EPIDEMIOLOGICAL TYPING

A three-way ribotyping scheme for *Salmonella* serotype Typhimurium and its usefulness for phylogenetic and epidemiological purposes

BEATRIZ GUERRA, ELENA LANDERAS, M. ANGELES GONZALEZ-HEVIA* and
M. CARMEN MENDOZA

*Departamento de Biología Funcional, Area Microbiología, Universidad de Oviedo, 33206 Oviedo and
Laboratorio de Salud Pública, Principado de Asturias, Oviedo, Spain

Ribotyping of *Salmonella* serotype Typhimurium strains was optimised as a tool for epidemiological and phylogenetic purposes. Of five restriction endonucleases evaluated on a series of 84 isolates, *HincII*, *SalI* and *PvuII* were the most useful, generating 13, 9 and 9 ribotypes with 17, 11 and 18 polymorphic restriction sites, and attaining a discrimination index (DI) of 0.81, 0.53 and 0.59, respectively. The combination of results from tests with the three enzymes provided further discrimination (19 ribotypes, DI=0.84). It proved useful for clonal analysis, defining 19 clonal lines with a remarkable degree of genetic heterogeneity, that were grouped into two major clusters (including 12 and 7 lines, respectively) at a significance level of 0.65. When the attributes of this system were compared with those of phage typing, it was found that ribotyping showed higher typability and sensitivity, supporting its use as an appropriate molecular method. In tracing the molecular epidemiology of Typhimurium strains in Asturias, six lines were found that could be considered endemic and were represented by organisms implicated in salmonellosis throughout the period of study; another four lines included organisms isolated from meat, water or both.

Introduction

Salmonella enterica serotype Typhimurium (*S. typhimurium*) is associated with a wide variety of clinical manifestations [1], characterised by a wide dissemination among human and non-human sources; it is an important cause of food-borne infection in developed countries [2]. At the Spanish Reference Laboratory of *Salmonella* and *Shigella*, Typhimurium is second only to Enteritidis but its frequency has increased over the last few years, from 353 strains (13% of serotyped salmonellas) in 1988 to 1160 (25.1%) in 1994; it is also isolated more frequently than Enteritidis from non-avian meats [3, 4]. At the Principality of Asturias Public Health Laboratory (APHL, acting as *Salmonella* Asturian Reference Centre since 1990), during the period 1990–94, a total of 496 Typhimurium isolates (26% of salmonellas serotyped) was processed, associated with 469 human sporadic episodes and six outbreaks.

Epidemic strains of Typhimurium have been traced mainly by phage typing, although other phenotypic and molecular methods have provided useful epidemiological markers [5–10], including ribotyping by the analysis of restriction fragment length polymorphisms for highly conserved rRNA genes and associated sequences (rDNA region) [11, 12]. In the present study, an optimisation of the ribotyping procedure was attempted by the evaluation of five restriction endonucleases (REs) on a series of Typhimurium strains. A three-way ribotyping scheme (with *HincII*, *SalI* and *PvuII*) is proposed as a molecular method, and its value was tested both in terms of application to phylogenetics and the epidemiological surveillance of serotype Typhimurium.

Materials and methods

Bacterial strains and plasmids

This study included 84 isolates of Typhimurium from the collection of the APHL selected according to their date of isolation, place (in Asturian Health Area) of isolation, source and clinical and epidemiological presentation. The 72 human isolates included: 42 from

