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Usefulness of genetic typing methods to trace epidemiologically *Salmonella* serotype Ohio

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SUMMARY

Different genetic typing procedures were applied in an epidemiological study of *Salmonella* serotype Ohio. Isolates that generated identical DNA fingerprints (*Hin*cII ribotypes, ERIC and RAPD profiles) were clustered into the same lineage, and the addition of data from plasmid, integron and resistance profiles was used to differentiate types. Results led to the determination of the endemic and the emergent epidemic types at specific times, and to ascertain the clinical and epidemiological impact of each type. In the series analysed (47 clinical isolates and 3 non-clinical isolates) 11 lineages and 32 types were found. Two lineages were considered prevalent and endemic, and during an epidemiological alert (Spain, 1998) a re-emergence and spread of organisms mainly from the most frequent lineage had occurred. The combination of H-ribotype with ERIC profile, as primary markers, and resistance profile with plasmid profile, as secondary markers, was shown to be the most useful tool to trace epidemiologically Ohio.

INTRODUCTION

In July 1998 the Spanish National Centre of Epidemiology recommended the surveillance of *Salmonella enterica* ssp enterica serotype Ohio (antigenic formula 6,7,14:b:1,w) because an increase in salmonellosis episodes caused by Ohio, mainly in young children under 2 years, had been observed in previous weeks in several hospitals within different Spanish Regions. In the 'Laboratorio de Salud Pública' (LSP), which acts as the Asturias Reference Centre for *Salmonella*, this serotype was not reported during 1997 whereas it was involved in six episodes during February–July of 1998. It is noteworthy that the real incidence of cases infected with Ohio could be much greater than that reported here. This presumption is supported by the fact that in intestinal disease episodes only infants and adults with severe clinical symptoms usually require hospital attention, and salmonella organisms are mainly collected from such patients. In addition, not all the isolates collected in the Microbiology Laboratories are forwarded to the LSP.

The aim of this report was to ascertain the epidemic genetic types of Ohio that have been circulating and causing human salmonellosis in the Principality of Asturias before and during the epidemiological alert of 1998. For this purpose, together with antimicrobial resistance, several genetic typing methods were used to characterize Ohio isolates. Data from typing procedures were processed to cluster isolates into lineages and types, and to trace epidemiologically Ohio during a 9-year period (1990–8). In addition, each of the procedures used was evaluated for serotype Ohio typing in order to propose the most useful method, or combination of methods, for future epidemiological survey studies.

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MATERIALS AND METHODS

Salmonella strains

This study was carried out with 50 isolates of *Salmonella* serotype Ohio: 39 isolates (collected over 1990–8 in 6 Microbiology Laboratories of Public Hospitals situated in different localities of the Principality of Asturias) which have been registered as causal agents of 33 cases of salmonellosis (in 3 cases, more than 1 isolate from each patient was processed); 8 clinical isolates from other Spanish regions collected in 1998; and 3 strains from other sources (CECT 4379 and 4380 from food and sea water, respectively, and LSP 461/94 from sewage). *Salmonella* serotype Enteritidis ATCC 13076 was used as reference strain in DNA fingerprinting assays; Enteritidis ATCC 13076 and Typhimurium LT2 (carriers of virulence plasmids of about 60 and 90 kb, respectively) and Typhimurium LSP 14/92 (carrier of integrons with variable regions of 1000 and 1200 bp) as controls of virulence plasmids and class 1 integrons, respectively.

Antimicrobial susceptibility

Antimicrobial susceptibility was tested by disk diffusion using commercial disks (bioMérieux and Oxoid, Spain) [1]. The antimicrobials and concentrations in μg tested were: amikacin 30 (Am), ampicillin 10 (Ap), cephalotin 30 (Ce), ceftazidime 30 (Caz), chloramphenicol 30 (Cm), ciprofloxacin 5 (Cf), gentamicin 10 (Gm), imipenem 10 (Ipm), kanamycin 30 (Km), streptomycin 10 (S), nalidixic acid 30 (Nal), tetracycline 30 (Tc), trimethoprim 5 (Tp), sulphadiazine 300 (Su), spectinomycin 10 (Sp), and trimethoprim-sulphamethoxazole 1·25/23·75 (Sxt).

