Molecular characterization of *Streptococcus suis* strains by 16S–23S intergenic spacer polymerase chain reaction and restriction fragment length polymorphism analysis

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Abstract

We developed a new molecular method of typing *Streptococcus suis* based on polymerase chain reaction (PCR) amplification of a large fragment of rRNA genes, including a part of the 16S and 23S genes and the 16S–23S intergenic spacer region (ISR), followed by restriction fragment length polymorphism (RFLP) analysis with *Rsa*I or *Mbo*II endonuclease. The 16S–23S ISRs of 5 *S. suis* isolates were sequenced and compared. Size and sequence polymorphisms were observed between the S735 reference strain and the 4 wild-type strains. The genetic relationships between 138 independent *S. suis* strains belonging to various serotypes, isolated from swine or human cases, were determined. The discriminatory power of the method was > 0.95, the threshold value for interpreting typing results with confidence (0.954 with *Rsa*I and 0.984 with *Rsa*I plus *Mbo*II). The in vitro reproducibility was 100%. The strains isolated from humans were less genetically diverse than the strains isolated from pigs. For the first time, 2 molecular patterns (R6, M9) were significantly associated with *S. suis* serotype 2 strains. This genetic tool could be valuable in distinguishing individual isolates of *S. suis* during epidemiologic investigations.

Résumé

Une nouvelle méthode de typage moléculaire de Streptococcus suis *a été développée. Elle est basée sur l'amplification génique des régions intergéniques présentes entre les gènes codant pour l'ARNr 16S et 23S de* S. suis *(ISR) et sur une microrestriction du produit amplifié à l'aide des enzymes de restriction* RsaI *et* MboII*. Les régions intergéniques présentes entre les gènes codant pour l'ARN 16S et 23S de cinq isolats ont été séquencés et comparés. Des polymorphismes de taille et de séquence ont été observés entre la souche de référence S735 et les quatre souches sauvages. Les relations phylogénétiques entre 138 souches de* S. suis *ont été étudiées. Elles n'étaient pas reliées entre elles, étaient isolées chez l'homme ou chez le porc et appartenaient à différents sérotypes. La technique s'est révélée discriminante (indice de discrimination supérieur à 0,95 : valeur limite pour interpréter les résultats de typage avec confiance) et reproductible (*D = *0,954 avec* RsaI *et* D = *0,984 avec* RsaI *et* MboII*). Les profils génétiques obtenus à partir des souches humaines sont apparus plus homogènes que ceux des souches isolées chez le porc. Pour la première fois, deux profils (R6, M9) ont été significativement associés aux souches appartenant au sérotype 2. Cette méthode pourrait donc être fort utile pour identifier* S. suis*, lors de suivis épidémiologiques de l'infection en élevage.*

(Traduit par les auteurs)

Introduction

Streptococcus suis is a worldwide cause of a variety of porcine infections. One of the most important agents of swine meningitis, it also causes meningoencephalitis, septicemia, arthritis, endocarditis, pericarditis, polyserositis, and sudden death of weaning piglets as well as growing pigs (1). This pathogen is also responsible for meningitis, septicemia, arthritis, and endocarditis in humans, usually people who have worked with raw pork or had contact with pigs (2,3). More recently, several cases of human *S. suis* infection acquired from wild boars have been reported (4–6).

Streptococcus suis can be classified into types according to capsular polysaccharide antigens. To date, 35 serotypes have been described: 1, 2, 1/2, and 3 to 34. Serotype 2 has always been considered the most virulent and is the most prevalent type isolated from diseased pigs in most countries where the swine industry is important (7–9). However, *S. suis* serotype 2 strains have been isolated from healthy pigs, and *S. suis* strains of other serotypes can also cause disease in pigs (1,10). Moreover, the mere presence of the putative virulence factors (such as muramidase-released protein, extracellular protein factor, and hemolysin) does not necessarily define a strain as virulent (8,11).

Several molecular typing schemes have been developed to determine the relatedness of strains of *S. suis* associated with infection, including multilocus enzyme electrophoresis (12), restriction endonuclease analysis with *Hae*III (13), ribotyping (14,15), repetitive

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extragenic palindromic (REP) or enterobacterial repetitive intergenic consensus (ERIC) polymerase chain reaction (PCR) (16,17), arbitrarily primed PCR (18,19), and pulsed-field gel electrophoresis (PFGE) (20–22). Among these methods, PFGE has been shown to be highly discriminatory and reproducible (21). However, it is laborious and time-consuming. The recently introduced multilocus sequence typing scheme (23), a nucleotide-sequence-based method with a high discriminatory power and a high degree of reproducibility, is difficult to apply for routine diagnostic testing. Characterization of the 16S–23S ribosomal (r) DNA intergenic spacer region (ISR) has also been used to compare bacterial strains (24–27). Schlegel and colleagues (25) reported that ISR analysis is a powerful method for identifying species within the genus *Streptococcus*, including *S. suis*. However, this method, based on PCR amplification and restriction of a fragment of rRNA genes, including the 16S and 23S genes, and the ISR, was not used to differentiate *S. suis* strains. On the other hand, Hassan and associates (27) showed size and sequence variations of the ISR but with a limited number of *S. suis* strains.

The objective of our study was to combine these 2 last techniques to develop a new method of differentiating *S. suis* strains in an easily and rapidly reproducible and discriminatory manner. This new molecular typing method would be based on PCR amplification of a larger fragment of rRNA genes, including a part of the 16S and 23S genes, and the 16S–23S rDNA ISR, followed by restriction fragment length polymorphism (RFLP) analysis with *Rsa*I and *Mbo*II endonucleases. We then analyzed the genetic relationships between 138 *S. suis* strains of various serotypes, isolated from swine or human cases.

Materials and methods

Bacterial strains

We studied 138 epidemiologically unrelated strains of *S. suis*: 86 strains isolated from pigs with meningitis, septicemia, or arthritis; 22 strains isolated from clinically healthy pigs, the specimens being from the nasal cavity or palatine tonsil; 29 strains isolated from humans; and reference strain S735 serotype 2, isolated from a diseased pig by one of us (M.G.). Capsular typing revealed serotype 1 (1 strain), serotype 2 (98 strains, 29 from humans and 69 from swine, 1 of the 69 being the reference strain), serotype 1/2 (7 strains), serotype 3 (6 strains), serotype 4 (1 strain), serotype 5 (1 strain), serotype 7 (12 strains), and serotype 9 (10 strains), as previously reported (28); 2 strains were autoagglutinable and thus not typable. The specificity of the ISR-RFLP method was evaluated with 31 strains belonging to 25 other bacterial species related to *S. suis* or isolated from pigs (Table I).