Table 1. Sources and properties of Typhimurium strains used in this study

Strain ref. no.*	Year	Health area	Source	Antigenic formula	Phage type	Ribotype with		
						<i>HincII</i>	<i>SalI</i>	<i>PvuII</i>
81	1987	III	Faeces	4, 12 :i: 1, 2	104	H1	S1	P1
125	1987	III	Faeces	4, 12 :i: 1, 2	..	H1	S1	P1
445	1988	III	Faeces	4, 12 :i: 1, 2	UT	H1	S1	P1
515 (2)	1991	IV	Faeces†	4, 12 :i: 1, 2	104	H1	S1	P1
828 (2)	1991	IV	Drinking water	4, 5, 12 :i: 1, 2	104	H1	S1	P1
14	1992	IV	Faeces	4, 12 :i: 1, 2	104	H1	S1	P1
15	1992	IV	Faeces	4, 12 :i: 1, 2	104	H1	S1	P1
16	1992	III	Faeces	4, 12 :i: 1, 2	104	H1	S1	P1
27	1992	IV	Faeces	4, 12 :i: 1, 2	104	H1	S1	P1
28	1992	III	Faeces	4, 12 :i: 1, 2	UT	H1	S1	P1
36	1992	III	Faeces	4, 12 :i: 1, 2	UT	H1	S1	P1
24	1993	V	Faeces	4, 5, 12 :i: 1, 2	UT	H1	S1	P1
37	1993	IV	Faeces	4, 5, 12 :i: 1, 2	104	H1	S1	P1
57	1993	IV	Faeces	4, 12 :i: 1, 2	UT	H1	S1	P1
59	1993	IV	Urine	4, 12 :i: 1, 2	104	H1	S1	P1
75 (2)	1993	V	Faeces†	4, 12 :i: 1, 2	UT	H1	S1	P1
189	1993	IV	Abscess	4, 12 :i: 1, 2	104	H1	S1	P1
453 (6)	1993	I	Pork meat/faeces†	4, 12 :i: 1, 2	UT	H1	S1	P1
23	1994	IV	Sputum	4, 12 :i: 1, 2	UT	H1	S1	P1
209	1994	IV	Sputum	4, 12 :i: 1, 2	UT	H1	S1	P1
412	1994	VIII	Water‡	4, 12 :i: 1, 2	UT	H1	S1	P1
514	1994	VIII	Water‡	4, 12 :i: 1, 2	104	H1	S1	P1
520	1994	VIII	Water‡	4, 12 :i: 1, 2	104	H1	S1	P1
537	1994	VIII	Water‡	4, 12 :i: 1, 2	104	H1	S1	P1
104	1990	IV	Faeces†	4, 5, 12 :i: 1, 2	193	H2	S1	P1
18	1992	III	Faeces	4, 5, 12 :i: 1, 2	193	H2	S1	P1
404	1992	III	Faeces	4, 5, 12 :i: 1, 2	120	H2	S1	P1
43	1993	V	Tracheal aspirate	4, 5, 12 :i: 1, 2	193	H2	S1	P1
91	1993	III	Faeces	4, 5, 12 :i: 1, 2	193	H2	S1	P1
95	1993	III	Faeces	4, 5, 12 :i: 1, 2	193	H2	S1	P1
97	1993	III	Faeces	4, 5, 12 :i: 1, 2	193	H2	S1	P1
43	1994	I	Faeces	4, 12 :i: 1, 2	193	H2	S1	P1
13	1992	III	Faeces	4, 5, 12 :i: 1, 2	193	H2	S1	P1
29	1992	III	Faeces	4, 5, 12 :i: 1, 2	193	H2	S1	P1
