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RESEARCH ARTICLE

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Rapid identification of *Salmonella typhimurium*, *S. enteritidis* and *S. virchow* isolates by Polymerase Chain Reaction based fingerprinting methods

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Summary In this study we used and evaluated three rapid molecular typing methods for the identification of three frequent, clinically significant *Salmonella* serovars on the basis of the ease, simplicity and reproducibility of the chosen methods. We determined the genetic diversity among several isolates of *Salmonella enteritidis*, *S. typhimurium* and *S. virchow*, and compared them with other enterobacteria by using the repetitive extragenic palindromic (REP) sequences, the enterobacterial repetitive intergenic consensus (ERIC) sequences, and the 16S–23S rDNA intergenic spacer region (ITS1). The objective was to evaluate their potential application to discriminate among members of the species *Salmonella enterica* subspecies *enterica* using the genetic diversity of the group found by genomic fingerprinting. The three different serovars of *Salmonella* studied gave reproducible and distinguishable profiles using whichever of the above mentioned polymerase chain reaction (PCR) methods assayed. The conserved patterns in each serovar allowed for easy differentiation from other serovars of *Salmonella*.

Key words *Salmonella* · 16S–23S rDNA spacer region · Enterobacterial repetitive intergenic consensus (ERIC) · Molecular typing · Repetitive extragenic palindromic (REP) sequences

Introduction

Salmonella is a genus of Gram-negative rod-shaped bacteria of the family Enterobacteriaceae. They cause a wide range of human diseases such as enteric fever, gastroenteritis and bacteremia. Gastroenteritis associated with food-borne outbreaks is probably the most common clinical manifestation of the infection [1, 2, 9, 11, 25]. The taxonomy of *Salmonella* has been controversial. Typically new isolates were named after the derived disease (or host) or after the name of the place where they were initially found. Following the scheme of Le Minor [21], enterobacteria of the genus *Salmonella* belong to a single species, *Salmonella enterica*, and seven subspecies have been recognized applying biochemical tests: I, II, IIIa, IIIb, IV, V, and VI [22].

Strains of *Salmonella* sp. are classified into serovars in accordance with the Kauffmann–White scheme [17], which gives serovar status to each antigenic type on the basis of wide diversity observed in somatic (O), capsular (Vi) and the flagellar (H) antigens. The most frequently isolated serovars of epidemiological importance belong to *Salmonella* serovar *enteritidis* (abbreviated hereafter to *S. enteritidis*), *Salmonella* serovar *typhimurium* (abbreviated hereafter to *S. typhimurium*), and *Salmonella* serovar *virchow* (abbreviated hereafter to *S. virchow*), and are members of named serovars of subspecies I [22–24, 31].

Established conventional methods to detect and identify *Salmonella* require selective enrichment and plating followed by biochemical tests. The diagnostic value of biochemical traits is generally combined with serological characterization, resistance to antibiotics, plasmid profiling, and phage-typing assays. The whole process requires several days and is likely to be replaced by molecular techniques [10]. In this study we wanted to use and evaluate three molecular typing methods on the basis of their ease, simplicity and reproducibility. We

determined the genetic diversity among several isolates of *Salmonella enteritidis, S. typhimurium* and *S. virchow*, and compared them with other enterobacteria by repetitive-element-sequence polymerase chain reaction (rep-PCR) [30] by using either repetitive extragenic palindromic (REP) sequences [26] or enterobacterial repetitive intergenic consensus (ERIC) sequences [15]. Furthermore, we applied a PCR identification method based on the 16S–23S rDNA intergenic spacer region (ITS1) which can be used to amplify regions between the 16S and 23S genes of the rRNA genetic loci [14].

Materials and methods

Bacterial strains The strains of *Salmonella* used in this work, and their sources, are included in Table 1. All strains were isolated at different times during 1995 from contaminated food and patients in hospitals. Most of the cases did not involve outbreak association. Samples were diluted and/or homogenized in TSB medium, and isolates obtained by *Salmonella* selective enrichment in Rappaport–Vassiliadis (RV) [29] medium after 24 h incubation at 43°C. Isolates were grown on

Table 1. *Salmonella* isolates and other Enterobacteriaceae used in this study.