Sixteen *S. suis* strains isolated from pigs at 5 farms were used to evaluate the reproducibility and stability of the ISR-RFLP patterns after 1 to 32 passages on artificial media.

New molecular typing method

Genomic DNA from each strain was prepared as described by Kellog and Kwok (29). Briefly, 20 colonies were suspended in a mixture of 250 μL of 10 mM Tris HCl (pH 8.3), 100 mM KCl, 2.5 mM $MgCl_2$, 1% (v/v) Tween 20 (Sigma-Aldrich Chimie, Saint Quentin Fallavier, France), 1% (v/v) Triton X-100 (Sigma-Aldrich Chimie), 0.01% (v/v) Nonidet P-40 (Sigma-Aldrich Chimie), and 120 μg/mL proteinase K (Sigma-Aldrich Chimie). The suspensions were incubated for 1 h at 60°C and then for 10 min at 95°C to inactivate the proteinase K; after cooling to room temperature, they were kept at −20°C. For each *S. suis* strain used to verify the stability of patterns, 2 independent extractions of DNA were performed.

The template DNA was then amplified with the forward primer 16S-489(f) (5′-TTCTCACTTGACGGTATCTTAC-3′), complementary to the 3′ end of the 16S rRNA gene (AF009477) (30), and the reverse primer 23S-206(r) (5′-GGTACCTTAGATGTTTCAGTTC-3′), complementary to the 5′ end of the 23S rRNA gene (31). The length of the PCR product was approximately 1714 base pairs (bp). The PCR mixture contained PCR buffer (67 mM Tris-HCl, 16 mM $[NH_4]_2SO_4$, 0.01% Tween 20, and 2.5 mM $MgCl_2$ [pH 8.8]), 1.5 mM of deoxyribonucleoside triphosphate (Eurobio, Les Ulis, France), 400 nM of each primer, 2 units of *Taq* DNA polymerase (Eurobio), and 5 μL of the DNA template in a total volume of 50 μL. The DNA template was replaced by double-distilled water for the negative control of the PCR step. Amplification was performed in a GeneAmp PCR system 9600 (Applied Biosystems, Courtaboeuf, France). The reaction procedure consisted of 40 cycles of amplification at 94°C for 30 s, 60°C for 30 s, and 72°C for 5 min, and then final extension at 72°C for 5 min.

Sequencing of the ISR

The 16S–23S rDNA ISR of 5 strains (field strains 298, 332, 347, 353, and reference strain S735) was sequenced. Strains 298 and 332 (serotype 2) had been isolated from cases of septicemia; strain 347 (serotype 2) and strain 353 (serotype 1/2) had been isolated from palatine tonsils of clinically healthy pigs. Characterization by PFGE with the use of *Sma*I endonuclease (21) showed that strains 298, 332, and 353 had the same pattern, whereas strain 347 and reference strain S735 had different patterns.

Nucleotide sequences were determined by cycle sequencing based on the dideoxynucleoside termination chain method described by Sanger (32). The PCR products were purified by means of the QIAquick PCR Purification Kit (Qiagen, Courtaboeuf, France), quantified spectrophotometrically, and sequenced by means of the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit and 373A sequencer (Applied Biosystems), according to the manufacturers' instructions. Two internal primers were designed to sequence totally the 1.7-kb ISR product: Rib-s2 (5′-TTTCTCTTCGGAGCATCGGTGA-3′) and Rib-as2 (5′-GACGTACAGGTTTCCATTTCC-3′).

Analysis of RFLP

To characterize the genetic variations in the ISR of the 138 *S. suis* strains, the ISR products were digested singly with *Rsa*I, *Mbo*II, *Ava*II, *Bsp1286*I, *Acc*II, *Bcn*I, and *Eco0109*I (Amersham, Les Ulis, France; Roche Diagnostics, Meylan, France). The reactions were performed with 15 μL of ISR products as described by the manufacturers.

The ISR products digested with *Mbo*II, *Ava*II, *Bsp1286*I, *Acc*II, *Bcn*I, or *Eco0109*I were separated in a 2% standard agarose gel in TBE buffer (90 mM Tris, 90 mM borate, 2.5 mM EDTA [pH 8]) for 2 h at a constant voltage of 125 V. The ISR products digested with *Rsa*I were separated in a 2.5% low-melting-point agarose gel in TBE buffer for 2.5 h at a constant voltage of 125 V. The patterns were detected by ultraviolet transillumination after ethidium bromide

Table I. Bacterial strains used to test the specificity of a new method of differentiating *Streptococcus suis*

Species	Strains	*n*
Streptococcus agalactiae	ATCC 13813	1
S. acidominimus	NCDO 2025	1
S. alactolyticus	ATCC 43077	1
S. anginosus	ATCC 33397	1
S. bovis	ATCC 33317	1
S. constellatus	ATCC 27823	1
S. difficilis	ATCC 51487	1
S. gordonii	ATCC 10558	1
S. hyointestinalis	CCUG 27888	1
S. intestinalis	ATCC 43492	1
S. pneumoniae	ATCC 33400	1
S. porcinus	ATCC 43138 and field strain	2
S. pyogenes	ATCC 12344	1
Escherichia coli	Field strains	2
Campylobacter jejuni	Field strain	1
Campylobacter coli	Field strain	1
Mycoplasma hyopneumoniae	ATCC 25934 and field strain	2
Mycoplasma hyosynoviae	ATCC 25591 and field strain	2
Mycoplasma hyorhinis	ATCC 17981 and field strain	2
Mycoplasma flocculare	ATCC 27399	1
Actinobacillus pleuropneumoniae[a]	ATCC 27088 and field strain	2
Actinobacillus lignieresii	ATCC 49236	1
Actinobacillus rossi	ATCC 27072	1
Pasteurella multocida	Field strain	1
Staphylococcus aureus	Field strain	1

ATCC — American Type Culture Collection, Rockville, Maryland, USA; NCDO — National Collection of Dairy Organisms, Shinfield, Reading, England; CCUG — Culture Collection, University of Göteborg, Göteborg, Sweden

[a] Serotype 1

staining. A 50-bp "ladder" was used as a molecular size standard (Pfizer, Paris, France).