6	1993	III	Faeces	4, 5, 12 :i: 1, 2	193	H2	S1	P1
47	1992	II	Faeces	4, 5, 12 :i: 1, 2	104	H3	S1	P1
22	1992	IV	Faeces	4, 12 :i: 1, 2	UT	H3	S1	P2
363	1991	III	Pus	4, 5, 12 :i: 1, 2	195	H3	S2	P2
365	1991	III	Urine	4, 5, 12 :i: 1, 2	195	H3	S2	P2
31	1993	V	Faeces	4, 5, 12 :i: 1, 2	104	H3	S2	P2
179	1993	IV	Faeces	4, 5, 12 :i: 1, 2	193	H3	S2	P2
106	1994	V	Faeces	4, 12 :i: 1, 2	UT	H3	S2	P2
57	1985	III	Faeces	4, 5, 12 :i: 1, 2	66	H3	S2	P2
34	1994	V	Faeces	4, 12 :i: 1, 2	5	H3	S2	P8
298	1988	III	Bile	4, 5, 12 :i: 1, 2	204	H4	S1	P1
346 (3)	1992	IV	Faeces†	4, 5, 12 :i: 1, 2	UT	H4	S1	P1
392	1992	IV	Lymphatic system	4, 5, 12 :i: 1, 2	UT	H4	S1	P1
13	1994	II	Faeces	4, 5, 12 :i: 1, 2	203	H4	S1	P1
8	1993	IV	Faeces	4, 5, 12 :i: 1, 2	UT	H4	S1	P1
722	1992	IV	Urine	4, 5, 12 :i: 1, 2	80	H4	S1	P1
166	1994	III	Faeces	4, 12 :i: 1, 2	80	H4	S1	P1
139	1987	III	Faeces	4, 12 :i: 1, 2	96	H4	S1	P4
415	1988	III	Faeces	4, 5, 12 :i: 1, 2	96	H4	S1	P4
122 (7)	1994	III	Faeces†	4, 12 :i: 1, 2	96	H4	S1	P4
14	1993	IV	Faeces	4, 5, 12 :i: 1, 2	104	H4	S2	P2
56	1984	III	Faeces	4, 12 :i: 1, 2	UT	H5	S3	P3
674	1991	IV	Blood	4, 5, 12 :i: 1, 2	UT	H5	S3	P3
80	1993	II	Faeces	4, 5, 12 :i: 1, 2	193	H5	S3	P3
150	1994	IV	Faeces	4, 12 :i: 1, 2	23	H5	S3	P3
323	1994	II	Blood	4, 12 :i: 1, 2	23	H5	S3	P3
23	1984	III	Faeces	4, 12 :i: 1, 2	UT	H6	S4	P5
100	1984	III	Faeces	4, 12 :i: 1, 2	UT	H6	S4	P5
72	1986	III	Faeces	4, 5, 12 :i: 1, 2	124	H7	S3	P6
611	1991	II	Drinking water	4, 5, 12 :i: 1, 2	28	H7	S3	P6
502	1994	VII	Water‡	4, 12 :i: 1, 2	UT	H8	S2	P2
15	1985	III	Faeces	4, 12 :i: 1, 2	..	H9	S6	P4
443	1994	VIII	Water‡	4, 12 :i: 1, 2	193	H10	S5	P7
94	1993	IV	Faeces	4, 5, 12 :i: 1, 2	193	H11	S7	P1
Reference strains								
247	1994	Spain	Blood	4, 12 :i: 1, 2	...	H1	S1	P1
BC-LT2	...	Germany		4, 12 :i: 1, 2	...	H3	S8	P2
ATCC 14028	...	USA		4, 12 :i: 1, 2	...	H12	S9	P9
BC-25268	1975	Germany		4, 12 :i: 1, 2	...	H13	S3	P3

