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Molecular epidemiology of drug-resistant *Salmonella* Typhimurium in Spain

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SUMMARY

One hundred and forty-seven Salmonella *serotype Typhimurium strains isolated in three provinces in the midwest of Spain were studied. Of these, 93.6% were drug resistant. There were two predominant resistance phenotypes: 43 isolates (29.3%) were resistant to amoxicillin, tetracyclines, chloramphenicol, streptomycin and sulphamethoxazole and 27 isolates (18.4%) to amoxicillin, amoxicillin/clavulanic acid, tetracyclines, chloramphenicol, streptomycin and sulphamethoxazole. Randomly amplified polymorphic DNA (RAPD) analysis and pulsed field gel electrophoresis (PFGE) were performed for molecular typing. Thirty-six DNA band profiles were differentiated by RAPD, and 38 by PFGE. We found a high level of clonality; 27% of strains were identical by both methods. There were additional smaller clonal lines within every area. The highest discriminatory power was obtained with PFGE, but the greatest degree of genetic diversity was observed among* Salmonella *Typhimurium using both RAPD and PFGE.*

Key words: *Salmonella* Typhimurium - Resistant phenotypes - RAPD - PFGE - Epidemiology

Epidemiología molecular de Salmonella *Typhimurium multirresistente en España*

RESUMEN

Se estudiaron 147 de cepas de Salmonella *serotipificadas como* Typhimurium *procedentes de tres provincias españolas del medio-oeste. El 93,6% de ellas eran resistentes a los antimicrobianos. Hubo dos fenotipos de resistencia predominantes: 43 cepas (29,3%) fueron resistentes a amoxicilina, tetraciclinas, cloranfenicaol, estreptomicina y sulfametoxazol, y 27 (18,4%) a amoxicilina, amoxicilina-ácido clavulánico, tetraciclinas, cloranfenicol, estreptomicina y sulfametoxazol. Los distintos patrones de resistencia se determinaron por técnicas de biología molecular: RAPD (*Randomly Amplified Polymorphic DNA*) y PFGE (*Pulsed Field Gel Electrophoresis*). Por RAPD se diferenciaron 36 patrones de bandas, y por PFGE 38. Se encontró una proporción alta de clones: el 27% de las cepas fueron idénticas por ambos métodos. Además, en cada área se encontraron algunos clones diferentes adicionales. Con PFGE se obtuvo el mayor poder discriminatorio, pero el mayor grado de diversidad genética se observó usando ambas técnicas conjuntamente.*

Palabras clave: *Salmonella* Typhimurium - Fenotipos resistentes - RAPD - PFGE - Epidemiología

INTRODUCTION

Salmonellosis is one of the main causes of gastroenteritis in our area. Resistance to antibiotics such as tetracyclines, chloramphenicol, fluoroquinolones and cephalosporins has increased in recent years (1-4). Knowledge of the epidemiological strains is very important in order to know the most probable mechanisms for resistance spreading and to suggest control measures.

Typing methods based on phenotypic differences are inherently limited by the capacity of microorganisms to alter underlying genes expression (5). Serotyping is usually inadequate as a single typing method for epidemiological purposes because exposed antigens can provide little information concerning the genetic relationships. In the case of *Salmonella*, most human salmonellosis episodes and outbreaks are caused by a few serotypes, thus discriminatory capacity of serotyping is clearly insufficient.

Problems associated with most phenotypic techniques boosted the development and refinement of DNA-based typing methods, which are now preferred for typing most common bacterial pathogens (6-9). Randomly amplified polymorphic DNA (RAPD) segment analysis and pulsed field gel electrophoresis (PFGE) have been proposed as two tools useful for characterizing a variety of bacterial pathogens.

