	Sporadic (1)	1994

* Ribotypes with *Sal* I, *Pvu* II, *Hinc* II, *Hin*d III, *Eco*R I and *Bgl* II.

clinical isolate or one reference strain. With each enzyme one ribotype was clearly more frequent than the others.

With the six REs the reproducibility (using visibly well cleaved DNA from the agarose gels) and typeability of the method reached 100%. The results with each 1 of the 6 REs were also used to evaluate the discrimination power of the method, which was calculated using 2 parameters: number of ribotypes (the results have been described above) and calculation of a discrimination index. For the latter, the total number of unrelated strains considered was 38. The 8 organisms tested from the Oviedo outbreak were considered as 2 strains (O-I and O-II) which differed in their pattern of resistance, plasmid content [8, 9] and *Hinc* II ribotypes (Table 1); the 4 Luanco outbreak isolates as another strain and the 4 Blimea outbreak isolates as another one, because isolates from each outbreak showed the same pattern of resistance, plasmid content [9] and ribotypes with the 6 REs. The calculation of the DI of each RE ranging between 0·14 with *Bgl* II and 0·54 with *Hin*d III (Table 1).

The variation of the DI with the use of 2, 3, 4, 5 or 6 REs was also evaluated; and so the data obtained with each RE were combined. Each combination of REs resulted in a different distribution of the strains into a certain number of CRTs. Results yielding the highest discrimination were: in combinations of 2 REs, *Sal* I–*Hin*d III; *Sal* I–*Hinc* II and *Hin*d III–*Hinc* II with 7, 7 and 5 CRTs and DIs of 0·76, 0·78 and 0·82, respectively. The combination of these 3 REs reached to 10 CRTs and DI = 0·86. Two combinations of 4, *Sal* I–*Hin*d III–*Hinc* II–*Pvu* II and *Sal* I–*Hin*d III–*Hinc* II–*Eco*R I, reached 12 and 14 CRTs and DI of 0·89 and 0·84, respectively. The combinations of 5 REs did not increase these values,

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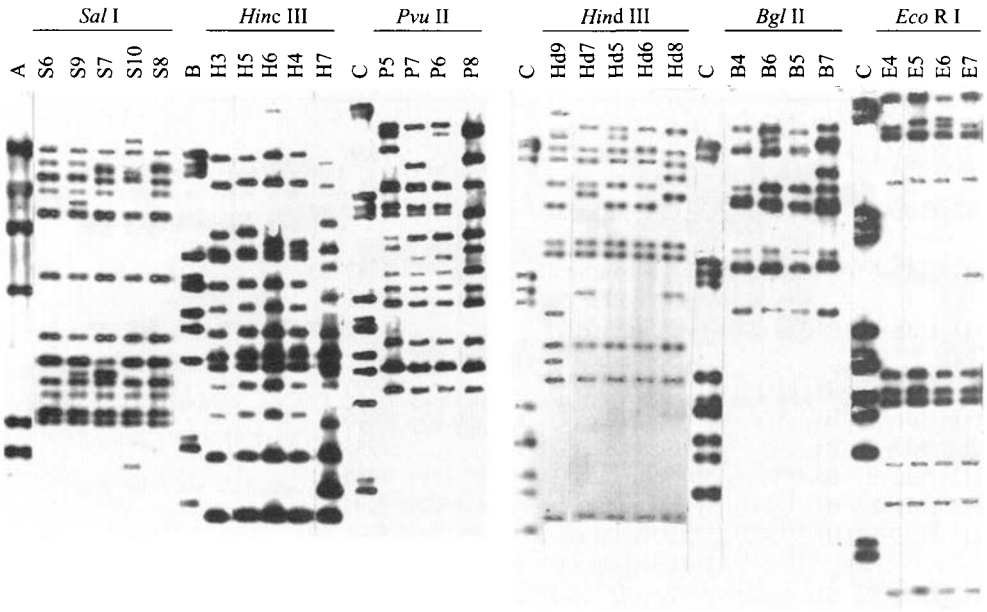

Fig. 2. Ribotypes from *S. flexneri*. Lanes A, B and C lambda DNA cleaved with: A, *Hind* III (sizes of fragments in Kb from top to bottom are 27·5, 23·1, 9·4, 6·6, 4·4, 2·3 and 2·0); B and C: *Pst* I (sizes of fragments in Kb from top to bottom are 14·4, 11·6 – these two fragments not shown in B – 5·1, 4·6, 4·5, 2·8, 2·6, 2·5, 2·1, 1·9, 1·7, 1·1 and 0·8).

