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PCR Ribotyping for Characterizing *Salmonella* Isolates of Different Serotypes

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The 16S-23S intergenic spacer region in 218 strains of *Salmonella* isolated from four Italian hospitals during the period from 1977 to 1994 was analyzed by PCR ribotyping. This molecular typing technique allowed for the identification of seven different and specific electrophoretic profiles for the seven serovars *S. enteritidis*, *S. london*, *S. anatum*, *S. panama*, *S. heidelberg*, *S. agona*, and *S. goldcoast*. Otherwise, the spacer region appears to be polymorphic in *S. typhimurium*, *S. infantis*, and *S. derby* since we could identify eight, six, and four different ribotypes, respectively.

All strains of *Salmonella* appear to be at least 70% related to each other by DNA hybridization (6) and may therefore be considered members of a single genospecies (16). Nevertheless, for historical reasons, this important human and animal pathogen is currently described as a genus in the family *Enterobacteriaceae*. Its classification inside the genus is based on the Kauffmann-White serotyping scheme. More than 2,300 serotypes have been described on the basis of the antigenic structure of the cell surface lipopolysaccharide (O antigen) and flagellar proteins (H antigen). The ability of *Salmonella* isolates to transfer, acquire, and recombine the genes for phase I flagellin (*fliC*) and for antigen O (*rfb* cluster genes) explains the existence of such a large number of serotypes, but it also represents a major limit for the serological scheme. In fact, phylogenetic studies, and in particular, multilocus enzyme analysis, demonstrate that strains in the same serovar may be distantly related (4, 5, 15, 20).

Intraserovar differentiation is essentially designed for epidemiological investigations during outbreaks, and for a long time it was carried out by classic techniques, such as phage typing, biotyping, resistotyping, and plasmid profiling (26).

Modern typing methods are based on genotype characterization; moreover, most of them appear to be suitable for studying both population genetic structure and evolutionary relationships within the genus. Fingerprinting of rRNA coding sequences, i.e., ribotyping (1, 3, 10, 18, 19), and hybridization with oligonucleotide probes (7) have been used to type different *Salmonella* isolates, but the most common molecular approach has been IS*200* fingerprinting, which was used for intraserovar discrimination of many *Salmonella* serotypes, i.e., *S. enteritidis* (19, 25), *S. typhimurium* (2, 3, 22), *S. heidelberg* (23), *S. berta* (24), *S. typhi* (27), *S. paratyphi* B, and *S. java* (11).

However, both IS*200* fingerprinting and ribotyping are complex techniques, since they require several crucial steps which are beyond the technical ability of many laboratories. A more promising and simpler technique seems to be PCR ribotyping, which is based on the amplification of the spacer sequences between the 16S and 23S genes in the rRNA transcriptional units (14). rRNA loci are present in 2 to 11 copies on the chromosomes of most bacterial species. Whereas a high degree of sequence homology exists for rRNA genes, the intergenic spacer regions show extensive sequence and length variations which can be used to characterize bacteria at the genus (13), species (9, 13), and subspecies (8, 14) levels.

In the present work we investigated the possibility of differentiating *Salmonella* strains both at the serotype level and at the intraserovar level on the basis of the polymorphism of the 16S-23S rRNA intergenic spacer region.

MATERIALS AND METHODS

Bacterial strains. The strains of *Salmonella* included in the present study (Table 1) were isolated during the period from 1977 to 1994 from patients with diarrhea and from food of poultry origin in four Italian laboratories (Trieste, Udine, Rome, and Padua).

Isolation was carried out by standard procedures, and identification was achieved through biochemical and serological characterization. All the strains were grown on Luria-Bertani (LB) medium and were stored frozen at −40°C in 8.75% (vol/vol) dimethyl sulfoxide.

DNA extraction. Three milliliters of an overnight culture of each isolate in LB broth were centrifuged, washed once in 10 mM Tris-HCl–5 mM EDTA buffer (pH 8), and resuspended in 0.5 ml of the same buffer containing 0.3 mg of lysozyme per ml, and the mixture was incubated at 37°C for 1 h. After the addition of sodium dodecyl sulfate (0.5%) and proteinase K (100 μg/ml), the samples were incubated again at 55°C until the solution became clear. DNA was purified by two extractions with phenol-chloroform-isoamyl alcohol (25:24:1) and was collected by precipitation with ethanol. The DNA was resuspended in 10 mM Tris-HCl–1 mM EDTA buffer (pH 8) and was stored at 4°C until it was used. Phenol extraction may be omitted and substituted by the less laborious treatment at 95°C for 10 min in order to inactivate the proteinase K. The DNA samples extracted by the two methods gave, in our hands, identical results.