RAPD analysis, also known as arbitrarily primed-polymerase chain reaction (AP-PCR), is based on the presence of priming sites on the genome close enough to permit PCR amplification using single primers with arbitrary nucleotide sequences. It is a PCR-based assay that has been developed to detect polymorphisms in genomic DNA (10-12). PFGE is based on chromosomal DNA digestion with restriction enzymes which cut DNA (typically <20 restriction sites). Since most resulting DNA fragments are too large to be resolved by conventional agarose gel electrophoresis, PFGE uses a multidirectional electrophoresis that continually changes the positive charge location. The DNA molecules respond by continually reorienting their migration direction through the agarose gel. By varying both the direction and the duration of the electric field, PFGE allows the separation of DNA molecules well over 1,000 Kb (13, 14). The purpose of this study was to investigate the clonal relationship among drug-resistant *Salmonella* Typhimurium in three different areas of the midwest of Spain, and the potential of RAPD analysis and PFGE, alone and combined, for these investigations.

MATERIAL AND METHODS

Bacterial strains

The study was performed on 147 drug-resistant *Salmonella* Typhimurium isolates obtained between December 2000 and March 2001, in the microbiology services of three hospitals in the midwest of Spain: Hospital Universitario, Salamanca (54 strains, area 1); Hospital Virgen de la Concha, Zamora (70 strains, area 2); and Hospital Nuestra Señora de Sonsoles, Ávila (33 strains, area 3). All strains were isolated from fecal specimens of outpatients and inpatients with diarrhea. Strains were identified by conventional biochemical analysis and by serotyping with different antisera (*Difco*™). It was not possible to study the phage type for most strains.

RAPD analysis

For the RAPD analysis, DNA was extracted as described previously (15). DNA purity and concentration were determined by optical density at 260 and 280 nm using a Zuzi 52 calculator (Auxilab, Spain).

Amplification reactions were prepared in 25 µl volumes containing 40 ng of total *Salmonella* DNA, 25 pmol of primer 5, and one small bear (*Ready to Go*®, Amersham Pharmacia Biotech) containing Taq DNA polymerase, dNTPs (0.4 mM each dNTP), BSA (2.5 µg) and buffer (3mM $MgCl_2$, 30 mM KCl and 10 mM Tris, pH 8.3). The primer used was 5'-d[AACGCGCAAC]-3'. It was selected from among six different primers because of the clear and distinct banding patterns obtained.

The PCR procedure was performed in a Progene thermal cycler (Techne Ltd.) subsequent the following program: 95 ºC for 5 min; (1 min at 95 ºC, 1 min at 36 ºC, 2 min at 72 ºC) × 45 cycles; 72 ºC for 5 min.

RAPD products were visualized by ethidium bromide staining after electrophoresis in a 2% agarose gel at constant voltage, and photographed with ultraviolet illumination from a fixed video camera position. Gels were analyzed with the software program *Lane Manager 2.2*© (T.D.I., S.A.).

Pulsed field gel electrophoresis

PFGE was performed as described above (15). A single colony was inoculated into 3 ml trypticase soy broth and incubated at 37 ºC on a roller drum for 16–18 h. The cells were collected by centrifugation, washed in buffer (75 mM NaCl, pH 8.0; 25 mM EDTA, pH 8.0), and the optical density of the cells at a wavelength of 610 nm was adjusted to 1.40. Chromosomal-grade agarose was prepared in 10 mM Tris and 0.1 mM EDTA to a final concentration of 1.2% and maintained at 55 ºC. Plugs were formed by mixing 0.5 ml of cell suspension with 0.5 ml of agarose, and this mixture was then transferred into plug molds. Once solidified, the plugs were transferred to lysis buffer (50 mM Tris, pH 8.0; 50 mM EDTA, pH 8.0; 1% sarcosine; 1 mg of protein-

ase K/ml) for a 16–20 h incubation in a 55 ºC water bath. The lysis buffer was removed, and the plugs were washed in 5 ml of sterile distilled water for 5 min and then with 3 ml of TE (10 mM Tris, pH 8.0; 1 mM EDTA, pH 8.0) for 5 min at room temperature. A final set of four washes, 30 min each, was done with 3 ml of TE per wash. Two 1-mm–thick slices of plugs were preincubated with 200 µl of 1 × *XbaI* buffer (Boehringer Mannheim Corporation) for 30 min. The buffer was then removed and replaced with a fresh mixture containing 50 U of *XbaI* restriction enzyme in 1 × restriction buffer and incubated at 37 ºC for 16–20 h. The plugs were soaked in 0.5 × Tris-borate-EDTA (TBE) prior to electrophoresis or stored at 4 ºC in 0.5 ml of TE.