Genomic DNA isolation, ribotyping and PCR-based procedures

Isolation of genomic DNA and ribotyping were carried out as previously described [2, 3]. *Hin*cII was selected to perform ribotyping because it detects a high number of restriction sites in the *rrn* loci and yields a good discriminatory power within several salmonella serotypes [3–5]. In the PCR-based procedures DNA was obtained from 15 μl, of a 10-fold distilled water dilution of a Luria–Bertani broth overnight culture as previously described [6]. The primers and basic performance conditions have been referenced: PCR ribotyping [7, 8]; enterobacterial repetitive intergenic consensus (ERIC) sequences analysis [9], randomly amplified polymorphic DNA (RAPD) segment analysis [6]; virulence genes (*pho*, *stn*, *inv*E/A, and *spv*C) [10–13]; presence of class 1 integrons and the *sul1* gene in these integrons [14, 15]. The 1600-bp variable regions in integrons from different strains were tested: (i) by restriction fragmentation pattern analysis using *Taq*I, *Pvu*I and *Hin*cII, following the manufacturer's recommendations (Amersham Pharmacia Biotech, Spain) and (ii) for the content of *aadA1a*, and *dfrA* genes by PCR, using 15 μl of a 10-fold water dilution of the 1600-bp amplicons as template DNA; *dfrA*-primers [forward/backward: 5′GTGAAACTATCACTAATGG-3′/5′-CCCTTTTGCCAGATTTGG-3′](GenBank/EMBL acc. no. Z83311) and *aadA*-primers and performance PCR conditions as in ref. [15].

Plasmid analysis

Plasmid content was determined following the alkaline lysis method [16]. Plasmids of similar size collected from different strains were analysed by restriction fragmentation pattern using *Bgl*I and *Hin*cII, following the manufacturer's recommendations (Amersham Pharmacia Biotech, Spain). Genetic plasmid transfer by transformation was performed by the calcium chloride procedure [16] using *Escherichia coli* Max Efficiency DH10™ Competent Cells (Gibco–BRL).

Epidemiological and statistical analysis

The Principality of Asturias is an autonomous community in the North of Spain covering an area of about 10565 km^2 divided into eight health areas, each attended by one or more public hospitals. The population in 1996 was 1093937 inhabitants (48·3% males), with the following distribution into age groups: $<$ 1 year old (8271 inhabitants); 1–15 years (254024); 16–60 years (592302); and $>$ 60 years (239340). As a measure of the incidence density (ID) for each year, period, sex, and age groups the following equation was used: number of cases/person-year at risk $\times$ 10^5 inhabitants. Lineages and types were categorized as: endemic when they included several isolates from different patients collected throughout the study period; epidemic when several isolates from different patients were collected during a

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defined time period, ranging from days to months; and sporadic when one or more isolates were obtained from a single case, or from several cases separated by long time periods.

The discrimination index (DI), i.e. the probability that two unrelated strains obtained from the population would be placed into different typing groups [17] was calculated for 43 strains, because isolates showing identical features and epidemiologically relatedness (collected from members of a single family or from a single patient at different times) were assigned to a single strain.

RESULTS

Salmonella serotype Ohio as a causal agent of human salmonellosis in the Principality of Asturias

During the period 1990–8, the LSP processed a total of 3255 salmonella isolates, which had been implicated in 2848 sporadic cases and 75 outbreaks of human salmonellosis. Of these, no outbreaks and only 33 cases (of which 3 cases, affecting < 1 year infants, occured as persistent or recurring salmonellosis) were associated with serotype Ohio, representing an ID = 3·01. Some epidemiological features of the cases infected with Ohio are compiled in Table 1. Two facts should be noted: the increase in the number of cases in 1998 with regard to the previous years; and the higher incidence in infants under 1 year compared to the other age groups.