Species / Strain (Source)	Antibiotic Resistance	Locality
Salmonella arizona:		
1749 (human[2])	Te^r, Sxt^r	Palma de Mallorca
Salmonella derby:		
1169 (meat)	S	Ibiza
Salmonella enteritidis:		
968 (human[2])	Amp^r	Palma de Mallorca
1103 (pork sausage); 1672, 1675, 1677, 1679 (human[2])	S	Palma de Mallorca
1671, 1673 (human[2])	Amp^r, Cr^r; Cb^r	Palma de Mallorca
1689, 1690, 1691 (human[1])	S	Ciudadela, Menorca
Salmonella typhimurium:		
869 (food)	S	Palma de Mallorca
999 (chicken); 1001, 1064 (water)	S	Inca, Mallorca
1169 (meat)	S	Ibiza
1498 (fish); 1530 (hamburger); 1606, 1627 (human[2])	S	Palma de Mallorca
953 (chicken)	Amp^r; Cb^r,Gm^r	Inca, Mallorca
963 (chicken)	Cr^r	Inca, Mallorca
1024 (egg)	Cr^r	Palma de Mallorca
964 (pork sausage); 1382 (sobrasada); 1710 (human[2])	Te^r	Palma de Mallorca
1584 (human[2])	Amp^r; Cb^r, Sxt^r, Te^r	Palma de Mallorca
554, 1586, 1588, 1602, 1612, 1619, 1670, 1701, 1704, 1706, 1708, 1713, 1720, 1735 (human[2])	MR	Palma de Mallorca
1085 (pork sausage)	MR	Ibiza
1714, 1728, 1743 (human[2])	S	Palma de Mallorca
1749 (human[2])	Sxt^r, Te^r	Palma de Mallorca
Salmonella virchow:		
618 (human[4]); 622 (human[2])	MR	Palma de Mallorca
811 (human[2])	S	Palma de Mallorca
868, 874, 881, 887 (chicken)	S	Ibiza
904 (chicken)	S	Inca (Mallorca)
1028 (sea water)	S	Alcudia (Mallorca)
1716 (human[2])	Amp^r; Cr^r; Cb^r, Sxt^r, Te^r	Palma de Mallorca
1727 (human[2])	Amp^r	Palma de Mallorca
Escherichia coli 7/49 (human[3])		
Citrobacter freundii CECT 401		
Enterobacter aerogenes CECT 684		
Enterobacter cloacae CECT 194		
Klebsiella pneumoniae K7 774		
Proteus mirabilis DSMZ 4479		
Proteus vulgaris CECT 484		
Shigella dysenteriae CECT 584		
Shigella flexneri CECT 585		
Yersinia enterocolitica DSMZ 4780		

Notes: CECT = Colección Española de Cultivos Tipo, Valencia, Spain. DSMZ = Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany. The clinical isolates were obtained from [1]Clinica Salus (Mahon, Menorca), [2]Hospital of Son Dureta (Palma de Mallorca), [3]Hospital Universidad Sevilla (Sevilla, Spain), and [4]Majadahonda (Madrid, Spain). The food isolates were obtained periodically from different shopping centres, and hotels. Amp = ampicillin. C = chloramphenicol. Cr = cephalothin. Gm = gramicidin. Te= tetracycline. Sxt = clotrimoxazol. MR = resistant to ampicillin, carbenicillin, chloramphenicol, and tetracycline. ^{r}= resistant. S = Sensitive to ampicillin, amoxicillin, carbenicillin, cephalothin, chloramphenicol, gramicidin, clotrimoxazol, tetracycline and norfloxacin.

Table 2. Description of primers used

Primer pair*	Sequence 5' → 3'	Length (nucleotides)
REP		
REP-A	TCM GGC CTA C	10
REP-B	GNC ATC MGG C	10
ERIC		
ERIC1R	ATG TAA GCT CCT GGG GAT TCA C	22
ERIC2	AAG TAA GTG ACT GGG GTG AGC G	22
rrn		
rrn16S	GAA GTC GTA ACA AGG	15
rrn23S	CAA GGC ATC CAC CGT	15

*See text for description.
A, adenine; G, guanine; T, thymine; C, cytosine; M, adenine or citosine; N, adenine, citosine, guanine or thymine.

Xylose–Lysine–Deoxycholate (XLD) medium, for isolation of enteric pathogens [27]. Media included a H_2S indicator, and incubation was extended to 48h to increase visibility of H_2S production. Presumptive *Salmonella* isolates were grown in Selenite–Cystine [3] broth at 37°C for 24 h. *Salmonella–Shigella* (SS) agar plates were inoculated from positive orange tubes and incubated at 37°C for 24–48 h. Colorless non-lactose-fermenter colonies were used to inoculate Kligler's iron agar tubes [18, 19], and incubated at 37°C for 24 h. Alkaline/acid, H_2S, with/without gas isolates were tested on Christensen's urea agar [8] for the detection of urease activity after 24 h incubation at 37°C. Differentiation between *Salmonella* subspecies was made using biochemical tests [21]. The serotyping of *Salmonella* strains followed the Kauffmann–White scheme [17]. Dispens-O-Disc, susceptibility test system (Difco Laboratories) was used to observe antibiotic resistance. All strains were preserved in Luria–Bertani broth amended with 15% glycerol and stored at –70°C.