Reference strains were from: BC, Bayer A.G., Research Centre, Wuppertal, Germany; ATCC American Type Culture Collection. Strains are listed in order according to their ribotypes (RTs) beginning with *HincII*. UT, phage untypable by the scheme of Anderson *et al.* [15].

*Number of isolates tested (in parentheses) if >1.

†Outbreak.

‡Treated sewage water.

sporadic episodes of acute enteritis; 12 from sporadic episodes of extra-intestinal infection; and 18 faecal isolates from six different food-borne outbreaks. All 72 isolates came from persons requiring treatment in Asturian Hospitals during the period 1984–94. In addition, there were three isolates from pork meat implicated in one of the outbreaks, three from drinking water and six from treated sewage water. Four Typhimurium strains from other collections were used as reference strains (Table 1); one strain (93/92) of Enteritidis belonging to the prevalent clonal line in the Principality of Asturias [13] was used as an out-group strain.

Plasmid pKK3535, which carries the *rrnB* operon from *Escherichia coli* cloned in the *Bam*HI site of pBR322 [14], was used as the source of probe DNA. The probe was a 7.5-kb *Bam*HI fragment labelled with 11-d-UDP-digoxigenin according to the instructions of the kit manufacturer (Boehringer-Mannheim).

Serotyping and phage typing

Serotyping of strains was performed by slide-agglutination tests with commercial somatic (O) and flagellar (H) antisera (Sanofi Diagnostic Pasteur, France) and they were confirmed as belonging to serotype Typhimurium (4,[5],12:i:1,2). Phage typing, by the extended phage-typing scheme of Anderson *et al.* [15], was performed in the Spanish Reference Laboratory of *Salmonella* and *Shigella*, Instituto de Salud Carlos III, Majadahonda, Madrid.

Ribotyping

Chromosomal DNA was obtained and purified by an SDS-phenol isolation method [16]. Samples were cleaved with REs and tested by Southern blot hybridisation, performed as described previously [17, 18] with a non-radioactive DNA labelling and detection kit (Boehringer-Mannheim). In the analysis of each RE, isolates representing the different banding patterns were tested at least three times to evaluate the reproducibility of the method. The patterns of bands containing rRNA gene sequences were designated ribotypes. The polymorphic restriction sites (PRS) were deduced by the presence or absence of bands among the total ribotypes from each RE as described before [18, 19]. Strains showing identical ribotypes after testing with *Hinc*II, *Sal*I and *Pvu*II, were ascribed to the same clonal line.

Statistical analysis

The discrimination index (DI), 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 [20].

For phylogenetic analysis, the totality of the ribotypes

generated with each of the enzymes *Hinc*II, *Sal*I and *Pvu*II was first analysed; then the combined data from all three REs were processed as described earlier [18]. Thus, clustering analysis between combined ribotypes was carried out by Jaccard's distance coefficient (D) and the unweighted pair group method with arithmetic averages (UPGMA) in the software Programme MVSP (Multivariate Statistics Package, 2.0a).

Results

Value of five restriction endonucleases in ribotyping Typhimurium

A total of 84 Asturian isolates of Typhimurium and four reference strains was analysed by ribotyping. Total genomic DNA from each was separately cleaved with five REs: *Hinc*II, *Sal*I, *Pvu*II, *Eco*RI and *Bgl*I, and the different results in rDNA restriction patterns were observed for each. Data from the ribotyping of organisms associated with six outbreaks showed that, in all cases, the isolates from each outbreak yielded identical ribotypes with the five REs, allowing them to be considered as six different strains. Thus, the 84 Asturian Typhimurium isolates were grouped into 68 strains, as listed in Table 1. All strains could be typed, and with each RE one ribotype was clearly more frequent than the others. For the remaining stages of the present study, results with *Eco*RI and *Bgl*I were discarded, as they were not considered sufficient for meaningful discrimination or of value as a typing tool: *Eco*RI generated only three ribotypes (represented by 63, 8 and 1 strains, respectively) and showed a very low discriminatory power; *Bgl*I was discarded because it frequently generated partial digestions and its banding patterns were also difficult to interpret, due to the close situation of fragments in the region 3.5–2.5 kb.

With *Hinc*II, strains were discriminated into 13 ribotypes (labelled H1–H13) including 13–16 fragments of small size (6.5–0.5 kb). When the ribotypes were compared, six fragments common to all were found, and the analysis of the uncommon fragments led us to establish 17 PRS (Fig. 1). With *Sal*I, nine ribotypes (S1–S9) were generated, with 11–15 fragments (sizes 23–2 kb), seven common fragments and 11 PRS (Fig. 2). With *Pvu*II, nine ribotypes (P1–P9) including 13–15 fragments (sizes 14.2–1.7 kb), six common fragments, and 18 PRS were obtained (Fig. 3). The banding patterns yielded with each of the REs in Typhimurium strains were very similar to one another and, albeit with a lower number of common fragments, to Enteritidis ribotypes (Figs 1–3).