The patterns were digitized and analyzed with use of the Biogene package (Vilber-Lourmat, Marne la Vallée, France) as previously described (21). The unweighted pair group method with arithmetic mean was used with a confidence interval of 8%. The numerical index of discrimination described by Hunter and Gaston (33), calculated with *Rsa*I or *Mbo*II alone or in combination, was used to rate the discriminatory power of the ISR-RFLP method.

The relationships between the patterns of strains isolated from diseased subjects versus clinically healthy pigs, including serotype and origin (pig or human), were analyzed with the Fisher exact test ($n \leq 5$) or the chi-squared test ($n > 5$) of independence in 2×2 tables by means of the Systat 9.0 program for Windows. Differences were considered statistically significant when the *P*-value was less than 0.05.

Results

Specificity of the ISR-RFLP method

When the microorganisms listed in Table I were used as DNA templates, no amplification product was observed (data not shown).

Characterization of the 16S–23S ISR of 5 *S. suis* strains

Sequences spanning the 3′ end of the 16S gene, the 16S–23S rISR, and the 5′ end of the 23S gene of the 5 *S. suis* strains studied were deposited in GenBank under the accession numbers AY585196, AY585197, AY585198, AY585199, and AY585200. The sequencing allowed us to determine the exact size of each ISR: for strains 298, 332, and 353, 424 bp; for strain 347, 425 bp; and for strain S735, 401 bp. The multiple-alignment result of the 5 nucleotide sequences is shown in Figure 1. The 5 sequences were 84% identical; the sequences of strains 298, 332, 347, and 353 were 98% identical.

The ISR had distinct subregions with an ordered relationship. As previously described (24), these regions were defined after multiple alignment with the ISR sequence of *S. equi* (AF489598). The number of regions varied from 7 to 8. Region R1 (positions 1016 to 1039 of the multiple alignment), following the end of the 16S rRNA gene, and region 2 (positions 1040 to 1066) were present and identical in all 5 strains. Region R3 was not found among the 5 isolates. Region R4 (positions 1067 to 1140) was present in all 5 strains and was 100% identical to the sequence for the transfer (t) RNA gene for alanine found in several other streptococcal ISRs and 97% identical to the DNA sequence of *Staphylococcus aureus* coding for $tRNA^{Ala}$

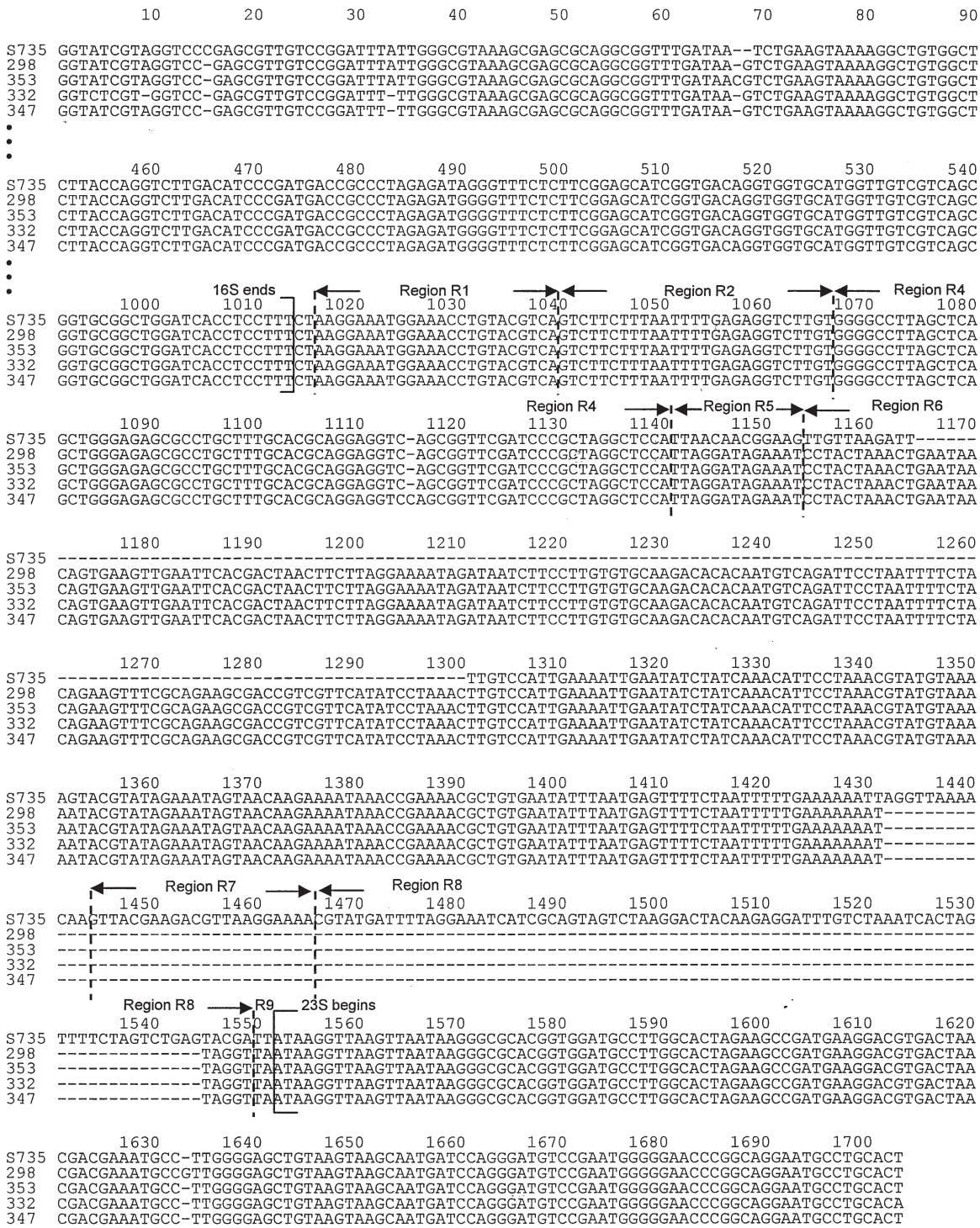


Figure 1. Multiple-sequence alignment of 5 strains of *Streptococcus suis*. The nucleotide sequence numbers are given from a consensus alignment. Dashes indicates spacers between adjacent nucleotides introduced for maximum alignment. Arrows indicate the 3′ end of the 16S ribosomal (r) DNA gene (position 1013), the 5′ end of the 23S rDNA gene (1552), and the 8 regions of the 16S–23S intergenic spacer region (ISR) (1014 to 1551): R1 (1016 to 1039), R2 (1040 to 1066), R4 (1067 to 1140; identical to the transfer RNA^{Ala} gene), R5 (1141 to 1153), R6 (1154 to 1443), R7 (1444 to 1465), R8 (1466 to 1549), and R9 (1550 to 1551), as defined by Chanter and coworkers (24).