Genomic DNA extraction DNA was isolated from late-exponential phase cells by lysis with sodium dodecyl sulfate (SDS)–proteinase K and treatment with cetyltrimethylammonium bromide (CTAB) [32]. The DNA concentration was determined by measuring the absorbance at 260 nm and adjusted to 1 µg/µl with sterile-filtered water. A rapid method to isolate DNA from bacterial colonies was also used. Colonies of at least 1 mm diameter from each isolate were individually picked from the culture plates. Bacterial cells were transferred into microfuge tubes containing 100 µl suspension of 5% Chelex-100 sodium form (100–200 mesh) in sterile TE (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]) buffer. Samples were vortex-mixed, boiled for 15 min, and then centrifuged for 5 min at 12,000 ×g. The supernatant was stored at 4°C for further analysis. One microliter was added to the PCR assay.

PCR primers and amplification conditions The REP, ERIC and ITS1 primers used are described in Table 2. The REP-A and REP-B primers were designed from the ten nucleotides of the conserved ends of the palindrome consensus REP sequences in opposite orientations described in detail previously by Versalovic et al. [30]. At ambiguous positions in the REP consensus sequence (positions where degeneracy must be considered), REP-A and REP-B primers contained multiple nucleotides (A, C, G, or T). The ERIC primers were designed as described elsewhere [30]. Finally, the ITS1 primers were designed from conserved areas of aligned rRNA bacterial sequences [16]. We used two highly-conserved target sequences [20], primer rrn16S at the 3'-end of 16S rRNA (position 1491–1505) [6], and primer rrn23S near the 5'-end of 23 rRNA (position 21–35), following the *Escherichia coli* numbering [5]. All the oligonucleotide primers were synthesized on a Pharmacia Gene Assembler Plus (Pharmacia Biotech, Barcelona, Spain).

PCR reactions were carried out in a 100-µl reaction volume, overlaid with sterile mineral oil. The PCR reaction mixture consisted of 10 mM Tris-HCl (pH 9.0)–1.5 mM $MgCl_2$–50 mM KCl, 200 µM each of the four deoxynucleotide triphosphates (Boehringer-Mannheim, Barcelona, Spain), 0.5 µM of each of the two opposing primers, 100 ng of genomic DNA or 1 µl supernatant of the boiled whole-cell suspension as template bacterial DNA, and 2.5 U of *Taq* DNA polymerase (Pharmacia Biotech, Barcelona, Spain). A biphasic PCR was run in REP-PCR. The first step consisted of two cycles with the following profile: 94°C, 5 min; 33°C, 5 min; 68°C, 5 min. The REP-PCR was completed by 30 cycles of denaturation (94°C, 1 min), annealing (45°C, 1 min), and extension (68°C, 2 min), followed by a single final extension (68°C, 16 min). ERIC- and ITS1-PCR amplifications were performed by 30 cycles running under the following profile: denaturation at 94°C for 1 min, primer annealing (ERIC primers, 45°C, 1 min; ITS1 primers, 52°C, 1 min), and DNA extension at 72°C for 2 min. A final elongation step at 72°C for 10 min was carried out. Ten microliters of amplified DNA were analyzed in 1.5% (w/v) NuSieve (FMC Bioproducts) agarose gel electrophoresis for REP- and ERIC-PCR products; or 3% (w/v) for ITS1-PCR products. The sizes

of the fragments produced in the amplifications were calculated from their positions relative to the positions of the molecular weight markers.

Results

The PCR products obtained from whole cells without previous isolation and purification of DNA were less visible or reproducible than those obtained from isolated and purified DNA. Reproducibility was evaluated in terms of duplicate tests within a single PCR run, and between runs of duplicate DNA preparations from the same strain. Therefore all amplification reactions were performed with isolated DNA. After optimization of the PCR variables for each set of primers, PCR patterns from isolated DNA were repetitive for each species and strain with REP, ERIC, and ITS1 based primers (data not shown). Clear genomic patterns were obtained for all *Salmonella* and enterobacterial strains. The three PCR-based typing methods were applied to all the isolated strains and their patterns were digitalized and stored in an image database. These upgrowing database has been used to recognize successfully new unknown isolates for classification of controversial strains not readily differentiated by biochemical and serotyping tests.

REPs Reproducible profiles on agarose gels were obtained from amplified fragments using 10-mer primers REP-A and REP-B under defined conditions (Fig. 1). A biphasic PCR with a first step consisting of two cycles with long times of denaturation, annealing, and extension, and annealing at low temperature (33°C) resulted in an optimal amount of PCR product with REP primers. We compared these profiles and found identical fingerprints for strains included in the same serovar of *S. enteritidis* and *S. typhimurium*, and similar but non-identical patterns for *S. virchow* isolates. These patterns were easily distinguishable from those obtained in non-*Salmonella* strains, and their interpretation was not complicated (Fig. 1A and 1B). Comparative analyses of fingerprints generated by REP-PCR allow us also to distinguish strains belonging to different serovars. Besides, all strains identified as *S. enteritidis*, *S. typhimurium*, and *S. virchow* present at least one bright common 1.8 kpb band (Fig. 1 B). A second band (≈0.8 kpb) seems to be also common to the three serovars, although it is weak in *S. typhimurium*. *S. typhimurium* and

