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IS*200* fingerprint of *Salmonella enterica* serotype Typhimurium human strains isolated in Sardinia

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SUMMARY

A collection of *Salmonella enterica* serotype Typhimurium human strains isolated in Northern Sardinia (Italy) was examined for the insertion sequence IS*200*, phage type, antibiotic profile, ribotyping polymorphisms and plasmid profile. All clinical isolates studied contained from 4 to 10 copies of the IS*200* element. IS*200* permitted to discriminate Typhimurium strains and to identify five IS*200* types, some of them circulating in Sardinia at least since 1900. Strains belonging to phage DT104 predominated and correlated with a specific IS*200* pattern.

INTRODUCTION

Salmonellosis in humans is most often caused by consumption of contaminated food such as meat, dairy products, eggs or fluids. It is one of the most frequent examples of an enteric disease that is transmitted from animals to humans [1]. Epidemiological studies of the disease require the application of simple typing systems for serovars and strain identification (for reviews see [2, 3]).

The conventional method for epidemiological investigations in salmonellosis caused by the most common serotypes is phage typing. Alternatively, the determination of antimicrobial resistance patterns has commonly been used to evaluate and trace epidemic strains. In the last decade, with the development of molecular biology techniques, new approaches have become available. Widely used are plasmid analysis, chromosomal fingerprinting by Southern hybridization, and macro restriction analysis of chromosomal DNA by pulse-field electrophoresis. IS*200*, a 707 bp insertion element, found in many isolates of salmonella [4–6] with the exception of Agona, some strains of Arizonae [5], Bovismorbificans [7], Dar-es-salam [8] and Choleraesuis [9] is another commonly used molecular probe. The use of this insertion sequence epidemiologically and phylogenetically significant observations have been made in several salmonella serovars (i.e. Enteritidis, Bovismorbificans, Abortusovis, Heidelberg, Dublin, Typhimurium, Brandeburg, Typhi) [3, 7, 9–15]. The element is also present in certain isolates of *Shigella* sp. and *Escherichia coli* but a suitable salmonella-specific probe is available to overcome this inconvenience [5]. The number of the IS*200* copies found in the genome of various salmonella serovars analysed ranges from 1 to more than 25 [5]. When several copies are present, IS*200* can be easily used as a fingerprint marker. The fact that IS*200* rarely transposes makes the assay highly reliable [16].

The aim of this study was to investigate the distribution of *Salmonella* species in clinical samples isolated in the year 1989–94, to monitor antimicrobial susceptibility. Furthermore, we have characterized at molecular level the clinical isolates of Typhimurium,

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the most common salmonella serotype causing human infection in Northern Sardinia (Italy) in 1994. For this purpose, a DNA probe derived from the insertion element IS*200* has been used to discriminate easily and efficiently among Typhimurium strains.

We have determined the copy number and the distribution of the transposon in the genome of strains isolated since 1989, the variation at and around the 16S rRNA gene loci, plasmid content and we have detected the presence of salmonella plasmid virulence (*spv*) region located in the high molecular weight plasmid. Furthermore we have analysed and compared phage type to the IS*200* pattern.

MATERIALS AND METHODS

Bacterial strains, culture conditions, phage typing

Salmonella strains used in this study were human strains isolated in the period 1989–94 in Northern Sardinia (Italy). A total number of 379 strains of *Salmonella* spp. isolated from 21917 stools examined were obtained (Table 1). Most of the strains were isolated from patients affected by diarrhoea or from specimens obtained by routine coproculture recovered in paediatric and in obstetrics divisions.

The strains were collected at the Istituto di Igiene e Medicina Preventiva, University of Sassari. All isolates represented sporadic cases (i.e. isolates from cases not associated with a known outbreak). Salmonellae were identified biochemically by the Enterotube system assay (Roche) and were serologically characterized by using specific anti-salmonella antisera (Difco Laboratories). The antigenic profile was in accordance to the method of Kauffman [17] for cell wall (O) and flagellar (H) antigen identification. Phage typing was carried out at CEPIS (Centro Enteropatogeni Italia Settentrionale, Milan) by using standard technique [18]. Strains were stored at −80 °C. IS*200* analysis of Typhimurium was performed for all the strains isolated in 1994 [32] and were compared to the following randomly chosen strains isolated in the previous years: 1989 (8/11); 1900 (10/35); 1991 (9/47); 1992 (15/68); 1993 (12/36).