Table 1. *Epidemiological data of cases infected with Ohio in the Principality of Asturias, Spain*

Parameter	Cases	(ID)
Year		
1990	3	(0·27)
1991	2	(0·18)
1992	1	(0·09)
1993	4	(0·37)
1994	7	(0·64)
1995	4	(0·37)
1996	3	(0·27)
1997	0	(0)
1998	9	(0·82)
Sex		
Male	16	(3·11)
Female	17	(2·93)
Age groups		
< 1 year	9	(108·81)
1–15 years	9	(3·54)
16–60 years	5	(0·84)
> 60	3	(1·25)
Unknown	7	

DNA fingerprinting analysis

The 50 Ohio isolates and Enteritidis ATCC 13076 were analysed by DNA fingerprinting procedures and the distribution of isolates into profiles can be seen in Table 2. All experiments were carried out at least twice to ensure the reproducibility of the results and only the reproducible and well defined fragments were used to define ribotypes and amplicon profiles. Ribotyping performed with *Hin*cII differentiated the Ohio isolates into eight (H1–H8) banding profiles and yielded a DI = 0·68. The H-ribotypes included 10–20 bands (of sizes 0·9–6·3 kb), 2 of which were categorized as matching, and 25 as mismatching or polymorphic fragments. The Enteritidis ATCC 13076 generated a different profile to Ohio (Fig. 1). By amplification of the spacer region between 16S and 23S rRNA genes, the Ohio strains were differentiated into only two SR profiles (SR1 and SR2) which included 2 matching and 1 mismatching bands (Fig. 2). SR1 was generated by all but 2 isolates; while SR2 was generated by CECT 4390 and 1 Asturian isolate, as well as Enteritidis ATCC 13076. This procedure yielded DI = 0·09. Combining data from *Hin*cII ribotyping and PCR-ribotyping, nine combined ribotypes were obtained but no increase in the DI.

By the amplification of ERIC sequences, 4 (E1–E4) profiles were generated (DI = 0·3). Repetitive analysis showed that the profiles generated always shared some well defined bands (3 of these present in the 4 profiles) but it was difficult to know the total number of polymorphic bands due to the low intensity of some of these. Enteritidis ATCC 13076 generated a profile different to those obtained from Ohio (Fig. 2).

By RAPD typing, performed with the primers labelled S, B and C, the isolates of Ohio were subdivided into 4 (S1–S4), 2 (B1–B2) and 3 (C1–C3) profiles, respectively (Fig. 3), yielding DIs of 0·22, 0·13 and 0·18, respectively. The 2 B-profiles included 3 matching and 3 mismatching bands. The 3 C-profiles did not included matching bands, and they included, at least, 8 mismatching bands. Combining data from the 3 procedures a further differentiation into 5 RAPD-profiles (DI = 0·22) was registered. Enteriditis ATCC 13076 yielded profiles different to those obtained from Ohio with the 3 primers.

Organisms showing identical ribotypes and amplicon profiles were considered to be closely related and

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Table 2. *Grouping of Ohio isolates into lineages and types*