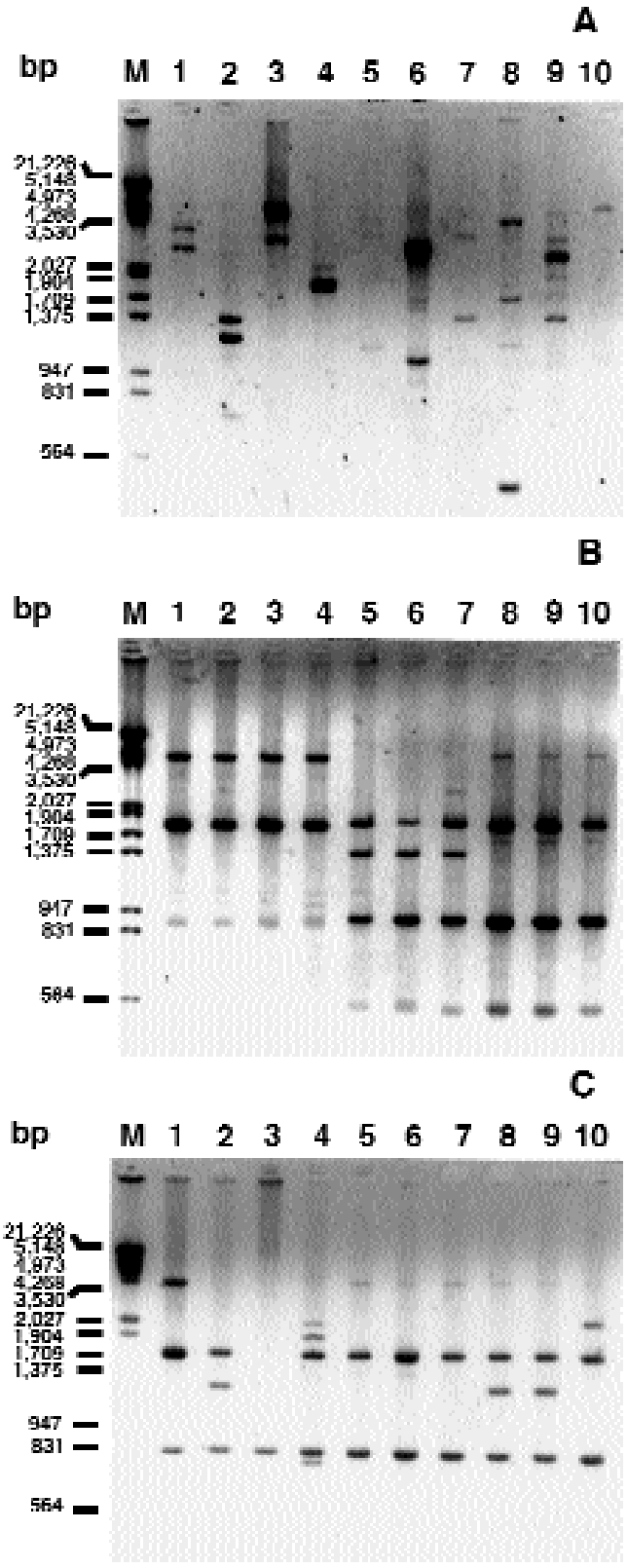

Fig. 1 DNA fingerprints generated by REP-PCR products (A, B) on agarose gels (1.5%). Lanes are as follows; gel A: 1, *Enterobacter cloacae* CECT 194; 2, *Escherichia coli* 7/49; 3, *Proteus vulgaris* CECT 484; 4, *Yersinia enterocolitica* DSMZ 4780; 5, *Klebsiella pneumoniae* K7 774; 6, *Citrobacter freundii* CECT 401; 7, *Proteus mirabilis* DSMZ 4479; 8, *Enterobacter aerogenes* CECT 684; 9, *Shigella flexneri* CECT 585; 10, *Shigella dysenteriae* CECT 584; gel B: 1, *Salmonella typhimurium* 554; 2, *S. typhimurium* 1708; 3, *S. typhimurium* 1085; 4, *S. typhimurium* 869; 5, *Salmonella enteritidis* 1689; 6, *S. enteritidis* 968; 7, *S. enteritidis* 1671; 8, *Salmonella virchow* 874; 9, *S. virchow* 881; 10, *S. virchow* 1727; gel C: 1, *S. typhimurium* 1708; 2, *S. enteritidis* 1689; 3, *Salmonella darwin* 1169; 4, *Salmonella arizona* 1749; 5, *S. virchow* 874; 6, *S. virchow* 868; 7, *S. virchow* 1727; 8, *S. virchow* 618; 9, *S. virchow* 622; 10, *S. virchow* 1727. M, molecular marker (lambda digested with *Eco*R I and *Hin*dIII)

S. virchow shared the highest molecular weight band (≈5.0 kpb). In contrast, *S. enteritidis* and *S. virchow* have in common a ≈0.5 kpb band, weak but constant in the fingerprints of those serovars. Specific bands can be seen in both *S. enteritidis* (bright band, 1.4 kpb) and *S. virchow* (weak band, 1.6 kpb).