DNA amplification. Oligonucleotide primers complementary to the conserved regions of the 16S and 23S rRNA genes were synthesized on a Millipore Cyclone Plus apparatus. The sequences of P1 (5′-TTGTACACACCGCCCGTCA-3′) and P2 (5′-GGTACTTAGATGTTTCAGTTC-3′) have previously been described by Kostman et al. (14). Amplification was performed in a 100-μl volume and contained 10 mM Tris-HCl (pH 8.3); 50 mM KCl; 200 μM (each) dATP, dGTP, dCTP, and dTTP; 0.5 μM (each) primer; 3 mM $MgCl_2$; 2.5 U of *Taq* polymerase; and about 100 ng of target DNA. The amplification mixtures were submitted to 35 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C. These conditions were chosen on the basis of preliminary experiments performed to optimize amplification of *Salmonella* target sequences. Each amplification included a blank, which contained all reagents but not the target DNA. The amplification products were separated by gel electrophoresis in 4% agarose gels (NuSieve 3:1; FMC BioProducts, Rockland, Maine), stained with ethidium bromide, and photographed. Analysis of the patterns was performed by visual inspection of negative films on a white light transilluminator. Samples showing similar patterns were run in the same gel for direct comparison. Two isolates were said to have the same electrophoretic profile when their band patterns were identical. Minor differences in band intensity were not considered.

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TABLE 1. Origins of the strains of *Salmonella* considered in the present work

Serotype	No. of strains from:				Total no. of strains
	Trieste	Udine	Rome	Padua	
S. agona		14	15		29
S. enteritidis	30		6	5	41
S. panama		5	17		22
S. anatum	3	6	1		10
S. goldcoast	4	1	4		9
S. london		5	16		21
S. heidelberg		5	13		18
S. derby	1	16	5		22
S. infantis	3	9	6		18
S. typhimurium	5	16	7		28

RESULTS

In the present work we characterized 218 strains of *Salmonella* belonging to 10 different serotypes (Table 1) by a PCR ribotyping procedure which allowed for the identification of 16S-23S rRNA intergenic spacer region profiles.

Electrophoretic analysis of the amplified products disclosed four to eight bands ranging from 700 to 1,100 bp in each sample (Fig. 1 and 2). Some of the bands appeared weak, consisting probably of secondary products. However, this was not considered a problem because, as already observed and discussed for PCR ribotyping of *Staphylococcus aureus* (8), the electrophoretic patterns were very constant and reproducible. Indeed, when PCR ribotyping was performed more than once with the same isolate, identical patterns were always obtained. This was true also when, to evaluate stability, three isolates were tested before and after 10 serial subcultures (data not shown).

For each of the serovars *S. agona*, *S. enteritidis*, *S. panama*, *S. anatum*, *S. goldcoast*, *S. london*, and *S. heidelberg* the procedure allowed for the identification of a single and specific spacer region profile. All strains belonging to these serotypes, even when they were isolated at different times and places, showed amplification products with characteristic electrophoretic patterns, as reported in Fig. 1.

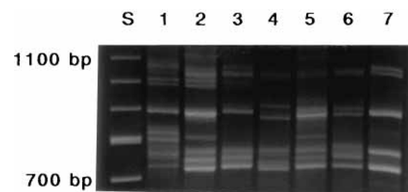

FIG. 1. Representative electrophoretic patterns of PCR ribotypes of *Salmonella* strains. Lanes: S, 100-bp ladder; 1, *S. goldcoast*; 2, *S. agona*; 3, *S. heildelberg*; 4, *S. panama*; 5, *S. anatum*; 6, *S. london*; 7, *S. enteritidis*. Ten microliters of the postamplification reaction mixture was run in a 4% agarose gel.

In a few cases we found electrophoretic patterns slightly different from those of the other strains within a serotype. We did not categorize these profiles as new ribotypes since the differences were only due to the intensity of one of the bands rather than its presence or position; moreover, it was found only in one strain each of *S. goldcoast* and *S. london* and in two strains of *S. heidelberg* (data not shown).

On the other hand, each one of the three serovars *S. derby*, *S. infantis*, and *S. typhimurium* exhibited more than one PCR ribotype. As shown in Fig. 2, we identified four different spacer region profiles for *S. derby*, six for *S. infantis*, and eight for *S. typhimurium*. The distributions of the PCR ribotypes among the strains of the various serovars and the geographic regions where they were isolated are summarized in Table 2. The most frequent patterns for *S. derby* were d1 (45%) and d2 (40%); together, they characterized the great majority of the strains in this serovar. Profile i3 (55%) was predominant among *S. infantis* strains. The profile distribution among strains of *S. typhimurium* appeared to be more heterogeneous; in fact, besides the presence of the higher number of patterns, the most frequent one identified only 37.7% of the *S. typhimurium* strains.

No relationship was found between the PCR ribotype and the geographic origin of the strains.

TABLE 2. Distributions of *Salmonella* strains among different PCR ribotypes and hospitals

Serotype	PCR ribotype	No. of strains from hospitals in:		
		Udine	Rome	Trieste
S. derby	d1	8	2	0
	d2	6	2	1
	d3	0	1	0
	d4	2	0	0
S. infantis	i1	1	0	0
	i2	1	0	0
	i3	5	5	0
	i4	0	0	1
	i5	2	0	2
	i6	1	0	0
S. typhimurium	t1	8	2	0
	t2	2	0	0
	t3	0	0	1
	t4	4	2	1
	t5	1	3	0
	t6	1	0	0
	t7	0	0	2
	t8	0	0	1

DISCUSSION

Two regions can be distinguished in 16S-23S transcriptional units: the highly conserved genes for rRNA and the sequences of the intergenic spacer region. Genes for rRNA are so conserved that the same primers can recognize sequences in most bacteria. On the other hand, the variability observed within the spacer could assume very diverse significance. For instance, each one of the numerous species included in the genus *Acinetobacter* has its own specific spacer profile (9). At variance, the spacer in *S. aureus* exhibits such a high degree of polymorphism that it could be regarded as an excellent target for subspecies typing. Dolzani et al. (8) found 22 different spacer region profiles among 74 randomly collected strains of *S. aureus* and suggested that the procedure be applied as a molecular biology-based approach for epidemiological investigations.