	Lineage features*						Type features†					Isolates origin‡		
	Ribotypes			RAPD								Clinical		
L	H	SR	ERIC	S	B	C	Type	Plasmids (kb)	Int. (bp)	Resistances	No.	cases	Geographic area	Isolation year
1	H1	SR1	1	1	1	1	t1	Free	Negative	Susceptible	1	1	A–III	1990
2	H1	SR1	4	1	1	1	t1	Free	Negative	S	1	1	A–III	1990
3	H2	SR1	1	1	1	1	t1	Free	Negative	Susceptible	7	3	A–III, IV	1991–94
							t2	Free	Negative	Su	2	2	A–III, IV	1991; 94
							t3	Free	Negative	ApGmSTc	1	1	A–III	1994
							t4	Free	Negative	ApGmSSuTc	1	1	A–III	1990
							t5	Free	1600	CmKmSSpSuSxtTcTp	1	1	A–IV	1998
							t6	Free	1600	ApKmSSpSuSxtTcTp	1	1	A–V	1998
							t7	2·5	Negative	Sp	1	1	Castellón	1998
							t8	4·7	Negative	SSpSuSxtTp	1	1	A–IV	1993
							t9	4·7	Negative	ApGmSSuTc	1	1	A–I	1994
							t10	4·7	Negative	ApGmSSpSuTc	1	1	A–III	1994
4	H3	SR1	1	1	1	1	t1	1·8	Negative	Susceptible	1	1	A–III	1992
5	H4	SR1	1	1	1	1	t1	Free	Negative	Susceptible	2	2	A–VIII, V	1995; 98
							t2	Free	Negative	Sp	1	1	A–V	1993
							t3	Free	Negative	Su	1	1	A–V	1996
							t4	Free	Negative	SpSu	3	1	A–V	1995
							t5	Free	Negative	SpSuTp	1	1	A–VII§	1994
							t6	Free	1600	ApSSpSuSxtTcTp	3	3	A–IV, V	1996
							t7	2·75	Negative	Susceptible	6	5	A–IV, V, Navarra Orense	1998
							t8	2·75	Negative	Sp	2	2	Tarragona, Valladolid	1998
							t9	3·9	1600	ApCmKmSSpSuSxtTcTp	1	1	Madrid	1998
							t10	4·7	Negative	Susceptible	1	1	A–V	1993
							t11	4·6–2·95	Negative	Susceptible	1	1	A–I	1995
							t12	2·95–2·4–1·9	1600	ApSSpSuSxtTcTp	1	1	Zaragoza	1998
6	H4	SR1	3	1	1	1	t1	2·75	Negative	Susceptible	1	1	A–III	1998
7	H5	SR1	1	1	1	1	t1	Free	Negative	Susceptible	1	1	A–V	1994
8	H6	SR1	2	2	1	1	t1	3	Negative	SSpSuTcTp	1	1	A–III	1994
9	H7	SR1	3	2	2	2	t1	5·2–4·8–3·4–2·8–2·2	Negative	NalSSpTc	1	1	Barcelona	1998
10	H7	SR2	3	3	2	2	t1	Free	Negative	Susceptible	1	1	A–IV	1998
							t2	Free	Negative	NalSSpSu	1	1	Ref.4380§	1992
11	H8	SR1	2	4	1	3	t1	Free	Negative	Sp	1	1	Ref.4379‖	1992

* L, lineage. H, *Hin*cII. SR, spacer region. S, B, and C, indicate the primers used in RAPD.

† Int. variable region in class 1 integrons. Ap, ampicillin; Cm, chloramphenicol; Gm, gentamicin; Km, kanamycin; S, streptomycin; Nal, nalidixic acid; Sp, spectinomycin; Su, sulphadiazine; Sxt, trimethoprim-sulphamethoxazole; Tc, tetracycline; Tp, trimethoprim. No., number of isolates.

‡ A–I to A–VIII indicate different Asturian Health Areas.

§, ‖ strains collected from sewage and food, respectively.

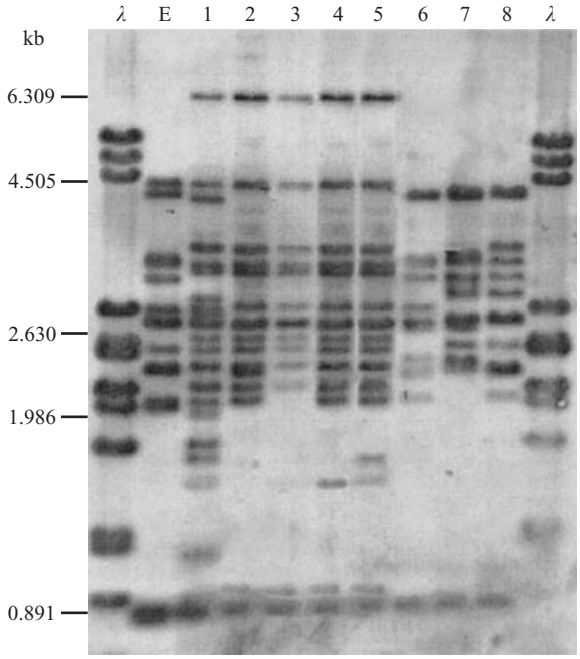

Fig. 1. Ribotypes generated with *Hin*cII in *Salmonella* serotype Ohio strains. Lane λ, DNA of phage λ digested with *Pst*I. Lane E, H-ribotype generated by Enteritidis ATCC 13076. The distribution of serotype Ohio isolates into H-ribotypes is shown in Table 2.