Antibiotic susceptibility test

All the strains isolated from 1989–94 were screened for their antibiotic susceptibility both by disk diffusion method on Mueller–Hinton agar (Oxoid Ltd) and with ATB antibiogram (Biomerieux). The following antibiotics were used: ampicillin (Ap) 10 μg; amoxicillin and clavulanic acid (Av) 30 μg; chloramphenicol (Cm) 30 μg; mezlocillin (Mz) 75 μg; tetracycline (Tc) 30 μg; sulphamethoxazole-trimethoprim (Sxt) 23·75 + 1·25 μg; amikacin (Am) 30 μg; ceaphalothin (Ce) 30 μg; cefotaxime (Cf) 30 μg; ceftazidime (Caz) 30 μg; gentamicin (Gm) 10 μg; tobramycin (Tm) 10 μg; ciprofloxacin (Cip) 5 μg. The ciprofloxacin was inserted in the routine antibiogram since 1991. Strains belonging to phage type DT 104 were tested for their resistance to streptomycin (Sm) 10 μg and sulphonanides (Su) 10 μg.

DNA extraction and purification

Genomic DNA was isolated according to Ausubel and colleagues [19]. Plasmid DNA was extracted by alkaline lysis as previously described [20]. The approximate molecular weight of the plasmid was determined by mobility to plasmid with known molecular weight [21, 22].

Conjugation procedure

Transfer of plasmid from Typhimurium isolates to *E. coli* CSH26 nalidixic acid resistant by conjugation was carried out on sterile membrane filters (Millipore) by standard procedure. Selection of transconjugants were performed in MacConkey agar (Difco) containing the following antibiotics: ampicillin (50 μg/ml), mezlocillin (75 μg/ml) tetracycline (50 μg/ml), chloramphenicol (50 μg/ml), sulphamethoxazole (40 μg/ml) and nalidixic acid (40 μg/ml).

Hybridization with IS*200* probes

DNA was digested with the restriction enzyme *Pst*I (Promega), which does not cut within IS*200* [5]. Agarose (Bio-Rad) for gel electrophoresis was used at concentration of 0·8%. Plasmid pIZ46 (kindly provided by Dr Josep Casadesus, University of Seville, Spain) contains a tail-to-tail dimer of the *Eco*RI–*Hin*dIII fragment of IS*200* [5]. The probe was labelled with digoxygenin (Boehringer). Southern hybridization was carried out under high stringency conditions as described [9].

Hybridization with 16S rRNA gene

A probe to detect restriction fragment length polymorphisms around the 16S *rrn* locus was generated by PCR according to Stanley and colleagues [12]. The probe was used to hybridize genomic DNA, digested with *Pst*I (Promega), in Southern blot analysis.

Table 1. *Most common salmonella serotypes isolated in Sardinia in 1989–94*

Serovars	1989	1990	1991	1992	1993	1994	Total
S. typhimurium	25	35	47	66	36	33	242
S. enteritidis	34	6	40	43	19	26	168
S. derby	5	1	4	1	1	3	15
S. kimuenza	5	1	5	0	0	0	11
S. virchow	2	0	2	1	3	1	9
S. bovismorbificans	2	2	1	1	1	0	7

Table 2. *Characterization of human S. Typhimurium strains isolated in Northern Sardinia in 1994*