(NC_002758). The size of R4 was 73 bp for strains S735, 298, 332, and 353 and 74 bp for strain 347. The 13-bp region R5 (positions 1141 to 1153) was present in all 5 strains, was 54% homologous for all the strains, and was identical in strains 298, 332, 347, and 353. Region R6 (positions 1154 to 1443) was the longest (154 to 278 bp) and was identical in all the strains except S735, which showed a deletion in positions 1165 to 1300 and an insertion in positions 1432 to 1443. The 22-bp region R7 (positions 1444 to 1465) was present only in strain S735. Region R8 (positions 1466 to 1549) was present in all the strains but was only 5 bp in strains 298, 332, 347, and 353, whereas it was

Table II. Number of restriction sites of the endonucleases considered for restriction fragment length polymorphism (RFLP) analysis observed in the 16S–23S intergenic spacer region (ISR) of 5 strains of *S. suis*

	Recognition sequence	Strain; number of sites				
Endonuclease	5′–3′	S735	298	332	347	353
*Rsa*I	GT↓AC	8	5	5	5	5
*Mbo*II	$GAAGA(N)_8$↓	4	4	4	4	4
*Ava*II	G↓G(A,T)CC	2	2	2	3	2
*Bsp*1286I	G(A,G,T)GC(A,C,T)↓C	3	2	2	2	2
*Acc*II	CG↓CG	4	4	3	4	4
*Bcn*I	CC(G,C) ↓GG	4	4	4	4	4
*Eco0109*I	(A,G)G↓GNCC(C,T)	4	3	3	3	3

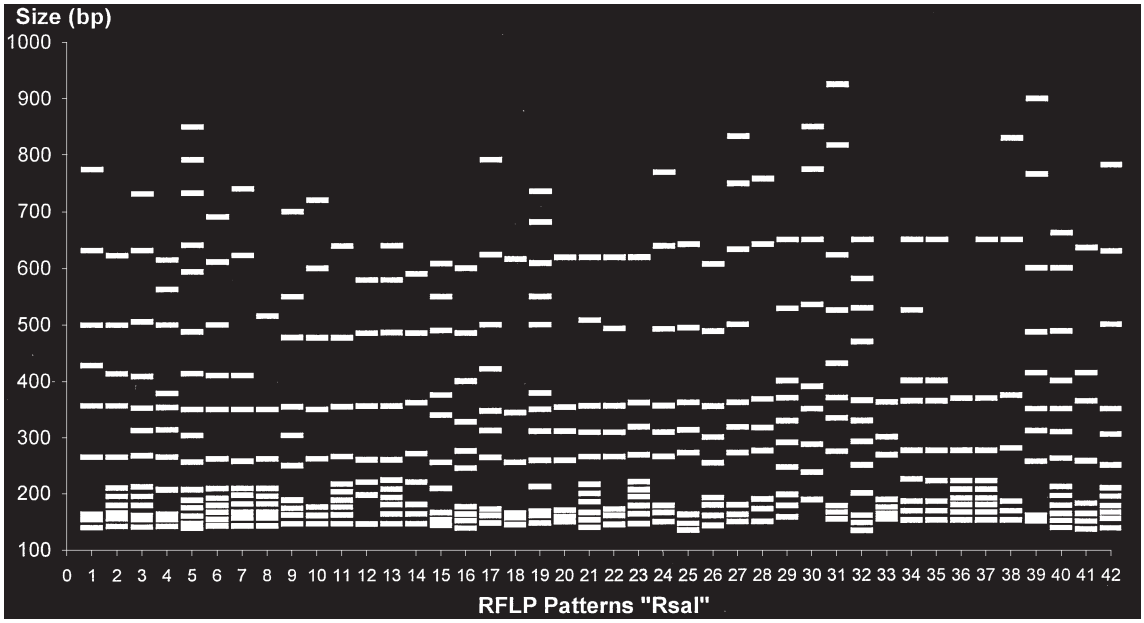


Figure 2. Schematic representation of the 42 patterns obtained by restriction fragment length polymorphism (RFLP) after restriction of the 16S–23S ISR of 138 *S. suis* strains with the endonuclease *Rsa*I. bp — base pairs.

84 bp in strain S735. Region R9 (positions 1550 to 1551) was present and identical in all 5 strains.

The ISR sequences of the 5 strains did not share significant homology with the sequences of any proteins in GenBank.

Choice of endonucleases for RFLP analysis

The positions of the *Rsa*I, *Mbo*II, *Ava*II, *Bsp1286*I, *Acc*II, *Bcn*I, and *Eco0109*I restriction sites were determined in the 16S–23S ISR of the same 5 strains (Table II). Only endonucleases with 4 or 5 restriction sites in the ISR (*Rsa*I, *Acc*II, *Bcn*I, *Eco0109*I, and *Mbo*II) were selected for the RFLP analysis. To further select endonucleases that would give a good distribution of restriction length fragments, the ISR-RFLP method was initially performed on 10 epidemiologically unrelated *S. suis* strains. All patterns obtained with *Acc*II, *Bcn*I, and *Eco0109*I digestion were composed of 1 to 4 fragments, whereas *Rsa*I and *Mbo*II digestion resulted in fragments of a large range of sizes and was therefore chosen for typing the 138 *S. suis* strains.