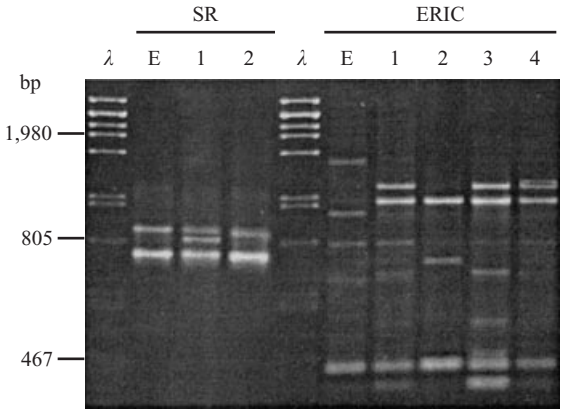

Fig. 2. Amplicon profiles in *Salmonella* serotype Ohio strains. SR, profiles generated by amplification of the spacer region between 16S and 23S rRNA genes. ERIC, profiles generated by amplification of ERIC sequences. Lane λ, DNA of phage λ digested with *Pst*I. Lane E, profiles generated by Enteritidis ATCC 13076. The distribution of serotype Ohio isolates into amplicon profiles is shown in Table 2.

members of a single lineage. In total, 11 lineages (L1–L11) were defined. Lineages L5 and L3 were observed most often (46 and 34% of the isolates, respectively, of the complete Ohio series and 41% of the clinical Asturian isolates). Lineage L10 was represented by one Asturian clinical isolate and CECT

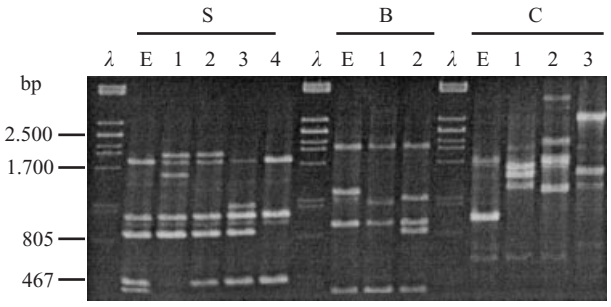

Fig. 3. RAPD profiles in *Salmonella* serotype Ohio strains generated with S, B and C primers. Lane λ, DNA of phage λ digested with *Pst*I. Lane E, RAPD profiles generated by Enteritidis ATCC 13076. The distribution of serotype Ohio isolates into RAPD profiles is shown in Table 2.

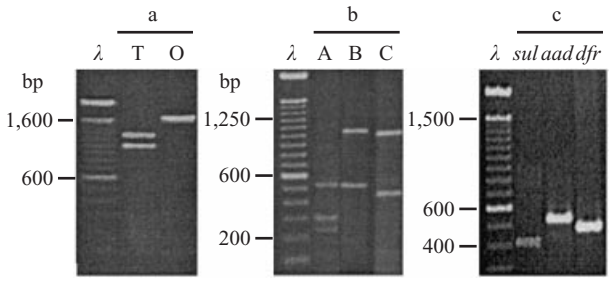

Fig. 4. Class 1 integrons and resistance genes in salmonella strains. (*a*) Integron profiles. T, Typhimurium 14/92; O, Ohio isolates. (*b*) RFLP of 1600 bp variable region in class 1 integrons from Ohio isolates: A, *Taq*I; B, *Pvu*I and C, *Hin*cII. (*c*) Amplification of resistance genes in 1600 bp variable region of class 1 integrons from Ohio isolates: *sul*, *aad*, and *dfr* corresponding to *sul1*, *aadA1a*, and *dfrA* genes, respectively.