Strain number	Antibiotics resistance*	IS*200* copy number	Plasmid content (kb)	*spv* R gene	Phage type†
50	ApMzCmTcSmSu	9 (G1A)	100–1·6	+	104
51	ApMzCmTcSmSu	9 (G1A)	100–1·6	+	104
77	ApMz	9 (G1A)	100	+	ND
81		9 (G1A)	100	+	12
79	ApMzCmTcSmSu	9 (G1A)	100–1·6	+	104
72	ApMzCmTcSmSu	10 (G1B)	100–4·4–1·6	+	104
65	ApCmTmSmSu	10 (G1C)	100–1·6	+	104
57	ApMzCmTcSmSu	10 (G1D)	100–1·6	+	104
53		7 (G2)	100–6·4	+	124
54		7 (G2)	100–6·4	+	124
78	Tc	5 (G3)	100	+	99
80		5 (G3)	100	+	99
82		5 (G3)	100	+	173
56	TmSxt	8 (G4A)	90	–	193
59	Tc	8 (G4A)	90	–	NT
60	TcSxt	8 (G4A)	90	–	193
68	TcSxt	8 (G4A)	100	–	ND
76	TcSxt	8 (G4A)	90	–	ND
58	Tc	9 (G4B)	120–6·4	–	NT
61		4 (G5A)	100	+	135
63		4 (G5A)	100	+	193
64		4 (G5A)	100	+	135
67		4 (G5A)	100	+	NT
69		4 (G5A)	100	+	ND
71		5 (G5B)	100	+	ND
52		11	—	–	12
55	ApMzTc	7	100	+	193
62	ApMzTc	7	100–3	+	193
66		7	—	–	12
70		7	100	+	NT
73		7	100	+	ND
75	ApMzCmTmTc	8	0·5–1	–	NT

* Ap, ampicillin; Mz, mezlocilin; Tc, tetracycline; Sxt, sulphamethoxazole-trimethoprim; Tm, tobramicin; Su, sulphonanides; Sm, streptomycin; Cm, chloramphenicol.

† ND, not determined; NT, not typable.

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Construction of oligonucleotide gene probes and PCR for *spv* region

Two oligonucleotide sequences corresponding to the *spv*R gene of the Typhimurium virulence plasmid were synthesized with a Pharmacia oligosynthetizer according to Caldwell and Gulig [23]. Sequences selected were 5′-CCCCGGGAATTCGCTGCATA-AGGTCAGAAGG-3′ and 5′-CCCCGGGATCCA-TGGATTTCTTGATTAATAAA-3′. PCR was performed in a 25 μl reaction volume containing 2·5 μl of heat-denatured bacterial DNA solution (20 ng), 25 pmol of each primer, the four deoxynucleotide triphosphates at final concentration of 0·5 mM and 0·2 Units of *Taq* DNA polymerase (Promega) in a reaction buffer consisting of 50 mM Tris-HCl pH 8·5, 20 mM KCl and 15 mM $MgCl_2$. After 5 min of preheating at 94 °C thermocycling was performed on samples for 30 cycles of denaturation (94 °C, 1 min), annealing (1 min, 45 °C), and elongation (2 min, 72 °C). Plasmid pGTR061 (kindly provided by P. Gulig, University of Florida, Gainesville), was used as positive control for *spv* region.

Data analysis

The patterns produced by IS*200* fingerprinting were evaluated with the commercial Image Master Software (Pharmacia). Dendrogram was constructed by the UPGMA clustering method using the Dice coefficient.

RESULTS

Salmonella serovars isolated in Northern Sardinia from humans

Analysis of the prevalence of salmonella strains isolated (228) in stool specimens from 1989–94 in Northern Sardinia, is reported in Table 1. Typhimurium was the most common serovar isolated (47·8%), followed by Enteritidis (29·9%). However, it must be noted that during the years under study the isolation of Enteritidis increased dramatically after 1990, thus reaching a percentage close to Typhimurium.

Analysis of IS*200* fingerprints in *Typhimurium*

We have analysed the IS*200* pattern of Typhimurium strains isolated in 1994 and compared to reference strains isolated since 1989. *Pst*I analysis of genomic DNA showed that the 32 strains isolated in 1994