DNA fragments were separated by using the CHEF system *Gene Navigator*® (GE Healthcare). Gels were run by using 0.5 × TBE buffer (45 mmol Tris base, 45 mmol boric acid and 1 mmol EDTA) at 14 ºC, with a linear increase in switching times (2.16–54.1 sec) for a period of 25 h at 200 v.

The gels were stained for 30 min in 1 l of sterile water containing 100 µl ethidium bromide (10 mg/ml), and then were photographed with ultraviolet illumination from a fixed camera position. Gels were analyzed with *Lane Manager 2.2* ©.

The discrimination index was calculated for both techniques using the Simpson's index of diversity. The genetic distance was calculated with dendrogram analysis, using a factor of comparison standard of 1 with *Lane Manager 2.2* ©.

Antibiotic susceptibility

Amoxicillin, amoxicillin/clavulanic acid, cefotaxime, cefepime, nalidixic acid, ciprofloxacin, levofloxacin, tetracycline, chloramphenicol, gentamicin, streptomycin, sulphamethoxazole and trimethoprim plus sulfamethoxazole were provided as standard powder by their respective manufacturers.

Minimal inhibitory concentrations were determined by the agar dilution method, according to Clinical Laboratory Standard Institute (CLSI) guidelines.

RESULTS

One hundred and fifty-seven *Salmonella* Typhimurium isolates were obtained during this period; 147 (93.6%) were resistant to one or more antibiotics. Susceptibility results, including resistance rates, appear in Table 1. Amoxicillin, tetracyclines, chloramphenicol, streptomycin and sulphamethoxazole resistance proportions ranged from 70–5%. Amoxicillin/clavulanic acid was much more active than amoxicillin alone, co-trimoxazole more than sulphamethoxazole alone and gentamicin more than streptomycin. *In vitro* resistance to first- and second-generation cephalosporin was very low, though these are not recommended for the treatment of salmonellosis, and no resistance to third- and fourth-generation cephalosporins, aztreonam and imipenem was found. No strains had phenotypes suggesting extended-spectrum beta-lactamases. There was

Table 1. Antibiotic susceptibility of *Salmonella* Typhimurium isolates.

Antibiotic	MIC_{50}	MIC_{90}	Susceptibility range	% resistance (susceptible/total)
Amoxicillin	>128	>128	0.5–>128	76.4 (120/157)
Amoxicillin-clavulanate	8	16	0.2–32	4.45 (7/157)
Cefazolin	2	4	1–>128	0.6 (1/157)
Cefuroxime	8	16	2–>128	1.9 (3/157)
Cefotaxime	0.1	0.2	0.03–1	0
Cefepime	0.1	0.5	0.016–4	0
Aztreonam	0.1	0.1	0.06–0.2	0
Imipenem	0.2	0.2	0.06–1	0
Nalidixic acid	8	64	1–>128	12.73 (20/157)
Ciprofloxacin	0.03	0.06	0.008–0.2	0
Levofloxacin	0.06	0.2	0.03–0.5	0
Moxifloxacin	0.06	0.5	0.03–0.5	0
Tetracyclines	32	>128	0.5–>128	75.15 (118/157)
Chloramphenicol	128	>128	0.06–>128	71.33 (112/157)
Gentamicin	0.5	8	0.06–128	6.36 (10/157)
Streptomycin	32	128	1–>128	73.8 (116/157)
Sulphamethoxazole	>512	>512	8–>512	73.88 (116/157)
Cotrimoxazole	0.1	0.2	0.016–>128	8.2 (13/157)

Table 2. Main susceptibility patterns of multidrug-resistant *Salmonella* Typhimurium isolates (isolates resistant to at least four antibiotics).