The Typhimurium strains, shown in Table 1, were used to evaluate the discriminatory power of the method with each of three chosen REs, by means of two parameters: number of ribotypes generated and calculation of a discrimination index (DI), which

Fig. 1. Ribotypes generated by *HincII* in Typhimurium. Lane A, lambda DNA cleaved with *PstI*; sizes of fragments (kb) from top to bottom are 5.08, 4.65, 4.5, 2.84, 2.58, 2.44, 2.14, 1.98, 1.70, 1.16, 1.09, and 0.81. HE, ribotype pattern of Enteritidis strain 93/92.

takes into consideration both the number of types defined by the typing method and their relative frequencies. The DIs obtained were: 0.81 with *HincII*, 0.53 with *SalI* and 0.59 with *PvuII*. The increase in discriminatory power by combination of data from two and three REs was also evaluated, resulting in a different distribution of strains; the number of combined ribotypes (CRTs) and DIs obtained with the combinations of REs were: *HincII* with *PvuII* 17 CRTs, DI = 0.83; *HincII* with *SalI* 16, 0.83; *SalI* with *PvuII* 13, 0.61; and *HincII* with *PvuII* and *SalI*, 19, 0.84. These data allowed us to propose and to evaluate as a molecular tool a three-way ribotyping scheme with *HincII*, *SalI* and *PvuII*, because it yielded the highest discrimination. The 19 CRTs were considered as clonal lines (labelled I–XIX.). The distribution of strains into lines is also shown (Fig. 4). The most frequent was line I (34.72%), represented by 24 Asturian strains and the reference strain labelled 247/94, a clinical isolate that had caused septicaemia in a hospitalised patient from another Spanish city (Burgos). Fifteen lines (II–XVI) were represented by

Asturian strains only, the other three lines (XVII–XIX) by each of the other reference strains: Typhimurium LT2, ATCC 14028 and 25268, a highly virulent clinical isolate from Germany [21]. Enteritidis strain 93/92 was ascribed to line XX.

Correlation between the three-way ribotyping scheme and phage typing

The results obtained from the three-way ribotyping scheme were compared with phage-typing results [15]. Whereas all strains could be ribotyped, 20 (of 66) phage-typed strains were phage untypable (UT). Strains grouped in the two most frequent phage types were divided by ribotyping: 16 strains of PT104 into clonal lines I, III, V, IX and 14 strains of PT193 into clonal lines II, V, X, XV and XVI. Three strains of PT23 were ascribed to line X, and three strains of PT96 to line VIII. On the other hand, it is noteworthy that the most frequent line (I) included only strains of PT104 or that were UT; the second most frequent line (II) included strains of PTs 193 and 120 only.

Fig. 2. Ribotypes generated by *SalI* in Typhimurium. Lane B, lambda DNA cleaved with *HindIII*: sizes of fragments (kb) from top to bottom are 23.13, 9.42, 6.56, 4.36, 2.32 and 2.03. SE, ribotype pattern of Enteritidis strain 93/92.

Clonal analysis of Typhimurium by the three-way ribotyping scheme

With the data from the analysis of the fragments generated by *HincII*, *SalI* and *PvuII*, an index of similarity between clonal lines was calculated and a dendrogram (Fig. 4) was constructed from the similarity matrix. The 19 clonal lines of Typhimurium showed genetic distance coefficients (*D*) between 0.16 and 0.64 and, at a significance level of 0.65, they were grouped into two major clusters (labelled A and B) each with two subclusters. Cluster A grouped 12 lines and cluster B grouped the other seven lines. All strains of Typhimurium appeared loosely related ($D = 0.78$) to the out-group strain 93/92 of Enteritidis.