Contrasting fingerprints of strains of *S. enteritidis* and *S. typhimurium* serovars did not allow clear differentiation of strains of the same serovar, with some unique exceptions (data not shown): strain 964 (Te^r) of *S. typhimurium* showed a double band in the upper range instead of the characteristic single band (5.0 kpb), and another band of 2.5 kpb. The multidrug resistant strain 1701, showed a 0.4 kpb band (different to the 0.5 kpb band observed in *S. enteritidis*). Finally, the 5.0 kpb upper band characteristic in *S. typhimurium* was missing in strains 1064, 1704, 1706, and 1498 of *S. typhimurium*. The correlation of the REP fingerprints of these strains with the REP fingerprint of *S. enteritidis* was low since the expected specific bands for this serovar: 1.4 and 0.5 kpb, were also missing.

Variations were observed in isolates of *S. virchow*. Although strains belonging to this serovar exhibited also reproducible and conserved bands, additional and polymorphic bands were detected in correlation with the antibiotic resistance spectra (Fig. 1C). The isolates of *S. virchow* sensitive to drugs did not show the additional band present in multidrug-resistance *S. virchow* isolates. Furthermore the only ampicillin-resistant *S. virchow* strain 1727 observed had an additional band absent in the other strains but it lacked the characteristic band observed in multidrug resistant isolates.

ERICs Amplified polymorphic PCR patterns generated by using ERIC1R and ERIC2 primers were also obtained for all strains included in this study (Fig. 2). With this primer set, normal PCR cycling conditions of 30 cycles were applied, which always resulted in enough DNA signal to allow fingerprinting diagnostic. Analyses of these fingerprints indicate again that this technique can effectively distinguish between *Salmonella* strains representing different serovars and other enterobacteria . Although the fingerprints obtained by ERIC-PCR were more complex, in terms of the number of bands found per sample (i.e. in *Salmonella* fingerprints 15–19 bands were found, including the weaker bands), the patterns provided were not more discriminatory than those obtained when REP-based. The results obtained also suggested that there should not be correlation between drug resistance and the type of fingerprint, because no extra bands were seen for strains of the same serovar, independently of their drug resistance (Fig. 2B).