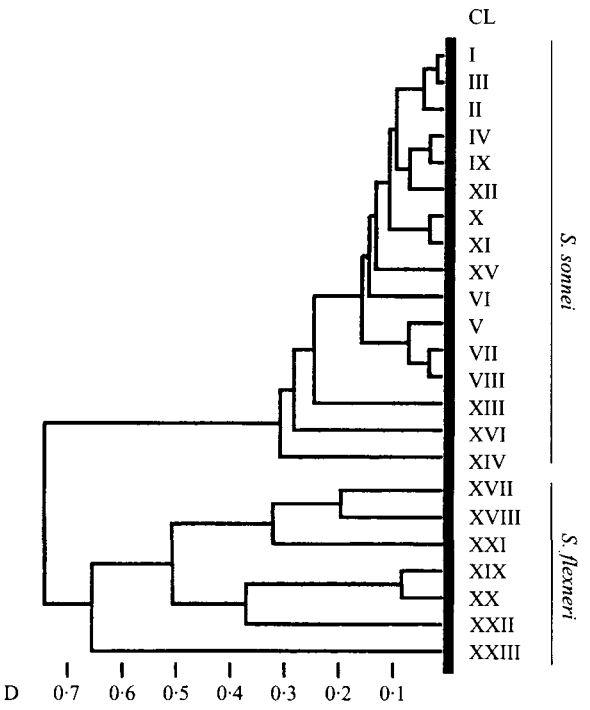

Fig. 3. Dendrogram of genetic relationships among clonal lines of *Shigella*. CL: clonal line. D: genetic distance.

but with the 6 REs 16 CRTs were reached resulting in a DI = 0·91.

Clonal lines were defined in terms of combined ribotypes with the 6 REs, the clinical isolates of *S. sonnei* being grouped into 16 lines (labelled I to XVI) which are shown in Table 2. Some of the lines were closely related given that only slight differences with a single RE were detected between them. It is noteworthy that line I included one clinical isolate and the reference strain CECT 542; whereas ATCC 25931 and 11060 strains were ascribed to lines XII and XV, respectively, which did not include clinical isolates. Seven lines only included 1 clinical isolate; 4 other ones (lines II, III, IV and X) each included organisms from sporadic episodes and one outbreak, all of them registered before or during 1987; each of the other 3 (lines V, IX and XIII), included 2 isolates from sporadic episodes registered in the last period (1993–4).

The 13 clinical isolates and the reference strain CECT 585 of *S. flexneri* were also ribotyped using the 6 REs. *Sal* I and *Hinc* II and *Hind* III each divide the organisms into 5 ribotypes, and the other 3 (*Pvu* II, *Bgl* II and *Eco* RI) into 4 (Fig. 2a, b and Table 1). The ribotypes generated with each RE showed differences in the number, size and distribution of fragments compared with the *S. sonnei* ribotypes, in all the cases with some common fragments in both species. The results from different REs showed that combinations of either *Sal* I, *Hinc* II or *Hind* III with *Eco*R I or *Pvu* II increase the number of CRTs to 6, and with the combination of both (*Eco*R I–*Pvu* II) the increase was to 7. Combinations of higher numbers of enzymes did not increase this number of CRTs. The DI for

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individual REs and the combination of REs was also calculated, ranging between 0·63 and 0·71 for individual REs (Table 1) and reaching a maximum (DI = 0·81) for the above cited combinations of 3 or more REs.

Results from the 6 REs allowed us to group the *S. flexneri* strains into 7 clonal lines (labelled XVII to XXIII) shown in Table 2. Three points of epidemiological interest should be noted: (i) 6 isolates collected before 1986 belonged to line XVII which did not include organisms collected during later years; (ii) 1 isolate collected in 1994 and the reference strain CECT 587 were ascribed to line XIX, which only differed in the size of one *Pvu* II fragment from line XX (Fig. 2, lanes P7 and P6, respectively), that included organisms isolated in 1987; and (iii) each of the 3 isolates collected during 1993–4 was ascribed to a different line, showing differences among them with 5 or 6 REs.