Discussion

The evaluation of a typing system for epidemiological purposes should have established a series of attributes including: stability, typability, reproducibility, sensitivity or discriminatory power [11, 22]. Three features of

Fig. 3. Ribotypes generated by *PvuII* in Typhimurium. Lane A, lambda DNA cleaved with *PstI*: sizes of fragments (kb) from top to bottom are 14.17, 11.51, 5.08, 4.65, 4.5, 2.84, 2.58, 2.44, 2.14, 1.98 and 1.7. PE, ribotype pattern of Enteritidis strain 93/92.

the three-way ribotyping scheme reached 100% (stability, typability and reproducibility). With respect to sensitivity, the series under study was differentiated into 19 groupings or clonal lines with a $DI = 0.84$, which is less than that ($DI > 0.95$) proposed as optimum by Hunter and Gaston [20]; however, if the limited size of the present series and the limited geographic region from which it was collected are taken into account, a DI of 0.84 may be considered satisfactory.

Ribotyping may be considered the universal eubacterial genetic typing method, because it uses common probes as well as available equipment, reagents and supplies. However, for different *Salmonella* serotypes, different REs have been found to be most useful in producing interpretable ribotypes [6, 9, 23, 24] and the present study identified *HincII* alone, or in combination with *SalI* and *PvuII*, as the most useful RE in subtyping Typhimurium. *HincII* ribotyping of

Fig. 4. Single linkage dendrogram showing the results of cluster analysis on the basis of *HincII*, *SalI* and *PvuII* combined ribotypes of strains of Typhimurium. CL, clonal line; CRT, combined ribotype; D, genetic distance coefficient; S, sporadic; O, outbreak. The earliest strain showing the cited combined ribotype is given as the type strain. Major clusters, A and B, are shown at a significance level of 0.65. Clonal line XX is Enteritidis strain 93/92.

Typhimurium has been reported by Nastasi *et al.* [6]; when results from that and the present series were compared, ribotypes numbered 8, 5, 1-14, 6, 2 and 16 found by Nastasi *et al.* [6] among Italian isolates seemed identical or very similar to ribotypes H1, H3, H4, H7, H13 and H11, respectively, among Asturian strains. It should be pointed out, however, that the rates of the most frequent ribotypes in the two series were different.

It is noteworthy that the three REs generated some very well differentiated uncommon fragments in the rDNA banding patterns and in some cases would enable the deduction of possible genetic changes undergone, and so appear to offer a good tool for phylogenetic analysis, which could be used together with other markers, as previously described [7, 9]. It must also be emphasised that *HincII* recognised ambiguous DNA sequences (GT(T/C)|(A/G)AC) that included the *SalI* recognition sequence (GTCGAC) as a subsequence. This implies that the ribotypes generated with *HincII* include fragments of a smaller size than *SalI*-ribotypes and that the patterns from both REs are very different. The differences were also translated into the number of ribotypes and in the distribution of the strains generated by each RE.

A comparison between phage-typing and the three-way

ribotyping scheme revealed a higher typability and sensitivity associated with the latter and also revealed some relationships. The most frequent clonal lines included only strains of the most frequent PTs or strains that were phage untypable. However, the most frequent PTs (104 and 193) could be differentiated into four and five lines, respectively. It should also be noted that three PT96 strains analysed (isolated in different years) were the only ones that were ascribed to line VIII. In this sense, all the data presented support ribotyping as both a complementary and alternative tool to phage typing for epidemiological studies.