4380. The remaining lineages were represented by a single strain.

Virulence, integron, plasmid, and antimicrobial resistance profiles

The presence of four virulence genes: *inv*E/A, *stn*, *pho* and *spv*C was tested for by PCR. All Ohio strains were negative for *spv*C which is a plasmid-located gene, but were positive for the genes of chromosomal location, apart from CECT 4380 which did not carry *inv*E/A or *stn* genes (figure not shown). Seven epidemiologically unrelated isolates carried class 1 integrons, with a variable region yielding a PCR product of 1600-bp (Fig. 4*a*). The 1600 bp products were cleaved with *Taq*I, *Pvu*I, and *Hin*cII which generated indistinguishable restriction patterns with each enzyme. (Fig. 4*b*).

A total of 20 Ohio isolates carried plasmids. The number and size of the plasmids carried by each strain, and the restriction fragmentation pattern

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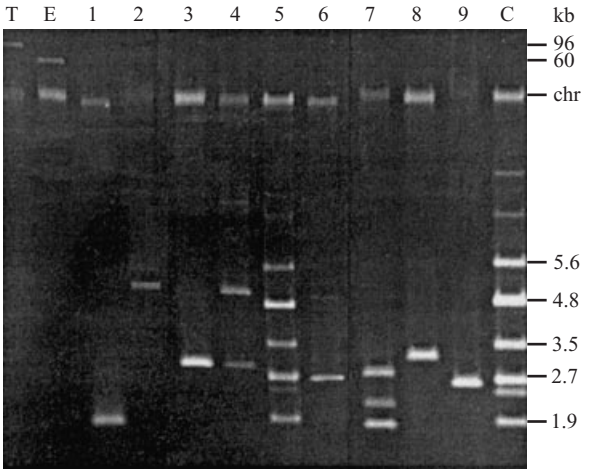

Fig. 5. Plasmid profiles in *Salmonella* spp. strains. Lane C, molecular size standard plasmids. Lane T, virulence plasmid from Typhimurium LT2. Lane E, virulence plasmid from Enteritidis ATCC 13076. Lanes 1–9, plasmid profiles from serotype Ohio isolates. Chr, chromosomic DNA. The distribution of serotype Ohio isolates into plasmid profiles is shown in Table 2.

analysis of plasmid DNA using separately *Bgl*I and *Hin*cII, led us to group isolates into nine plasmid profiles (Fig. 5, Table 2). The plasmids were more frequent among isolates from the second half (1995–8) than from the first half (1990–4) of the period under study (53·8 and 25%, respectively). To calculate the DI, plasmid-free strains were grouped into a plasmid profile. In this way, 10 plasmid profiles were considered (DI = 0·66).

All Ohio isolates were susceptible to amikacin, cephalotin, ceftazidime, ciprofloxacin and imipenem; 22 clinical isolates (46·8%) were susceptible to all the antimicrobials tested, a further 8 (17%) isolates showed a single resistance (sulphadiazine, streptomycin, or spectinomycin) and the remaining 17 (36·2%) included between 2 and 8 resistances, in different combinations (Table 2). The resistant isolates were grouped into 16 R-profiles, and to calculate the DI all the susceptible strains were grouped into another profile. The DI obtained was 0·83. Some other findings should be noted: (i) the most frequent resistances were spectinomycin (40%), streptomycin (32%), tetracycline (26%), and ampicillin (21%); (ii) gentamicin-resistance only appeared among isolates collected during 1994, while the other resistances appeared throughout the period under study; (iii) resistance to the antimicrobials used in the control of extraintestinal and severe intestinal salmonellosis; ampicillin, chloramphenicol and trimethoprim (which is used in combination with sulphamethoxazole under the name of cotrimoxazole) was less frequent in the first (16·6; 0·0 and 4·2%, respectively) than in the second half (23·1; 7·7 and 26·9% respectively) period.