Patterns[a]	No. isolates (%)
A Te C S Su	43 (29.3)
A Ac* Te C S Su	27 (18.4)
A Te S Su	5 (3.4)
A Ac Te C S Su	5 (3.4)
A Te S Su C G Sxt	3 (2.0)

[a]A: amoxicillin; Ac: Co-amoxiclav; Te: Tetracyclines; S: Streptomycin; Su: sulphamethoxazole; Sxt: sulphamethoxazole + trimethoprim; C: chloramphenicol; G: gentamicin.
*Intermediate susceptibility.
There were 22 other multidrug resistance profiles, each of which included one or two isolates.

12.7% resistance to nalidixic acid, but no strains were resistant to fluorinated quinolones. Among the 147 drug-resistant isolates, 115 (78.2%) were resistant to four or more antibiotics. There were two predominant resistance phenotypes: 43 isolates (29.3%) were resistant to amoxicillin, tetracyclines, chloramphenicol, streptomycin and sulphamethoxazole, and 27 isolates (18.4%) to amoxicillin, amoxicillin/clavulanic acid, tetracyclines, chloramphenicol, streptomycin and sulphamethoxazole (Table 2).

Thirty-six patterns were differentiated by RAPD analysis (Fig. 1). Each of these profiles included 4–12 fragments which were 250–1,200 bp. All patterns but one were specific for one area (*i.e.,* appeared in only one area). Nevertheless, the only pattern that was found in the three areas was largely predominant. RAPD pattern 3 (RAPD-3) was shown by 38 isolates in area 1 (70.4%), 37 isolates in area 2 (52.9%) and 16 isolates in area 3 (48.5%) (Table 3). On the whole, 61.9% of drug-resistant isolates found in the region were identical by RAPD analysis.

When studied by PFGE, 38 patterns were found (Fig. 2). As with RAPD analysis, all patterns except one were specific for one area, and the only pattern common for the three areas was predominant. In total, 72 isolates (49%) belonged to the major pattern PFGE-3 (Table 3). This pattern predominated in the three areas (44.4% in area 1, 54.3% in area 2 and 30.3% in area 3).

Both RAPD analysis and PFGE were able to differentiate patterns obtained by the other technique in subpatterns (Table 3). If we take into account both techniques, 59 drug-resistant isolates (40.1%) were identical by using both techniques combined (RAPD/PFGE). These 59 isolates may be grouped into four very similar resistance patterns:

1. Streptomycin, tetracyclines, chloramphenicol, amoxicillin, sulphamethoxazole (57.6%).

Table 3. Correlation between main RAPD and PFGE patterns in *Salmonella* Typhimurium.

RAPD patterns	PFGE patterns																									
	3	**5**	**6**	**7**	**8**	**10**	**11**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**21**	**22**	**24**	**26**	**27**	**28**	**37**	Total
3	59	2	1	1	2	1	1	5	2		3				1					3	9	91
4	1																					1
5	1																					1
10						1				1												1
11																						1
12								2											1			2
13	1									1												2
14	1																					1
16																						1
21	6																					6
23													8	1		2						11
27	1											1					7					9
29	1																					1
30	1																					1
37																					1	1
Total	72	2	1	1	2	2	1	7	2	2	3	1	8	1	1	2	7	1	1	3	10	130