Data from *HincII*, *SalI* and *PvuII* ribotyping were used to elucidate the genetic relationships found among the clonal lines of Typhimurium, as well as for an approach to its molecular epidemiology in the Principality of Asturias, Spain. The results enabled the detection of genotypic differences that provided the differentiation of clinical organisms into 14 clonal lines showing a high degree of genetic heterogeneity. It should be noted that 59.71% of the Asturian strains belonged to the three most frequent lines (I, II and VII), which may be considered to be closely related and, differing only in their *HincII* ribotypes, fall into cluster A. Organisms ascribed to these lines may be considered as highly ubiquitous, because they were

collected from clinical, food and water samples in different Asturian geographic areas, in different years and were associated with both sporadic episodes of salmonellosis and five outbreaks. Line I also included four strains isolated from treated sewage water, data supporting its capacity for survival in that environment. The fourth and fifth most frequent lines (V and X with 8.82 and 7.35% of Asturian strains, respectively), were represented only by organisms causing sporadic episodes of salmonellosis; line V belonged to cluster A and line X to cluster B. Both lines were less related to each other, and to the three most frequent lines cited above, differing in the ribotypes generated with the three REs. The five lines cited, together with line VIII (4.41% of Asturian strains) might be considered as endemic in Asturias, because they included organisms circulating and causing human salmonellosis throughout recent years. Organisms of other lines, which appeared at a low frequency, seem to play a less important role in human illness.

All these data suggest that the proposed three-way ribotyping scheme is a useful molecular technique which may be used for different epidemiological purposes: in epidemiological surveillance in both retrospective and prospective studies; in the diagnosis and surveillance of outbreaks; in studies of the evolution of clones through time and space; and in the surveillance of pathogenic clones in natural environments. It may also be useful at the level of the individual patient, for the diagnosis-surveillance of bacterial translocation, just as for the surveillance of excretion of bacteria from infected patients or healthy carriers.

We thank the following: Dr M. Altwegg for pKK3535 plasmid; Dr A. Aladueña for serotyping and phage typing Typhimurium strains; Dr H-P. Kroll, Dr Wauters, Dra. C. Marne and ATCC for reference strains; and the Microbiology Laboratories of Hospital Central de Asturias, Oviedo, Hospital San Agustín, Avilés, Hospital de Cabueñes, Gijón Hospital Comarcal de Jarrío and Hospital Carmen and Severo Ochoa, Cangas del Narcea, for clinical isolates. This work was supported by grants from Oviedo University (no. 94/128-2) and Fondo de Investigación Sanitaria (no. 95/0030).