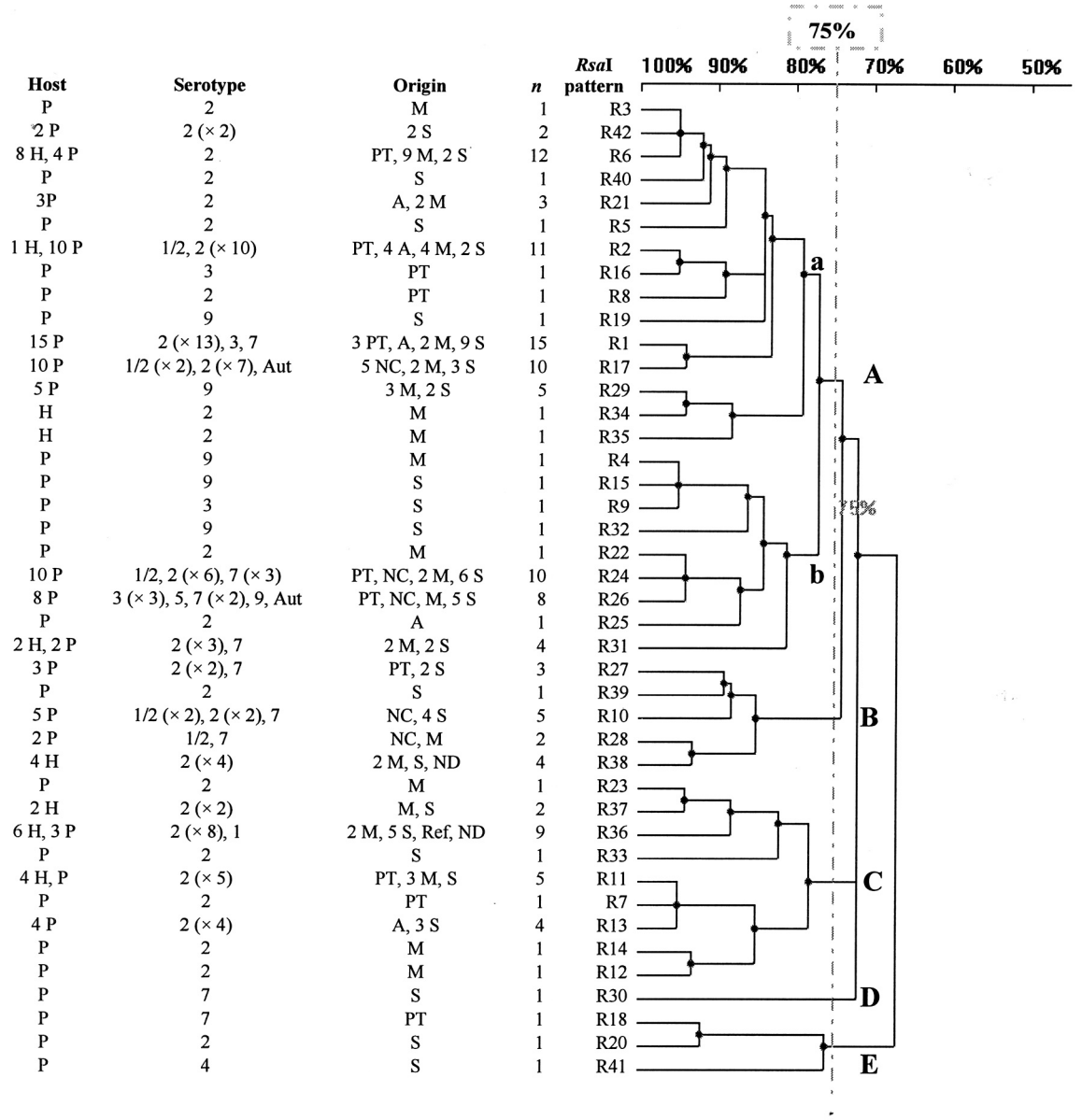
Stability of ISR-RFLP patterns

Under the conditions used, the in vitro stability of the ISR-RFLP patterns, estimated with strains isolated from different farms after 1 to 32 passages on artificial media, was 100% (results not shown). Moreover, this molecular typing method was reproducible: a similar pattern was shown for each strain after the 2 independent DNA extractions.

Genetic diversity of *S. suis* as defined by ISR-RFLP analysis

The *Rsa*I ISR-RFLP patterns were composed of 6 to 21 fragments of 134 to 925 bp; 42 patterns were identified among the epidemiologically unrelated 138 *S. suis* strains analyzed. Figure 2 schematically represents all the patterns, and Figure 3 presents the deduced relationships among the strains. The 138 strains diverged by up to 33% (67% homology). At 72% homology, 5 groups (A to E) were identified. At 75% homology, group A was divided into subgroups a and b. Some fragments were present in multiple patterns; there were 3 potential markers: a 700-bp fragment, a 500-bp fragment, and 6 fragments (sextuplet) of 140, 155, 165, 175, 195, and 224 bp (± 8%) (Figure 2).

The *Mbo*II ISR-RFLP patterns were composed of 1 to 9 fragments of 493 to 1717 bp; 24 patterns were identified among the epidemiologically unrelated 138 *S. suis* strains analyzed. Figure 4



Host	Serotype	Origin	*n*	*Rsa*I pattern
P	2	M	1	R3
2 P	2 (× 2)	2 S	2	R42
8 H, 4 P	2	PT, 9 M, 2 S	12	R6
P	2	S	1	R40
3P	2	A, 2 M	3	R21
P	2	S	1	R5
1 H, 10 P	1/2, 2 (× 10)	PT, 4 A, 4 M, 2 S	11	R2
P	3	PT	1	R16
P	2	PT	1	R8
P	9	S	1	R19
15 P	2 (× 13), 3, 7	3 PT, A, 2 M, 9 S	15	R1
10 P	1/2 (× 2), 2 (× 7), Aut	5 NC, 2 M, 3 S	10	R17
5 P	9	3 M, 2 S	5	R29
H	2	M	1	R34
H	2	M	1	R35
P	9	M	1	R4
P	9	S	1	R15
P	3	S	1	R9
P	9	S	1	R32
P	2	M	1	R22
10 P	1/2, 2 (× 6), 7 (× 3)	PT, NC, 2 M, 6 S	10	R24
8 P	3 (× 3), 5, 7 (× 2), 9, Aut	PT, NC, M, 5 S	8	R26
P	2	A	1	R25
2 H, 2 P	2 (× 3), 7	2 M, 2 S	4	R31
3 P	2 (× 2), 7	PT, 2 S	3	R27
P	2	S	1	R39
5 P	1/2 (× 2), 2 (× 2), 7	NC, 4 S	5	R10
2 P	1/2, 7	NC, M	2	R28
4 H	2 (× 4)	2 M, S, ND	4	R38
P	2	M	1	R23
2 H	2 (× 2)	M, S	2	R37
6 H, 3 P	2 (× 8), 1	2 M, 5 S, Ref, ND	9	R36
P	2	S	1	R33
4 H, P	2 (× 5)	PT, 3 M, S	5	R11
P	2	PT	1	R7
4 P	2 (× 4)	A, 3 S	4	R13
P	2	M	1	R14
P	2	M	1	R12
P	7	S	1	R30
P	7	PT	1	R18
P	2	S	1	R20
P	4	S	1	R41