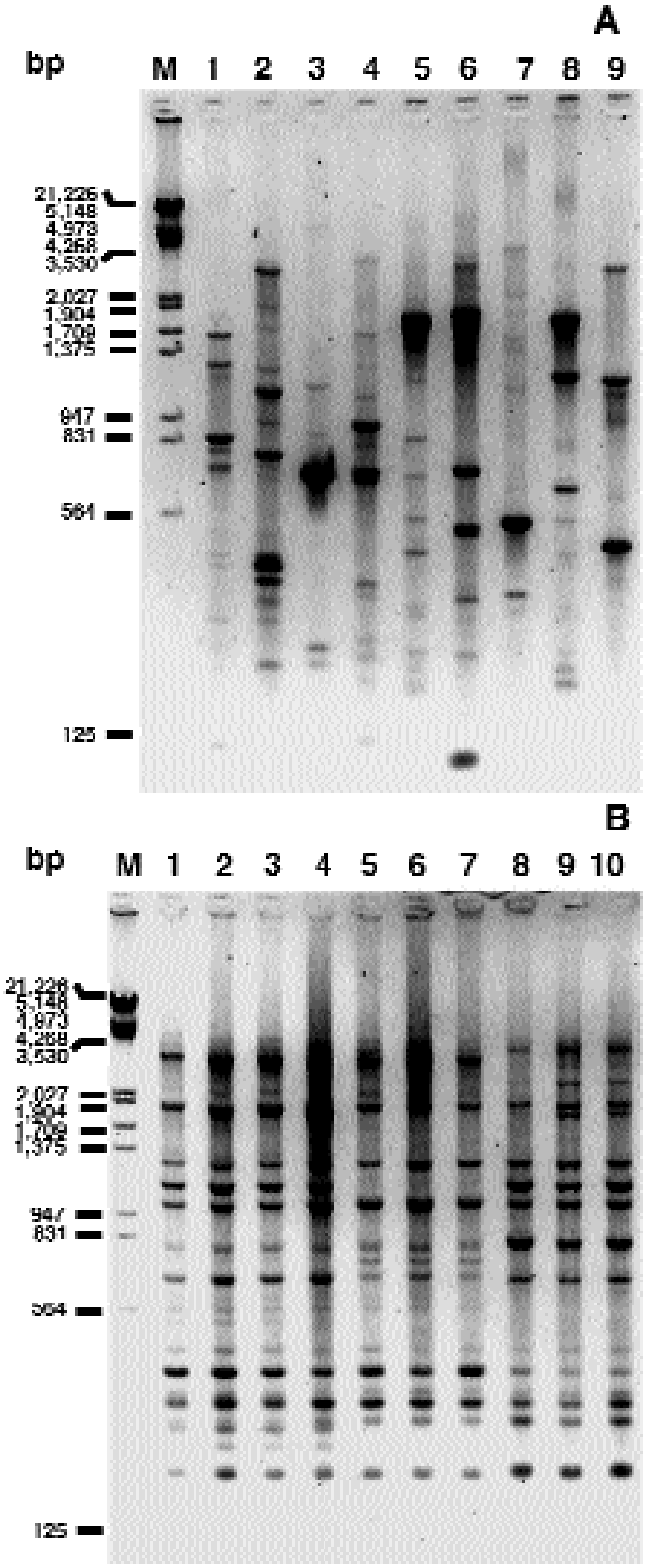

Fig. 2 Composite photographs of gels showing fingerprints generated by ERIC-PCR products (A, B) on agarose gels (1.5%). Lanes are as follows; gel A: 1, *Enterobacter cloacae* CECT 194; 2, *Escherichia coli* 7/49; 3, *Proteus vulgaris* CECT 484; 4, *Yersinia enterocolitica* DSMZ 4780; 5, *Klebsiella pneumoniae* K7 774; 6, *Citrobacter freundii* CECT 401; 7, *Proteus mirabilis* DSMZ 4479; 8, *Enterobacter aerogenes* CECT 684; 9, *Shigella flexneri* CECT 585; gel B: 1, *Salmonella typhimurium* 554; 2, *S. typhimurium* 1708; 3, *S. typhimurium* 1085; 4, *S. typhimurium* 869; 5, *Salmonella enteritidis* 1689; 6, *S. enteritidis* 968; 7, *S. enteritidis* 1671; 8, *Salmonella virchow* 874; 9, *S. virchow* 881; 10, *S. virchow* 1727. M, molecular marker (lambda digested with *Eco*RI and *Hin*dIII)

Therefore, three different types of fingerprints were obtained and these correlated with the serovar arrangements of the strains studied. *S. typhimurium* and *S. virchow* showed the most similar fingerprints and only a few bands allowed to distinguish both groups of strains. *S. enteritidis* fingerprints were clearly different from those exhibited by *S. typhimurium* and *S. virchow*. Two bands, one absent in *S. enteritidis* and the other present only in *S. enteritidis* offer a true discriminatory argument.

In the ERIC-PCR based profiles we found some deviations from a basic serovar profile. Excluding the *S. typhimurium* strain 1706, the rest of exceptions detected in REP profiles followed a typical ERIC serovar fingerprint. Strain 1706 had a particular ERIC profile with an additional band of 830 bp (data not shown). This additional band was also present in strains 963, 1024, and 1714 of *S. typhimurium*. Only strains 963 and 1024 were related by source and/or antibiotic resistance (Table 1).

ITS1 Fingerprints for all the strains were determined using the selected primers for 16S–23S interspacer region amplification (Fig. 3). Profiles based on the electrophoretic mobilities allowed to distinguish *Salmonella* strains from other enterobacterial species (Fig. 3A) and to classify them into the same fingerprinting ITS1-based group (Fig. 3B). *Salmonella* profiles had 4 to 6 bands. Profiles are conserved in *Salmonella*. Three of the bands (544 bp, 400 bp, and 227 bp) were present in all the strains of the three serovars investigated. These common bands were considered primary bands for the differentiation from other enterobacterial species.

Bands similar to the 544 bp band were found in *P. vulgaris*, *K. pneumoniae*, and *Enterobacter aerogenes* (Fig. 3A). The most similar bands in terms of electrophoretic mobility to the 400 bp band observed in *Salmonella* strains can be seen in *Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter aerogenes*, and *Shigella flexneri* fingerprints. The 227 bp band present in *Salmonella* is not observed in any other enterobacteria.

Finally, the presence or absence of each one of the rest of the bands (secondary bands) could distinguish strains of different serovars. One of the considered non-primary bands has a calculated size of 650 bp and is found both in *S. typhimurium* and *S. virchow*. The absence of this band in *S. enteritidis* is again a good criterion to differentiate this serovar from the other two, once an isolate has been identified by the presence of the three primary bands. A second non-conserved band (453 bp) is only observed in *S. typhimurium* multidrug