References

- Golberg MB, Rubin RH. The spectrum of *Salmonella* infection. *Infect Dis Clin North Am* 1988; **2**: 571-598.
- Pang T, Bhutta ZA, Finlay BB, Altwegg M. Typhoid fever and other salmonellosis: a continuing challenge. *Trends Microbiol* 1995; **3**: 253-255.
- Echeitia MA, Usera MA. Prevalence of *Salmonella* serotypes isolated in Spain from human and non human sources (1983-1987). *Microbiología* 1989; **5**: 95-103.
- Usera MA, Díez R, Echeitia A. Análisis de los serotipos de *Salmonella* sp aislados en España en el año 1994. *Boletín Epidemiológico Semanal* 1995; **3**: 165-172.
- Threlfall EJ, Frost JA. The identification, typing and fingerprinting of *Salmonella*: laboratory aspects and epidemiological applications. *J Appl Bacteriol* 1990; **68**: 15-16.
- Nastasi A, Mammina C, Villafrate MR. Epidemiology of *Salmonella typhimurium*: ribosomal DNA analysis of strains from human and animal sources. *Epidemiol Infect* 1993; **110**: 553-565.
- Stanley J, Baquar N, Threlfall EJ. Genotypes and phylogenetic relationships of *Salmonella typhimurium* are defined by molecular fingerprinting of IS:200 and 16s *rrn* loci. *J Gen Microbiol* 1993; **139**: 1133-1140.
- Hampton MD, Threlfall EJ, Frost JA, Ward LR, Rowe B. *Salmonella typhimurium* DT193: differentiation of an epidemic phage type by antibiogram, plasmid profile, plasmid fingerprint and *Salmonella* plasmid virulence (*spv*) gene probe. *J Appl Bacteriol* 1995; **78**: 402-408.
- Millemann Y, Lesage M-C, Chaslus-Dancla E, Lafont J-P. Value of plasmid profiling, ribotyping, and detection of IS200 for tracing avian isolates of *Salmonella typhimurium* and *S. enteritidis*. *J Clin Microbiol* 1995; **33**: 173-179.
- González-Hevia MA, Gutierrez MF, Mendoza MC. Diagnosis by combination of typing methods of a *Salmonella typhimurium* outbreak associated with cured-ham, an unusual infection source. *J Food Protec* 1996; **59**: 426-429.
- Pfaller MA. Typing methods for epidemiologic investigation. In: Balows A, Hausler WJ, Herrmann KL, Isenberg HD, Shadomy HJ (eds) *Manual of clinical microbiology*, 5th edn. Washington, D.C., American Society for Microbiology. 1991: 171-182.
- Stull TL, LiPuma JJ, Edlind TD. A broad-spectrum probe for molecular epidemiology of bacteria: ribosomal RNA. *J Infect Dis* 1988; **157**: 280-286.
- González-Hevia MA, Llaneza JJ, Mendoza MC. Usefulness of molecular genetic markers in the typing of *Salmonella enterica* serovar Enteritidis causing a food-borne outbreak. *Int J Food Microbiol* 1994; **22**: 97-103.
- Brosius J, Ullrich A, Raker MA *et al.* Construction and fine mapping of recombinant plasmids containing the *rrnB* ribosomal RNA operon of *E. coli*. *Plasmid* 1981; **6**: 112-118.
- Anderson ES, Ward LR, de Saxe MJ, de Sa JDH. Bacteriophage-typing designations of *Salmonella typhimurium*. *J Hyg* 1977; **78**: 297-300.
- Mak YM, Ho KK. An improved method for the isolation of chromosomal DNA from various bacteria and cyanobacteria. *Nucleic Acids Res* 1992; **20**: 4101-4102.
- Sambrook J, Fritsch EF, Maniatis T. *Molecular cloning: a laboratory manual*, 2nd edn. Cold Spring Harbor, NY, Cold Spring Harbor Laboratory Press. 1989.
- Mendoza MC, Alzugaray R, Landeras E, González-Hevia MA. Discriminatory power and application of ribotyping of *Yersinia enterocolitica* O:3 in an epidemiological study. *Eur J Clin Microbiol Infect Dis* 1996; **15**: 220-226.
- Karaolis DKR, Lan R, Reeves PR. Sequence variation in *Shigella sonnei* (Sonnei), a pathogenic clone of *Escherichia coli*, over four continents and 41 years. *J Clin Microbiol* 1994; **32**: 796-802.
- Hunter PR, Gaston MA. Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. *J Clin Microbiol* 1988; **26**: 2465-2466.
- Brunner H, Kroll HP. Reduced proliferative response of mouse spleen cells to mitogens during infection with *Salmonella typhimurium* or *Listeria monocytogenes*. *Microb Pathog* 1989; **6**: 365-276.
- Maslow JN, Mulligan ME, Arbeit RD. Molecular epidemiology: application of contemporary techniques to the typing of microorganisms. *Clin Infect Dis* 1993; **17**: 153-64.
- Esteban E, Snipes K, Hird D, Kasten R, Kinde H. Use of ribotyping for characterization of *Salmonella* serotypes. *J Clin Microbiol* 1993; **31**: 233-237.
- Landeras E, Gonzalez-Hevia MA, Alzugaray R, Mendoza MC. Epidemiological differentiation of pathogenic strains of *Salmonella enteritidis* by ribotyping. *J Clin Microbiol* 1996; **34**: 2294-2296.