The results from the totality of the distinct patterns, which were produced with the 6 REs, in both species were used to study the genetic distance among clonal lines by single linkage dendrogram (Fig. 3). The 23 lines were grouped into two well differentiated clusters at a significance level of 0·75. The 16 *S. sonnei* lines fell into a cluster, showing genetic distance coefficients between 0·02 and 0·30; and the 7 *S. flexneri* lines fell into another cluster, showing genetic distance coefficients between 0·07 and 0·65. It is also noteworthy that at a low significance level of 0·15, *S. sonnei* are grouped into a subcluster including 13 lines and 3 additional lines (XIII, XIV and XVI) more loosely related to their next subcluster.

DISCUSSION

Shigellosis or bacillary dysentery represents a major cause of diarrhoeal disease in industrial countries and a monumental public health problem in many developing countries. In developing and oriental countries *S. flexneri* is the most frequently isolated among the four *Shigella* species but in Europe and the U.S.A. *S. sonnei* is the most frequent [4–9, 16–22]. Even in industrialized countries where shigellosis is in decline its epidemiological surveillance continues to be important because: (1) As few as 100 organisms can lead to infection even in a well-nourished individual, hence, shigellas are among the most communicable bacterial agents of diarrhoeal disease. (2) The main infection sources are faecal contaminated food and water, being the cause of sporadic national or international epidemics and more frequently of small community outbreaks [8, 9, 16–22]. The person-to-person spread is also a factor particularly in institutional outbreaks such as schools and nurseries [21]. (3) The only important reservoir of *Shigella* species, which for taxonomic purposes can be considered as pathogenic clones of *E. coli*, is man (though higher apes are also a natural host and reservoir). The shigellas seem to have adapted to a specific niche, the human bowel, and so the evolution of clones in different human populations could be in response to differences in diet, in the background bacteria and bacteriophage flora, or the selective pressure due to bacteriocins and antibiotics [7]. These three points emphasize the importance of the use of accurate genetic typing methods in the epidemiological surveillance of shigellas.

In Spain, the number of outbreaks and cases of shigellosis reported over the period 1988–91 was 15 and 1758 respectively [22]. None of these outbreaks took place in the Principality of Asturias, although in this region between 1982 and 1984 three water-borne outbreaks of *S. sonnei* shigellosis occurred in three localities with different water supplies and sewage facilities.The cases registered were about 1000 in the Oviedo outbreak [8], 65 in the Blimea outbreak and 250 in the Luanco outbreak [9]. Fortunately, in the years following 1984 only family outbreaks and sporadic cases have been reported and shigellas have been scarcely isolated in clinical microbiology laboratories.

In this work, ribotyping, performed with 6 REs, was used as a genetic marker in a retrospective study on the molecular epidemiology of shigellosis in the Principality of Asturias between 1982 and 1994. The combination of results from the 6 REs led us to divide the *S. sonnei* clinical isolates into 16 clonal lines with a degree of similarity higher than 70% (13 of these higher than 86%). The *S. flexneri* clinical isolates were discriminated into 7 different clonal lines with a higher degree of genetic heterogeneity. In fact the similarity ranged between 94% for the most closely related lines and 36% for the most loosely related. Among these 24 clonal lines, none seems to be clearly endemic in the bowel of Asturian people, since some lines are represented by only one or two strains, and other lines group higher numbers of strains but these have been circulating only throughout the first years of the period under study. The most frequent, lines IV and X (which differed in the ribotypes with 3 REs: *Sal* I, *Hinc* II and *Hind* III), included isolates from the

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Luanco and Blimea outbreaks, occurring in 1982 and 1984, respectively, and isolates implicated in several sporadic shigellosis episodes occurring in the following years; but none of the organisms collected in 1993–4 belonged to these lines. Organisms of lines II and III, both implicated in an important water-borne outbreak occurring in the capital, Oviedo, in 1982 have not been collected since 1983, data suggesting either a low adaptability to survival in healthy carriers, or that they have suffered mutations or changes affecting the rDNA region. The latter hypothesis can be supported because lines I, III and IV, represented by organisms collected over the period 1982–7, showed more than 97% of similarity with line IX, represented by organisms collected in 1993.