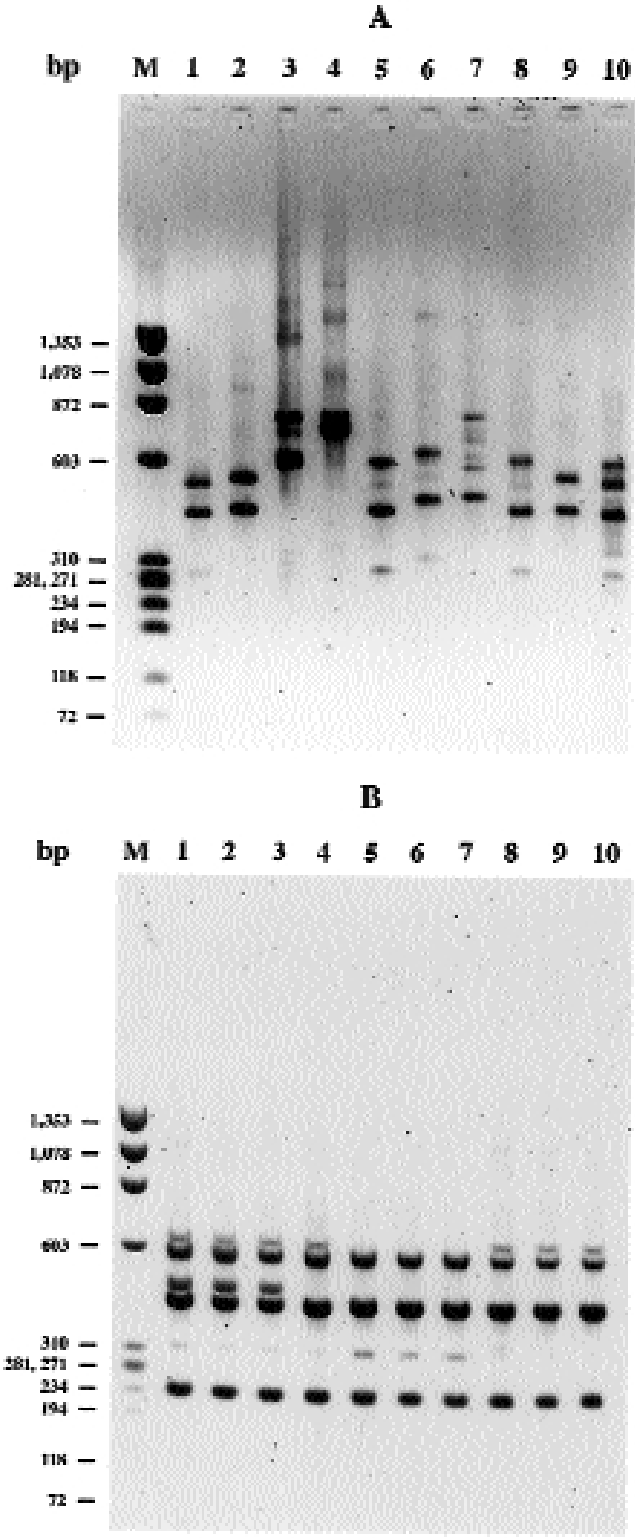

Fig. 3 Analysis of PCR-amplified 16S–23S intergenic spacer region (ITS1) products (A, B) on agarose gels (3.0%). Lanes are as follows; gel A: 1, *E. cloacae* CECT 194; 2, *Escherichia coli* 7/49; 3, *Proteus vulgaris* CECT 484; 4, *Yersinia enterocolitica* DSMZ 4780; 5, *Klebsiella pneumoniae* K7 774; 6, *Citrobacter freundii* CECT 401; 7, *Proteus mirabilis* DSMZ 4479; 8, *Enterobacter aerogenes* CECT 684; 9, *Shigella flexneri* CECT 585; 10, *Shigella dysenteriae* CECT 584; gel B: 1, *Salmonella typhimurium* 554; 2, *S. typhimurium* 1708; 3, *S. typhimurium* 1085; 4, *S. typhimurium* 869; 5, *Salmonella enteritidis* 1689; 6, *S. enteritidis* 968; 7, *S. enteritidis* 1671; 8, *Salmonella virchow* 874; 9, *S. virchow* 881; 10, *S. virchow* 1727. M, molecular marker (FX174 DNA digested with *Hae*III)

resistance strains. A third secondary band (310 bp), although apparently common to all *Salmonella* strains, was considered as secondary band due to its weak signal. Strain 874 of *S. virchow* showed an additional band (323 bp) that was not observed in any other *Salmonella* strain and could not be related to any special phenotypic trait.

Discussion

Many changes in taxonomy and nomenclature have resulted from the application of polyphasic approaches. These affect the traditional identification schemes used in the family Enterobacteriaceae and thus in *Salmonella* species. In the current classification of Enterobacteriaceae there is a tendency to conserve former designations and to separate or regroup previously existing groups, and also to the application of the most recent techniques, which comprise new biochemical tests, specific bacteriophages, DNA-related tests, and computer-assisted bacterial identification [7, 12]. In this work, we applied rapid genomic PCR typing methods based on several repetitive (REP, ERIC) and *rrn* operon conserved sequences. We tested these methods to evaluate their usefulness as molecular typing tools to differentiate *Salmonella* from other enterobacteria, and to distinguish strains of *Salmonella enterica* subsp. *enterica* between serovars *Salmonella* serovar *enteritidis*, *S.* serovar *typhimurium* and *S.* serovar *virchow*.