Figure 3. Genetic relationships among the 138 *S. suis* strains, estimated by clustering analysis of RFLP patterns obtained after restriction of the 16S–23S ISR with *Rsa*I. The human or swine origin, serotype, and tissue origin of the strains, number of strains for each pattern, and level of homology (%) among the strains are reported in the dendrogram. P — pig; H — human; Aut — autoagglutinable; M — meningitis; S — septicemia; PT — palatine tonsil; A — arthritis; NC — nasal cavity; ND — not done; Ref — reference strain.

schematically represents all the patterns, and Figure 5 presents the deduced relationships among the strains. The 138 strains diverged by up to 66% (34% homology). At 34% homology, 2 groups (A and B) were identified. At 72% homology, group A was divided into subgroups a, b, and c.

Table III presents the ISR-RFLP characterization of the 138 strains.

Distribution of ISR-RFLP patterns and groups in relation to *S. suis* tissue source

Among the 113 strains isolated from diseased pigs and humans, 38 *Rsa*I patterns and 23 *Mbo*II patterns were identified. After restriction with *Rsa*I, 78 of the 113 strains were observed to be in group A, and after restriction with *Mbo*II, 83 of the 113 were observed to be in subgroups a and b. No significant association was observed between the tissue from which the strains were isolated and *Rsa*I or *Mbo*II group.

Among the 22 strains isolated from the nasal cavity or palatine tonsil of healthy pigs, 14 *Rsa*I patterns and 11 *Mbo*II patterns were identified. In the 2 dendrograms, these strains were not clustered in a specific group. However, patterns R17 and M8 were significantly associated with the strains isolated from the healthy pigs ($P = 0.011$ and 0.028, respectively).

The 3 potential markers (700-bp fragment, 500-bp fragment, and sextuplet) observed after restriction with *Rsa*I were not significantly associated with the tissue source of the strains.

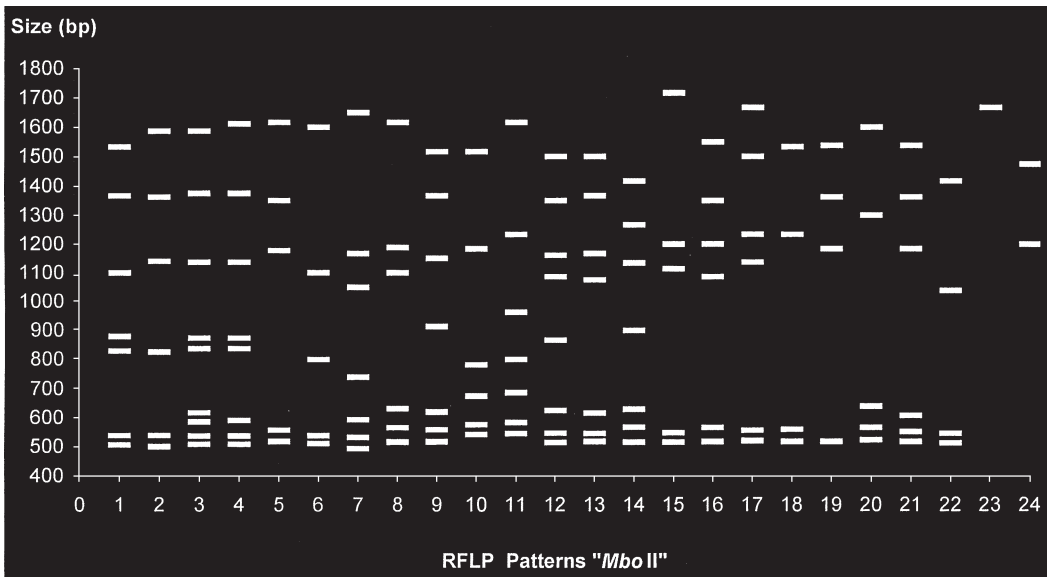


Figure 4. Schematic representation of the 24 RFLP patterns obtained after restriction of the 16S–23S ISR of the 138 *S. suis* strains with the endonuclease *Mbo*II.

Distribution of ISR-RFLP patterns and groups in relation to *S. suis* serotype

Among the 98 strains of serotype 2, 30 *Rsa*I patterns and 23 *Mbo*II patterns were identified. The dendrogram obtained after restriction with *Rsa*I (Figure 3) showed that 53 of the 98 strains were in subgroup a and 24 in group C; the relationships between these strains and groupings were significant ($P = 0.021$ and 0.002, respectively). The dendrogram obtained after restriction with *Mbo*II (Figure 5) showed that 32 of the 98 strains were in subgroup c, and the relationships between these strains and subgroup c were significant ($P = 0.006$). The patterns R6 and M9 were significantly associated with *S. suis* serotype 2 strains ($P = 0.019$ and 0.002, respectively).

The *S. suis* capsular types 1, 1/2, 3, 4, 5, 7, and 9 were clustered in subgroup b ($P < 0.001$) in the dendrogram obtained after restriction with *Rsa*I. Significant associations were observed between these strains and patterns R26 ($P < 0.001$), R29 ($P = 0.002$), M15 ($P = 0.018$), and M22 ($P < 0.001$).

The sextuplet observed in the *Rsa*I patterns was detected in 54 of the 98 *S. suis* serotype 2 strains and in 2 of the 38 *S. suis* strains of serotypes 1, 1/2, 3, 4, 5, 7, and 9 ($P < 0.001$). The other potential markers were not significantly associated with serotype.

Distribution of ISR-RFLP patterns and groups in relation to *S. suis* host species

The *S. suis* strains isolated from humans were genetically less diverse than those isolated from pigs. After restriction with *Rsa*I, 9 patterns were identified among the 29 strains isolated from humans, whereas 38 patterns were identified among the 109 strains isolated from pigs. Of the 29 strains isolated from humans, 12 (41%) were in group C, whereas of the 109 strains isolated from pigs, 96 (88%) were distributed among the other groups ($P < 0.001$). The patterns R6, R36, R11, and R38 were significantly associated with strains isolated from humans ($P < 0.001$, $P = 0.003$, $P = 0.007$, and $P = 0.002$, respectively), because they were identified in 22 (76%) of the 29 strains. The 700-bp fragment was detected in 8 (26%) of the 29 strains isolated from humans and in 5 (4%) of the 109 strains isolated from pigs ($P < 0.001$). The 500-bp fragment was observed in 13 (45%) of the 29 strains isolated from humans and in 88 (81%) of the 109 strains isolated from pigs ($P < 0.001$). The sextuplet was not significantly associated with the host species.