The spacer configuration in *Salmonella* strains somehow seems to be different from that in *Acinetobacter* species (9) and far different from that in *S. aureus* (8). Characterization of 218 strains of *Salmonella* demonstrated that most of the analyzed serovars (7 of 10) exhibited a specific PCR ribotype; consequently, in most cases, clustering either by spacer analysis or by serological procedures was consistent. On the other hand, we found serovars (3 of 10) with more than one spacer region profile, so that different PCR ribotypes could be identified

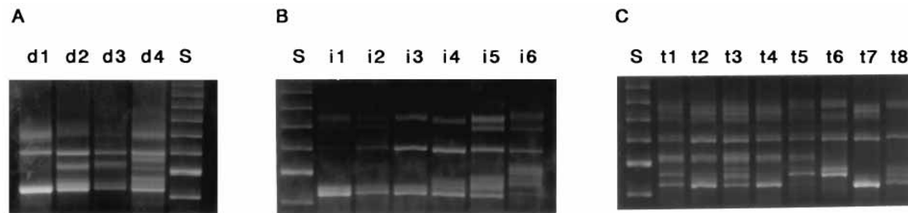

FIG. 2. Electrophoretic patterns of PCR ribotypes of *S. derby* (A), *S. infantis* (B), and *S. typhimurium* (C). Lanes: 1 to 8, electrophoretic patterns of the corresponding PCR ribotypes; S, 100-bp ladder (the more intense band corresponds to 800 bp). Ten microliters of the postamplification reaction mixture was run in a 4% agarose gel.

among strains which shared the same antigenic pattern. In other words, different serotypes of *Salmonella* were always characterized by different spacer region profiles, but the same profile was not always found in all strains of the same serotype.

Strains of *S. enteritidis* are invariably characterized by a single ribotype, even though they were collected in different regions of Italy and over a long period of time (1977 to 1994). The distinctive and constant organization of the spacer was, to some extent, unexpected in *S. enteritidis*, which is described as a polyphyletic serovar on the basis of multilocus enzyme electrophoresis, a technique which measures chromosomal genotypic diversity and the evolutionary relationship among different isolates (5). On the other hand, Gruner et al. (12) demonstrated, by analysis of the rRNA restriction patterns, that 41 randomly collected strains of *S. enteritidis* had a single ribotype; moreover, Stanley and Baquar (21) described for this serovar a high degree of genotypic homogeneity. In our hands, spacer region analysis appeared to be able to identify transcriptional units common to all the strains of *S. enteritidis* tested, and these units have apparently remained unchanged over time and probably originated from a single ancestor.

In addition, *S. derby* and *S. infantis*, described as polyphyletic serovars (5), exhibited more than one spacer region arrangement. This variability could arise by more profound modifications which might have occurred during phylogenesis and which apparently involved the whole genome, also including the rRNA transcriptional units. For these two serovars, PCR ribotyping probably recognized sequences which were derived not from their first ancestors but rather from their descendants. Thus, evaluation of the genetic relationship among the strains of the two serovars grouped in new PCR ribotyping clusters should prove to be very interesting.

S. typhimurium is described as monophyletic (5), since multilocus enzyme electrophoresis demonstrated that strains in this serovar are closely related to each other. Nevertheless, PCR ribotyping revealed the presence of different spacer region profiles. Analogous results were also obtained by the same technique by Nastasi and Mammina (17). At present we do not know how relevant these differences are and if they could correlate with the slight variations observed by multilocus enzyme electrophoresis analysis. It would probably be advantageous for us to compare the sequences of the spacer regions in the different *S. typhimurium* ribotypes.

As mentioned above, the possibility that *Salmonella* strains might exchange phase I flagellin and antigen O can, sometimes, cast doubt on the serological identification. Because of the high degree of specificity of spacer region profiles in *S. enteritidis*, *S. agona*, *S. panama*, *S. goldcoast*, *S. london*, and *S. heildelberg*, 16S-23S spacer region analysis could be very useful in confirming serological identification, in particular during outbreaks.

Nonetheless, PCR ribotyping can be considered a good technique for subtyping strains of *S. derby*, *S. infantis*, and *S. typhimurium*. It is rapid, easy to perform, and reproducible and has a good discriminatory power, so that it should be useful in epidemiological studies instead of (or together with) other, more complex subtyping techniques. Furthermore, we propose that PCR ribotyping is a method that can be used to identify within a serovar strain clusters more appropriate for subsequent phylogenetic investigations.

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