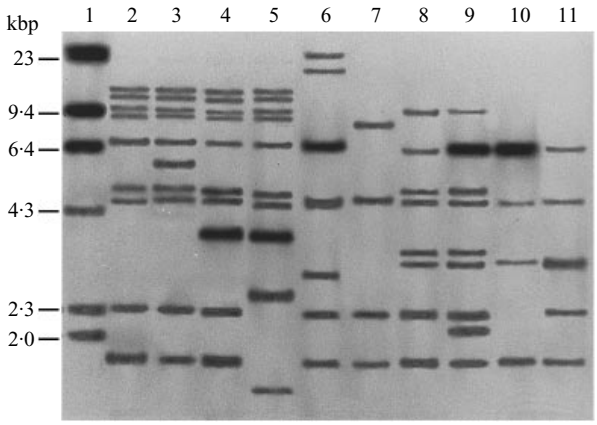

Fig. 1. IS*200* identification profile of Typhimurium clinical strains isolated in Northern Sardinia in 1994. 1, λ *Hin*dIII marker; 2, G1A; 3, G1B; 4, G1C; 5, G1D; 6, G2; 7, G3; 8, G4A; 9, G4B; 10, G5A; 11, G5B.

contained from 4 to 10 copies of the transposon (Table 2, Fig. 1). The IS*200* element was located in restriction fragments that ranged from 23 to 1·7 kb (Fig. 1). In addition to the 4·8 kb serovar specific band, common hybridization bands (2·4 and 1·9 kb) were observed in most of the strains. Figure 2 shows the relatedness among strains that belonged to different IS*200* types. We observed five groups of strains which possess the most conserved IS*200* type insertion homology and all isolates were joined to each other at a similarity of approximately 75% in the UPGMA clustering (Fig. 2). Group 1 (G1, 8 isolates) was the largest and possessed a profile type characterized by seven bands (kb 12·02, 10·08, 9·15, 8·60, 6·60, 5·69, 4·85). It was divided into four subgroups that showing homology ranging from 75 to 100% (Fig. 2). Subgroup 1A shared nine bands in common with strain 72 (G1:B) and strain 65 (G1:C) that carried also bands of 5·4 and 3·5 kb. Finally subgroup D (strain 57) was the most distantly related group with homology of about 75% to the group G1:A. Group 2 (strains 53, 54) exhibited the same profile with seven copies of the insertion sequence. Group 3 comprised strains (78, 80, 82) with the same pattern. Group 4 contained strains (56, 58, 59, 60, 68, 76) which shared eight bands and were closely related. Strain 58 carried an extra band of about 2·3 kb. Group 5 included six strains (61, 63, 64, 67, 69, 71) sharing four copies of transposon insertion. Strain 71 possessed an extra IS*200* copy of 2·4 kb. The remaining eight strains studied each showed a distinct IS*200* type from the other human isolates described.

Analysis of the IS*200* pattern of some strains randomly chosen isolated since 1989 (data not shown)

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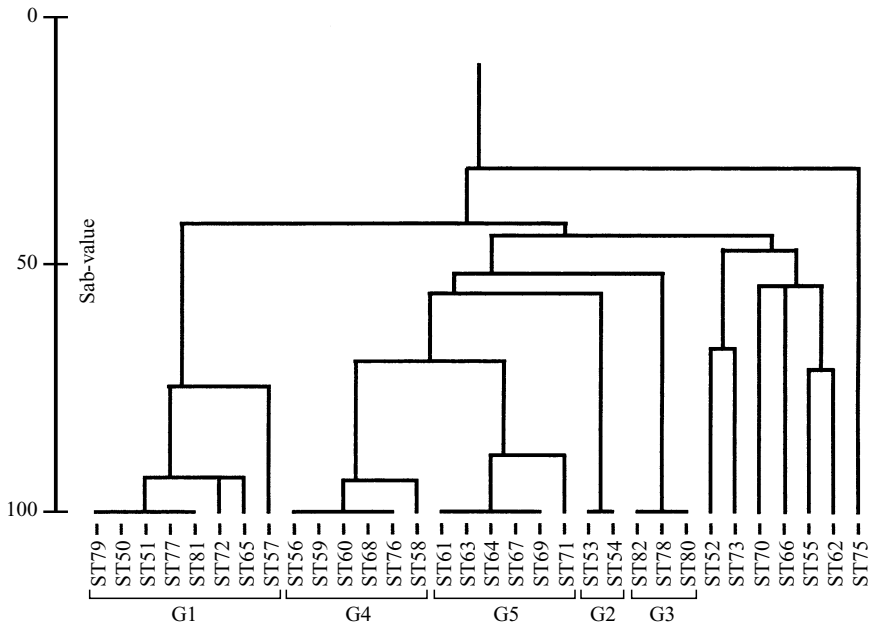

Fig. 2. Dendrogram showing the results of the cluster analysis on the basis of IS*200* genotypes of all Typhimurium strains.