After restriction with *Mbo*II, 8 patterns were identified in the 29 strains isolated from humans, whereas 21 patterns were identified in the 109 strains isolated from pigs. Of the 29 strains isolated from humans, 20 (69%) were in subgroup c ($P < 0.001$) and 24 (83%) had the patterns M10, M2, and M9 ($P < 0.001$, $P = 0.034$, and $P < 0.001$, respectively).

The new method as an epidemiologic tool

An epidemiologic tool would be valuable in investigating cross-colonization or possible epidemic invasive *S. suis* infections. Therefore, the discriminatory power of the ISR-RFLP method was evaluated for 3 classes of isolates: all *S. suis* strains; strains isolated from cases of meningitis, septicemia, or arthritis; and serotype 2 strains. Table IV shows that the index of discrimination was greater than 0.95 with *Rsa*I alone and with *Rsa*I and *Mbo*II in combination for the 3 populations.

Discussion

In our study, the ISR-RFLP method and the sequencing of 5 *S. suis* strains (the reference strain S735 and 4 field strains) showed a size polymorphism of the ISR: from 401 to 425 bp. Gel electrophoresis of the amplified ISR from these 5 strains revealed only 1 band, and the sequence data showed a quality compatible with the presence of a single template. This suggests that the copies of this operon in the chromosome of *S. suis* are homogeneous. In some bacteria, there

Table III. Characterization of the 138 *S. suis* strains by the ISR-RFLP method with the use of *Rsa*I and *Mbo*II[a]

Strain	Serotype	Origin	Host	*Rsa*I pattern	*Mbo*II pattern	Strain	Serotype	Origin	Host	*Rsa*I pattern	*Mbo*II pattern
4	2	S	P	R2	M23	144	2	A	P	R21	M5
9	2	S	P	R1	M8	145	2	M	P	R21	M5
12	2	M	P	R3	M9	147	2	M	P	R21	M5
13	9	M	P	R4	M5	148	2	M	P	R22	M15
18	2	S	P	R1	M18	149	2	M	P	R23	M5
20	2	M	P	R1	M8	152	2	NC	P	R24	M15
24	2	S	P	R2	M12	165	2	M	P	R14	M16
25	2	S	P	R1	M18	167	2	A	P	R25	M15
27	2	S	P	R5	M19	171	2	S	P	R1	M8
29	2	M	P	R2	M21	174	Aut	NC	P	R26	M18
30	2	M	P	R2	M8	176	7	PT	P	R27	M8
33	2	M	P	R2	M2	181	7	NC	P	R28	M20
36	2	S	P	R1	M8	191	9	S	P	R29	M5
39	2	S	P	R1	M8	193	9	S	P	R29	M15
40	2	A	P	R2	M21	194	9	M	P	R29	M15
41	2	A	P	R2	M13	195	9	M	P	R29	M16
42	2	A	P	R2	M21	196	7	M	P	R24	M15
43	2	A	P	R1	M8	198	7	M	P	R1	M15
44	2	A	P	R2	M8	199	7	M	P	R24	M15
54	2	M	H	R6	M5	201	9	M	P	R29	M5
56	1/2	PT	P	R2	M18	202	7	S	P	R24	M15
59	2	PT	P	R1	M17	203	7	S	P	R26	M22
62	2	PT	P	R1	M15	204	7	S	P	R30	M15
63	2	PT	P	R7	M23	205	7	S	P	R31	M15
64	2	PT	P	R6	M5	206	7	S	P	R26	M22
65	2	PT	P	R8	M18	208	9	M	P	R26	M22
69	3	PT	P	R1	M8	209	3	S	P	R26	M22
71	2	S	P	R1	M24	210	3	S	P	R26	M22
78	2	S	P	R1	M8	211	3	S	P	R26	M22
79	3	S	P	R9	M22	212	9	S	P	R32	M5
80	7	NC	P	R10	M8	215	1/2	M	P	R28	M15
81	1/2	S	P	R10	M15	261	2	M	H	R6	M9
86	2	S	P	R11	M17	262	2	M	H	R6	M9
89	2	A	P	R13	M5	263	2	M	H	R6	M9
90	1/2	S	P	R10	M15	264	2	M	H	R6	M9
91	2	S	P	R10	M20	265	2	M	H	R6	M9
93	2	M	P	R12	M5	266	2	M	H	R6	M9
100	1/2	S	P	R17	M8	267	2	M	H	R6	M9
102	9	S	P	R19	M12	268	2	M	H	R34	M9
103	2	S	P	R20	M15	269	2	M	H	R11	M9
104	9	S	P	R15	M13	270	2	M	H	R35	M9
105	2	S	P	R1	M8	271	2	M	H	R11	M9
106	3	PT	P	R16	M22	272	2	ND	H	R36	M9
109	1/2	NC	P	R17	M8	273	2	S	H	R36	M2
114	7	PT	P	R18	M20	274	2	M	H	R11	M2
117	2	S	P	R17	M8	275	2	M	H	R37	M9
118	2	S	P	R17	M8	276	2	M	H	R36	M9
127	2	NC	P	R17	M8	277	2	S	H	R37	M2
130	2	NC	P	R17	M8	278	2	M	H	R38	M10
131	Aut	NC	P	R17	M8	279	2	ND	H	R38	M10
132	2	NC	P	R17	M24	280	2	S	H	R38	M10
135	2	M	P	R17	M15	281	2	S	H	R36	M9
138	2	M	P	R17	M15	282	2	PT	H	R11	M9