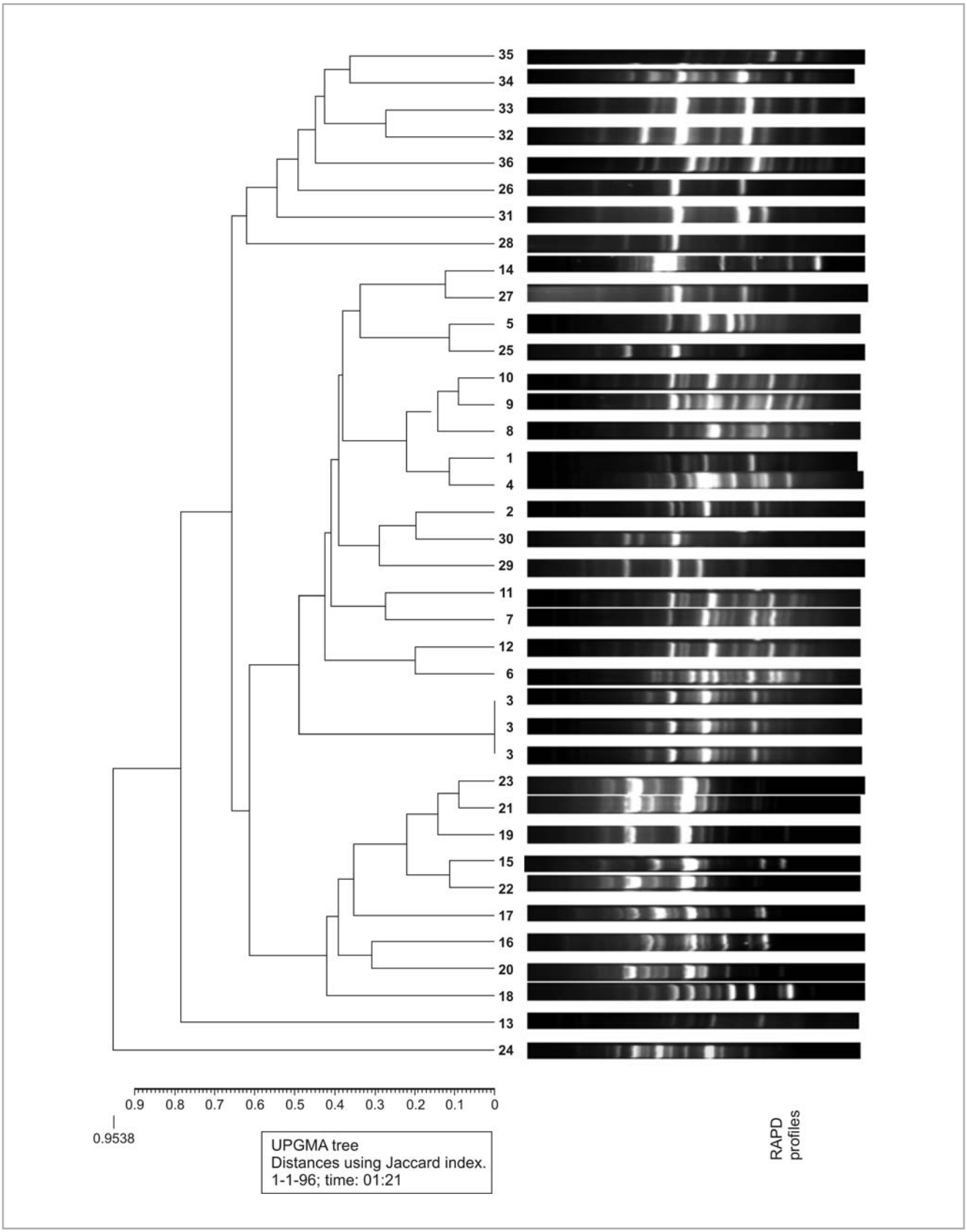

Figure 1. RAPD patterns obtained in every area and dendrogram. Pattern 3 was the most frquent pattern and the only pattern found in the three areas studied.