Table 3. *Antibiotic-resistance percentage among Typhimurium isolated in 1989–94*

Antibiotics	1989	1990	1991	1992	1993	1994	Total
Ampicillin	27·3	25·7	29·8	23·5	36·1	31·2	28·9
Amoxicillin + clavulanic acid	0	0	4·2	4·4	5·5	3·1	2·94
Mezlocillin	0	0	23·4	26·5	33·3	28·1	27·8
Cephalothin	9·1	2·9	4·2	4·4	22·2	0	8·56
Ceftazidim	0	0	0	0	0	0	0
Cefotaxim	0	0	2·12	0	0	0	2·12
Gentamicin	0	0	0	5·9	5·5	0	5·7
Tobramycin	0	0	2·1	7·3	5·5	3·1	4·52
Amikacin	0	0	0	1·5	2·7	0	2
Tetracycline	45·4	80	74·5	61·8	77·7	53·1	60·7
Chloramphenicol	27·3	17·1	8·5	25	25	18·7	19·3
Ciprofloxacin*	ND	ND	0	0	0	0	0
Sulphamethoxazole-trimethoprim	9·1	5·7	0	5·9	13·9	12·5	9·4

* ND, Not determined.

and comparison of them to the groups identified in 1994, several features were evident. Strains that belonged to groups G3 and G4 were circulating in Northern Sardinia until at least 1990, whereas the group G1 seems to be spread since 1991.

Antibiotic resistance profile and conjugation experiments

The antibiotic resistance among Typhimurium strains isolated in Northern Sardinia (1989–94) is reported in Table 3. 70·7% of the human isolates was resistant at least to one antibiotic. The most frequent antibiotic resistance detected was tetracycline (60·7%). All strains were susceptible to ciprofloxacin and cefotaxim. The most prominent multiresistance pattern was ApMzCmTc and correlated to IS*200* group 1 (Table 2). Isolates belonging to IS*200* group 4 were the only strains resistant to sulphamethoxazole with the exception of isolate 59 (Table 2).

Typhimurium strains that belonged to multiresistant IS*200* group 1 and sulphamethoxazole resistant IS*200* group 4 were mated with *E. coli* CSH 26 to investigate the linkage of antibiotic resistance determinants to plasmid DNA. Results of these

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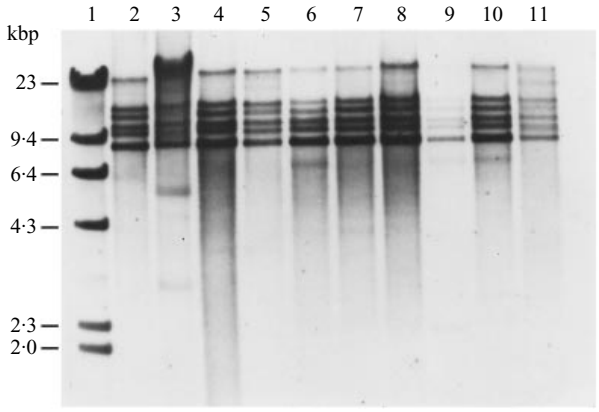

Fig. 3. *rRNA* profile of Typhimurium clinical strains. Genomic DNAs are digested with endonuclease *Pst*I. Lanes are as follows: 1, λ *Hin*dIII marker; 2, G1A; 3, G1B; 4, G1C; 5, G1D; 6, G2; 7, G3; 8, G4A; 9, G4B; 10, G5A; 11, G5B.

experiments showed that no antimicrobial resistance patterns were transferred by conjugation.