The direct analysis of colonies from agar plates would be advantageous for all the rapid methods described. However, as previously noticed [13], the amplification from whole cell extracts without a DNA purification step results in the absence of PCR products or non-amplification of some bands, leading to a progressive loss of reproducibility of the fingerprints. When we used unpurified *Salmonella* DNA for PCR and stored the spawned fragments instead of checking them immediately after the PCR had finished, we observe a loss of the banding pattern. This is attributable to thermostable endogenous *Salmonella* nucleases capable of withstanding the elevated temperatures during the PCR process [13]. In our case, DNA degradation might have been caused by the thermostable nuclease activity, since culture age was not responsible for this effect. Furthermore, this effect is not primer set dependent. In fact, we observed it even when PCR was run at low-stringency conditions with all three primer sets. Given that the procedure for PCR fingerprinting must offer the clearest and best reproducible banding pattern, we decided to use purified DNA for rapid PCR molecular typing of *Salmonella* and the other Enterobaceriaceae strains. We assigned to the same serovar *Salmonella* isolates which shared similar or identical profiles for either REP, ERIC and ITS1, and we did not find variations when we compared strains from different sources but included in the same serovar.

In some cases, the methods used in this work showed likely relationships between specific bands of the fingerprints obtained and antibiotic resistance; although this might depend on the combination of serovar-typing methods used rather than to be a standard behavior for all the serovars with a specific PCR method. In fact, when used to differentiate strains within the same serovar, REP-, ERIC-, and ITS1-PCR fingerprints did not show equal capabilities of discrimination. In general, a clear differentiation at strain level was not possible by using ERIC based primers, and with a few exceptions all strains of a specific serovar exhibited the same fingerprint. Additional polymorphic bands were present in *S. virchow*, however, when we used REP-based primers. We could detect possible relationships between the pattern identity of *S. virchow* isolates and the antibiograms observed for those strains showing either ampicillin resistance or multiple resistance. Additional bands were detected in these isolates in comparison with the basic and conserved bands detected in the initially analyzed strains of this serovar. Furthermore, the unique additional band viewed in multiple resistant strains was clearly different from the unique band observed in the ampicillin resistant strains. These isolates did not fall into different groupings since all of them show the other characteristic bands of strains identified as *S. virchow*. With the other PCR-based typing methods, we were not able to distinguish *S. virchow* strains on the basis of antibiotic resistance since all strains displayed a common PCR pattern, which was identical to that of the other strains of this serovar. The distribution of these specific bands seemed to be related to the distribution of antibiotic resistance. So, this typing method could provide information useful in epidemiological studies of this serovar.

When we compared the fingerprints of *S. virchow* strains which had multidrug resistance to fingerprints obtained for *S. enteritidis* strains, we found out that the band theoretically specific for *S. enteritidis* serovar was quite similar to that additional band observed in *S. virchow* strains with multidrug resistance (Fig. 1C). Although both amplicons have apparently the same molecular weight, their sequences are not necessarily identical, nor do they have similar functional contents. From the diagnostic point of view, this makes it even more difficult to distinguish between strains of *S. enteritidis* serovar and those *S. virchow* that have multidrug resistance. In any case, there are enough criteria (bands) to distinguish between strains of these serovars, including multidrug resistance strains of *S. virchow*. REP PCR was not useful to detect possible genomic alterations in *S. virchow* related to antibiotic resistance.

The fingerprints generated with the set of universal primers that anneal to conserved regions of both ends of the 16S and 23S rRNA genes of the *rrn* operon confirmed the existence of multiple operons in this species with certain degree of variation in length. Calculated lengths of 580 bp and 440 bp fragments are not quite different from the lengths observed for the spacer regions of other characterized operons in enteric bacteria [28]. Additionally, if we consider the three serovars as representative members of *S. enterica*, on the basis of the shared bands, the observed uniformity between the three serovars makes this approach

suitable also for classification at the subspecies level. Since this molecular typing method relies on DNA, a non-culture approach could be used for a preliminary diagnosis based on the presence of ITS1 specific bands for the serovars analyzed in the profiles obtained directly from a whole DNA sample recovered from blood, water, food, etc. This approach could be an alternative and/or a complement for the diagnostic of *Salmonella* with specific probes. Checking for the presence of the different and specific bands in a more complex pattern, due to the presence of other species in the samples —obtained by ITS1-PCR analysis— could be a reliable method for the determination of contamination by the serovars studied in this work.

The main consequence we can infer from the results is that the three methods described can be rapid diagnostic tools due to the reproducibility of the patterns by any of the three methods. Furthermore, we found some evidences that the generation of different fingerprints by the REP-, and ITS1-PCR methods is related to antibiotic resistance in the set of *S. virchow* and *S. typhimurium* isolates analyzed. The extent and significance of this phenomenon remains obscure and it could be apparently rather different in other serovars or simply it was not detected with the PCR conditions used. Nevertheless, the results obtained in *S. virchow* and *S. typhimurium* with REP and *rrn* operon based primers respectively, might be of interest, at least in these serovars, to know to what extent they are involved in the antibiotic resistance observed.

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