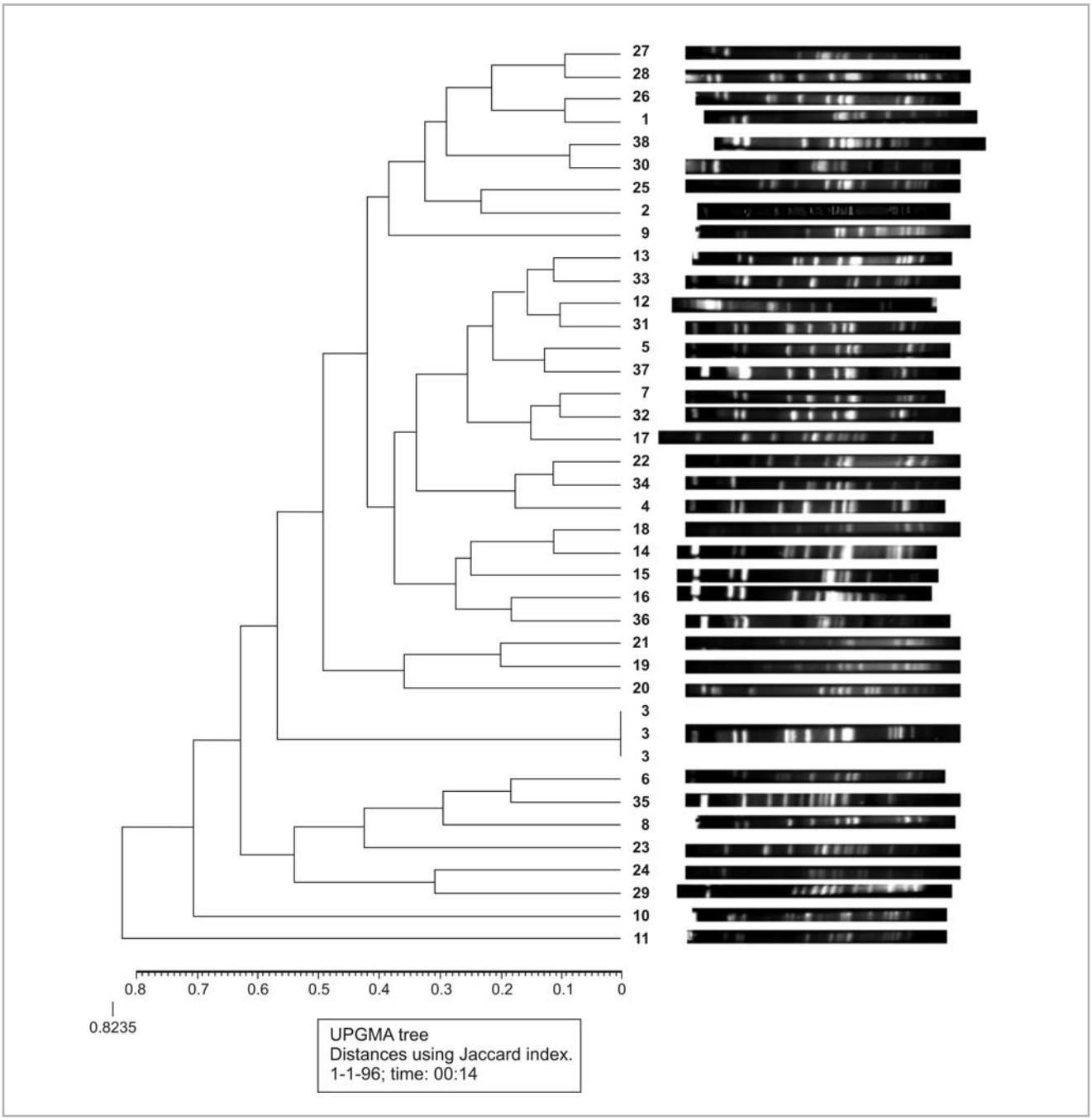

Figure 2. PFGE patterns obtained in every area and dendogram. Pattern 3 was the most frequent pattern and the only pattern found in the three areas studied.

2. Streptomycin, tetracyclines, chloramphenicol, amoxicillin, sulphamethoxazole, co-amoxiclav intermediate (37.3%).
3. Streptomycin, tetracyclines, chloramphenicol, amoxicillin, sulphamethoxazole, nalidixic acid (1.7%).
4. Streptomycin, tetracyclines, chloramphenicol, amoxicillin, sulphamethoxazole, co-trimoxazole (3.4%).

Dendrograms showed that, though a wide variety of patterns was found by both methods, genetic proximity among most patterns was high (Figs. 1 and 2).