Data from resistance to antimicrobials, plasmid and integron analysis were used to subdivide lineages into types. In total, 32 types were differentiated, 24 of them represented by Asturian clinical strains (Table 2). No clear relationship between plasmids and antimicrobial resistance was observed. Of the strains carrying plasmids, 50% were susceptible to all antimicrobials tested and the remaining strains showed seven different plasmid profiles. Transformation experiments with the total plasmid DNA from the resistant isolates were carried out and the results showed that no antimicrobial resistance from the Ohio isolates tested could be transferred to *E. coli*. Nor was any relation between the presence of class 1 integrons and plasmids revealed, because among the seven isolates carrying integrons only two carried plasmids. Conversely, it was found that the seven isolates carrying integrons were multiresistant, expressing some resistances in common [S-Sp, Su, Tc, Tp, and Sxt]. In these isolates the presence of *sul1*, *aadA1a* and *dfrA* genes in the integrons was analysed by PCR. Expected amplicons of about 400, 525 and 500 bp, respectively, were obtained (Fig. 4*c*).

Evaluation of the DNA fingerprinting procedures as typing methods within Ohio

The criteria to evaluate typing methods described in ref. [18] have been applied to procedures used in this work. All strains were tested at least twice with each procedure to ensure the reproducibility of the results. The typeability, accessibility of materials and reagents, and flexibility (they can be used in different bacteria species) are important advantages in all the procedures used. H-ribotyping showed the greatest reproducibility, ease of profile interpretation, and discrimination power (DI = 0·68). H-ribotyping can be carried out with basic electrophoresis equipment, but it is a complex, costly, and technically demanding procedure. Conversely, PCR-based procedures have the advantages of rapidity and ease of performance, whereas a thermocycler is necessary in addition to the basic electrophoresis equipment. It should be pointed out that the PCR procedures showed reproducibility in distributing strains into profiles, revealing themselves as adequate tools for epidemiological purposes.

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Moreover, the reproducibility and interpretation of amplicon banding profiles are shortcomings due to the fact that weak and/or not constant bands are frequently detected and this makes it necessary to choose the number and size of the fragments that define each profile. In a previous work, we commented that variations in amplicon profiles obtained in different laboratories could be found, but that each laboratory is free to establish which amplified fragments are considered in order to define profiles [6].

The epidemiological concordance and the *in vivo* stability were tested by comparing results from three groups of epidemiologically related isolates. The first group included five isolates, sequentially isolated from a single infant over a 5-month period, which showed identical features, being ascribed to L3t1. The second included two isolates, collected 2 months apart and were also associated with a persistent gastroenteritis in an infant, assigned to L5t7 which was the most frequent clone isolated during the epidemiological alert. The third group included three isolates ascribed to L5t4 which have been collected over a 5-month period from two infants who were brothers, one of which presented a recurrent salmonellosis.

The variation of the discrimination power with the use of two or more procedures was also evaluated. For this purpose the data obtained with each procedure were combined resulting in a different distribution of the organisms within a certain number of groupings or types. The combination of H-ribotyping, ERIC, resistance and plasmid typing yielded the same discrimination that the combination of all typing procedures: 32 types, DI = 0·98. Other interesting combinations were: H-ribotyping and resistance-typing, with 25 types, DI = 0·93; H-ribotyping, ERIC, and resistance typing, with 26 types, DI = 0·94; and H-ribotyping, resistance and plasmid typing, with 31 types, DI = 0·97.

DISCUSSION

Success in epidemiological surveillance studies of pathogenic bacteria can be related to the typing procedures applied to differentiate or to cluster organisms. In fact, the usefulness of a trait for typing is related to its stability in a given strain and its diversity among the strains forming one species. For this, the typing methods are based on the premise that clonally related organisms share traits that can differentiate them from other unrelated organisms. The term lineage could be used to design a genetic system that accumulates differences only through genetic processes that occur within single cells. In this work, traits screened by genetic procedures that analyse polymorphisms of chromosomal DNA (ribotypes, ERIC and RAPD profiles) were used as primary markers to cluster isolates into lineages. In addition, the presence or the profile of mobile and non-essential genetic elements (integrons and plasmids) together with the profile of resistance to antimicrobials were used as secondary markers to subdivide lineages into types. In this way, it was revealed that the series under study included organisms falling into 11 lineages and 32 types.