Phage type

The phage types present in Typhimurium strains isolated in 1994 were DT104 (the most prevalent group), DT124, DT173, DT193, DT135, DT12, and DT99 (Table 2). Some strains were not sensitive to the phages used (not-typable). All strains belong to IS*200* group 1 phage typed as DT104 and possessed multiple resistance with the only exception of strain 81, which phage typed as DT12, was susceptible to all antibiotics (Table 2). All DT104 strains were resistant also to sulphonamide and streptomycin as described elsewhere [24]. Some reference strains isolated in 1991 showing the IS*200* pattern corresponding to group 1, were also phage typed DT104.

Plasmid content

Plasmid analysis revealed the presence of a large plasmid (90–120 kb) in 29 out of 32 strains. Plasmids with a lower molecular weight were also observed in 11 strains. Two strains did not show any plasmid (Table 2).

To verify the presence of the virulence plasmid the bacteria were analysed by PCR with oligonucleotides for the *spv*R gene. All the strains that harboured a 100 kb plasmid, amplified a band of 890 bp (Table 2), with the exception of strain 68, corresponding to the *spv*R gene of the control sample, plasmid pGTR061, that carries the *spv* region [23].

Surprisingly, no amplification band corresponding to *spv*R gene was detected in strains belonging to the IS*200* group G4, which harboured a higher molecular-weight plasmid.

16S rRNA gene profile

Southern blot of genomic DNA restricted with *Pst*I and hybridized with the 16S rRNA gene showed basically a conserved profile among the strains studied (Fig. 3).

DISCUSSION

The most common salmonella serotypes isolated in Sardinia from human during the period 1989–94 were Typhimurium and Enteritidis. In particular, we detected an increase of Enteritidis since 1991. The reason of this increment is unclear although it may be due to contamination of food, poultry and meat products. Further investigation is needed to compare Enteritidis in chicken meat, eggs and human isolates in Sardinia in order to confirm that they originated from the same source.

We have used IS*200* as molecular probe to characterize a number of human clinical isolates of Typhimurium. IS*200* profiles permitted us to identify, among the strains isolated in 1994 five main related groups characterized by conserved and variable regions. Three of these groups (G1, G3, G4) were present in Northern Sardinia since at least 1990.

Although the method of choice for epidemiological studies of Typhimurium is phage typing [25], our study reinforced the concept that IS*200* is a suitable tool to study the epidemiology and the characterization of human isolates in epidemiological studies. Baquar and co-workers [13] found in phage type DT193 at least seven different IS*200* profiles among the strains examined. This was also evident in our study. We have isolated Typhimurium phage type DT193 strains with three different IS*200* patterns (strains belonging to group G4 and strains 55, 62), confirming the existence of a considerable genetic heterogenicity within DT193 strains. Similarly Olsen and colleagues [26] found five different IS*200* profiles among isolates that belonged to DT49.

Typhimurium phage type 104 has become increasingly common in cattle in England, Wales and Scotland since 1991 [24] and more recently in Germany [27]. It is responsible for the epidemic spread of multiresistance to Ap, Str, Sy, Tet, Cm. Interestingly, DT104, the most prominent phage type

isolated in Sardinia, belongs to IS*200* group G1 and it is multiresistant to antibiotics (with the exception of one strain sensitive to antibiotics and phage typed DT12) and could be responsible for the appearance of mezlocillin resistance. Furthermore, the IS*200* pattern of our DT104 corresponds to the strain described by Stanley and colleagues [12]. This datum is relevant from an epidemiological point of view since it shows that the same clone present in the UK has been circulating also in Sardinia since 1991. The circulation of this strain in our region might be related to introduction of cattle or bovine meat. An outbreak due to Typhimurium phage DT104 was associated with bovine product in Canada [28] and suggests the potential for salmonella spread by bovine meat and the importance of bovine as carrier for multiple resistant Typhimurium strains in imported beef.

16S *rrn* ribotyping, showed identical profiles in all the strains examined, and did not permit to identify any genetic variation. Although this may be due to the poor discriminatory power of the probe or restriction enzymes selected, the limitation of ribotyping in salmonella has been reported by other authors [26]. Although plasmid profile analysis has been previously found useful for salmonella epidemiology [29], we were unable to establish any epidemiological correlation by using this strategy. This may be due to the fact that almost all the strains under study harboured only one plasmid. In fact, according to Olkvik and colleagues [30], a unique plasmid cannot always be used to characterize a clone.

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