DISCUSSION

Salmonella non-Typhi is the principle cause of gastroenteritis in this region. Food from contaminated animal

sources is usually the origin of infections (16-19). Though resistance to antibiotics such as chloramphenicol (20-22) was described years ago, resistance to other antibiotics such as cephalosporins and fluoroquinolones has been increasing in recent years (23-28). The use of antibiotics in animal feeding is presumed to be one of the causes for this increase (29-32) because resistance to antibiotics has increased significantly in other infections with similar sources, such as campylobacteriosis (33-36).

Previous studies in Spain on the antibiotic susceptibility of *Salmonella* isolated from humans and animals show resistance rates similar to or moderately higher than those found in this study in isolates from human sources, while resistance rates among isolates from animal origin were higher (37). The main differences appear in aminoglycosides other than streptomycin, where resistance rates were >70% in animal isolates and <5% in human isolates, nalidixic acid (animal isolates 76%; human isolates, 6%) cephalothin (animal isolates, 24%; human isolates, 2%) and co-trimoxazole (animal isolates, 82%; human isolates, 19%). Nevertheless, some studies from human (38) and animal sources (39) show resistance rates for ampicillin/sulbactam and amoxicillin/clavulanate that are much higher (30-60%) than the rates found in our study for amoxicillin/clavulanate; further, these studies show very high resistance rates for cefazolin (21.8%) and cefuroxime (27.4%), while we found hardly any isolates resistant to any cephalosporin tested.

As in previous studies in Spain, the most frequent multidrug-resistant pattern comprised streptomycin, tetracyclines, chloramphenicol, amoxicillin and sulphamethoxazole; this included 29.3% of isolates. In previous studies this pattern was even more prevalent, including 76.6% of isolates and belonging in most cases to the phagotype DT 104 (37). Nevertheless, the second pattern in frequency in our study was not determined in the other studies, since amoxicillin/clavulanate was not tested. The third pattern in prevalence in our study, which included streptomycin, tetracyclines, amoxicillin and sulphamethoxazole, was also the next prevalent pattern in these studies.

Molecular techniques (40-44), such as PFGE, AFLP and RAPD analysis, are well recognized as tools that are useful in tracing the routes of transmission and environmental diffusion of different microorganisms, including *Salmonella* (45-48). In our study we used RAPD analysis and PFGE to investigate the epidemiological relationship between strains isolated in three different areas in the midwest of Spain. Previous studies have compared RAPD analysis and PFGE with other techniques. A recent study on *Salmonella* isolates obtained in the southeast of Spain showed that RAPD analysis was more efficacious than serotyping and phagotyping to differentiate isolates (37).

Though both methods are efficacious to differentiate isolates, the combination of both techniques increases the discrimination capacity. The higher efficacy of combined methods has been shown in previous studies of *Salmonella* (49-51). While RAPD analysis differentiated 36 patterns and PFGE differentiated 38, combining both techniques yielded 59 profiles. The combination of PFGE and ribotyping has also been found to be more efficacious than any of these techniques alone to differentiate *Salmonella* isolates (52).

Previous studies have shown that multidrug-resistant *Salmonella* Typhimurium isolated in Spain show very similar resistance patterns in most cases and belong to the same phagotype (mainly DT 104) but do not include more specific genetic analysis (18). According our study, multidrug-resistant *Salmonella* Typhimurium are also very close from a genetic point of view. In fact, 27% of the strains were indistinguishable even using RAPD analysis and PFGE combined, and most subtypes were separated by a small genetic distance. Both the dendrogram of Xba-1 digested genomic DNA separated by PFGE and the dendrogram of RAPD analysis patterns showed >65% similarity between most patterns.

The situation is different for other *Salmonella*; studies on *Salmonella* Enteritidis isolates from several European countries show an important genetic heterogeneity (52). These results suggest that in Spain most cases of human infection by multidrug-resistant *Salmonella* Typhimurium are caused by the epidemic spread of a multidrug-resistant clone or, at least, by a group of strains that are genetically very close, which are major contributors to the increasing resistance rates among *Salmonella* Typhimurium human isolates.

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