Organisms of *S. flexneri*, line XVII, seem to have been relatively frequent before 1986 but have not been found in the period following this year. On the other hand, line XXII includes organisms causing one sporadic episode of shigellosis registered in 1994, and the strain CECT 585 (NCTC 1) reputed to be the first case reported in France (Catalogue CECT). The 7 *S. flexneri* lines showed a higher degree of heterogeneity than *S. sonnei* lines. Between some pairs of lines a loose genetic relationship was found, this lower relationship between *S. flexneri* compared with *S. sonnei* could be a reflection of the fact that *S. flexneri* is an antigenically heterogeneous species comprising several serotypes, whereas *S. sonnei* is antigenically homogeneous having only one serotype.

When our findings for *S. sonnei* were correlated with those previously reported, we found that in an outbreak due to contaminated water ingestion occurring in 1991 in another Spanish city, Barcelona [19], all organisms tested by ribotyping showed similar *Sal* I-banding patterns to S1 ribotype of clinical isolates of our series. Also, the most frequent *Sal* I and *Pvu* II ribotypes found in our series were similar to ribotypes described by Hinojosa-Ahumada and colleagues [4] in strains from the United States and Karaolis and colleagues [7] in worldwide strains. These works identified *Sal* I as the most useful RE in *S. sonnei* ribotyping, which we confirm based on two features: its higher discrimination power and the generation of uncommon fragments of different sizes (between 12 and 2 Kb) and easy visual differentiation. On the contrary, neither of the two *Hinc* II ribotypes in our series is identical to any of the 13 *Hinc* II ribotypes previously reported in strains from Italy, France and Switzerland by Nastasi and colleagues [6]. However, in the latter series H1 and H3 ribotypes (which were found among the most frequent) show a high similarity with H1 and H2 ribotypes of our series; in fact they differ only in one fragment. In the paper of Nastasi and colleagues, a dendrogram of genetic relatedness among the 13 *Hinc* II ribotypes is presented, and the authors emphasized the presence of two major clusters of *S. sonnei* with a low degree of similarity. However, we think that the several additional fragments shown in ribotypes forming the second cluster (H2, H4 and H7) could be due to partial digestions. If our observation was correct, strains showing ribotypes H2, H4 and H7 should be grouped into H1, H3 and H6 respectively. Then, the totality of ribotypes from the series would form a single cluster with a similarity higher than 85%, data in line with those found in [6] and our series.

With respect to *S. flexneri*, the 5 *Hind* III ribotypes from our series appear as very similar to some of the 11 *Hind* III ribotypes described by Faruque and colleagues [5] in isolates from Bangladesh, a country where this species is strongly endemic.

In the present study the usefulness of using multiple REs for ribotyping in terms of epidemiological data gained was clear. However, the number of types and the DI were increased by the use of combinations of several REs as compared to the use of only one or two. On the other hand, the testing of many REs obviously increases the cost in terms of work, time and money and a six way ribotype would be an useful and accurate tool in outbreak diagnosis and short scale studies with a limited number of strains, but can be expensive or impractical in large scale studies. In the latter purpose, the data above supported the three way ribotyping using *Sal* I, *Hinc* II and *Hind* III as the better option for *S. sonnei* but not for *S. flexneri*. These enzymes, alone or in their different combinations, differentiated the *S. flexneri* strains into the same 5 groups, while the combination of each of them with *Eco*R I and *Pvu* II appeared as a more discriminative option.

To summarize, by using clinical isolates of *S. sonnei* and *S. flexneri* representing the strains causing shigellosis over the period 1982–94 in Asturias, we detected by means of ribotyping genotypic differences that: (i) provided a precise differentiation within both species in clonal lines, (ii) led us to establish the genetic relationship among organisms causing shigellosis and (iii) allowed us to ascertain the absence of clearly endemic lines or lines within which organisms have circulated over the last decade. This information could be used as a reference in prospective

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epidemiological surveillance of shigellosis as well as in studies of genetic variation within shigella clones.

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