The typing procedures were chosen because of their proved value to differentiate between and within salmonella serotypes [3–6]. Ribotyping determines the sequence divergence of the *rrn loci* which encode the ribosomal RNAs; ERIC typing reveals the presence of dispersed repetitive DNA sequences using consensus oligonucleotides, and RAPD typing detects sequence divergence of total DNA using arbitrary oligonucleotides. The separation of isolates into ribotypes, ERIC and RAPD profiles is based on differences in one or more DNA fragments due to single or multiple genetic events. For example, the two most frequent H-ribotypes (H2 and H4) showed only two mismatching fragments which could derive from a single insertion/deletion, whereas with regard to H1 they showed 11 and 7 mismatching fragments, respectively, fact that implies several genetic changes. The two most frequent lineages showed a closer relationship with one another as well as with other sporadic lineages differing only in a few mismatching bands generated by one of the typing procedures. On the other hand, these related lineages differed from some other sporadic lineages in the profiles generated by all (or by all except PCR ribotyping) typing procedures. These data support the fact that the isolates of Ohio can present a remarkable degree of genetic heterogeneity.

Epidemiologically interesting observations were: (i) two Ohio lineages could be considered endemic in Asturias, organisms falling into both were collected over 1990–8, and spread in other Spanish regions. (ii) Organisms falling into three epidemic types were circulating previously to the epidemiological alert of 1998, while organisms of a fourth epidemic type (L5t7) were only collected in 1998 in different Spanish regions. Organisms differing from L5t7 only in their resistance-profile, in their plasmid-profile, or in both

profiles were also collected during 1998 in different Spanish regions. These data support the fact that during the first half of 1998, the introduction of a new lineage had not taken place but rather the re-emergence of Ohio organisms belonging to an old lineage representing types with a narrow genetic relationship had occurred. (iii) The reservoir and food-vehicle of these types is unknown. It is noteworthy that to ascertain both of these, a hundred samples of foods (eggs, chicken, pork, beef, sausages, and hamburgers) were analysed for salmonella using standard procedures in our laboratories (data not shown). All samples were negative for Ohio although 22·7% were positive for other salmonella serotypes. (iv) It is also noteworthy that among Ohio organisms virulence plasmids were not found and that five multiresistant types (falling into two related lineages) carried integrons with variable regions of 1600 bp, generating identical restriction fragmentation and carrying *aadA1a* and *dfrA* genes, presumably forming a fused gene-cassette. This type of variable region in class 1 integrons has been found in our laboratory among several salmonella serotypes and in other laboratories among other enterobacteria species [19, 20].

From this study, the following main conclusions can be drawn. (i) The application of genetic typing procedures (ribotyping and PCR-based methods) has revealed that the Ohio isolates can present a remarkable degree of genetic heterogeneity, and this has also led us to determine the close relationship between the most frequent human pathogenic subtypes of this serotype. (ii) The addition of data from secondary typing procedures (plasmid, integron and resistance profiles) enabled us to differentiate types within lineages, to determine the sporadic and the emergent epidemic types at a specific time, as well as to ascertain the clinical and epidemiological impact of each type, e.g. during the Spanish epidemiological alert of 1998 a re-emergence of organisms assigned to different types falling mainly into the prevalent lineage had taken place. (iii) The evaluation of the different DNA fingerprinting procedures for Ohio typing showed that H-ribotyping was the most accurate procedure. Moreover, the combination of H-ribotyping with other typing procedures led to a more precise characterisation of the isolates and increased the discrimination power. Data from this work support H-ribotype alone or in combination with ERIC profile as a primary marker, and resistance profile alone or together with plasmid profile as a secondary marker.

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