Table III. *(concluded)*

Strain	Serotype	Origin	Host	*Rsa*I pattern	*Mbo*II pattern	Strain	Serotype	Origin	Host	*Rsa*I pattern	*Mbo*II pattern
298	2	S	P	R24	M8	365	5	PT	P	R26	M6
301	2	S	P	R33	M6	366	2	S	P	R27	M15
302	2	S	P	R36	M2	372	2	S	P	R27	M18
304	2	M	H	R36	M2	374	2	S	P	R42	M5
306	2	S	H	R36	M6	376	2	S	P	R42	M5
316	1	S	P	R36	M2	380	2	S	P	R39	M15
321	2	M	H	R31	M3	390	2	S	P	R13	M5
322	2	M	H	R31	M4	394	2	S	P	R24	M8
327	2	S	P	R6	M9	395	2	S	P	R13	M9
328	2	S	P	R6	M9	398	2	S	P	R24	M8
333	2	M	H	R38	M2	400	2	S	P	R24	M15
335	4	S	P	R41	M3	405	2	S	P	R40	M2
337	2	S	P	R24	M7	407	2	S	P	R13	M2
343	2	S	P	R10	M11	735	2	Ref	P	R36	M1
347	2	S	P	R31	M15	166'	2	M	P	R6	M16
353	1/2	PT	P	R24	M8	303	2	M	H	R2	M14

[a] P — pig; H — human; Aut — autoagglutinable; M — meningitis; S — septicemia; PT — palatine tonsil; A — arthritis; NC — nasal cavity; ND — not done; Ref — reference strain

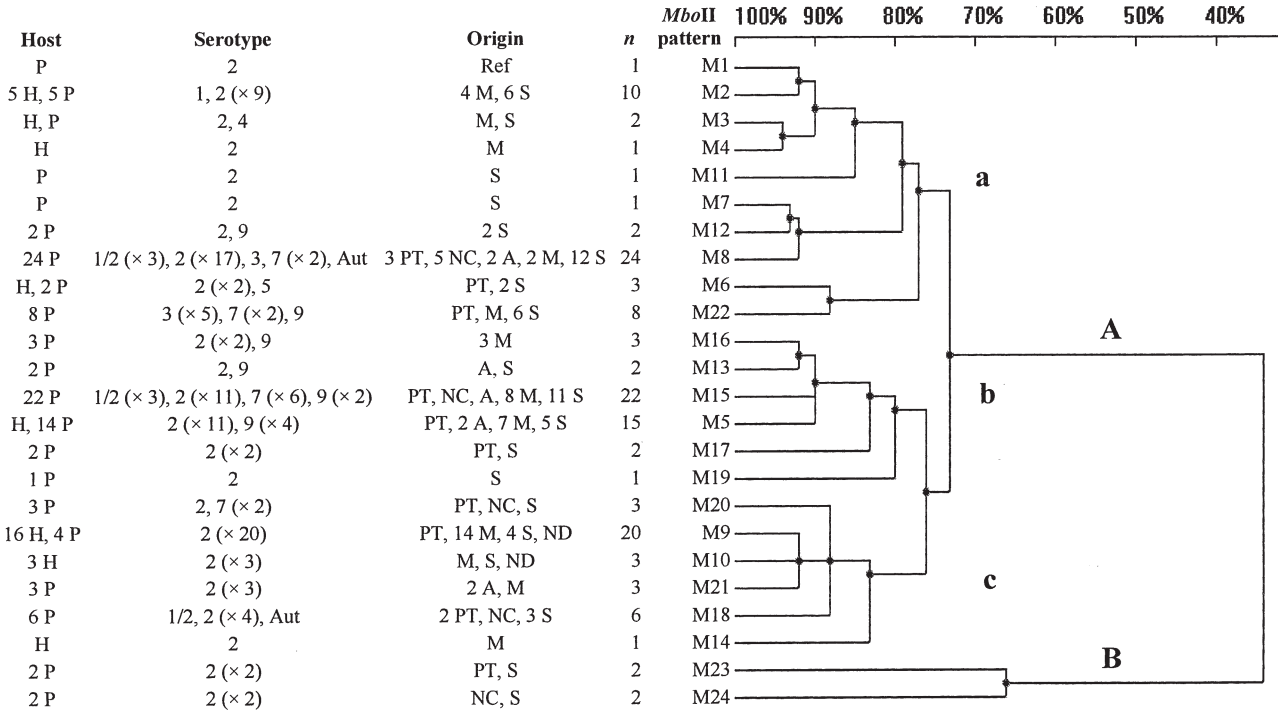


Figure 5. Genetic relationships among the 138 *S. suis* strains, estimated by clustering analysis of RFLP patterns obtained after restriction of the 16S–23S ISR with *Mbo*II.

are different versions of the operon in the same cell, so that there is more than 1 PCR product when DNA is amplified from primers in the 16S and 23S genes (34). In our study, the structure of the spacers appeared to be dominated by 3 variable regions: R6, R7, and R8. We noticed some differences between our results and those of Hassan and associates (27); for example, in the S735 strain, the ISR size (401 bp in our study versus 406 bp in the study of Hassan and associates), the size of regions R5 (13 bp versus 17 bp), R6 (154 bp versus 125 bp), R8 (84 bp versus 95 bp), and R9 (2 bp versus 45 bp), and the fact that region R7 (22 bp in our study) was not described by Hassan and associates. According to Chanter and coworkers (24), the occurrence of strains with very distinct spacer regions might be caused by DNA recombination. Moreover, the S735 strain is 40 y old and was subcultured many times. In our study, among the 4 field strains (serotype 2 or 1/2) isolated from disease cases or healthy pigs, no significant size or sequence polymorphism of the ISR was observed. The serotype 2 strain isolated from palatine tonsil of 1 healthy pig (strain 347), which had a PFGE pattern distinct from

Table IV. Numerical index of discrimination of the ISR-RFLP method, calculated from data for 1 or 2 endonucleases, in typing the total population and subsets of *S. suis* isolates

	Endonuclease; index		
Population	*Rsa*I	*Mbo*II	*Rsa*I + *Mbo*II
Total (n = 138)	0.954	0.904	0.984
Strains from disease cases (n = 113)	0.955	0.900	0.982
Serotype 2 strains (n = 98)	0.954	0.897	0.980

the patterns of the 3 other field strains, had an ISR of 425 bp (versus 424 bp in the other field strains) and a region R4, encoding the tRNA for alanine, of 74 bp (versus 73 bp). Therefore, molecular typing of *S. suis* strains on the basis of characterization of a fragment of rRNA genes, including a part of the 16S and 23S genes and the 16S–23S rDNA ISR, appears to be a potential method to characterize *S. suis* serotype 2.

Sequencing of the ISR cannot be used routinely with a large number of strains; thus, a PCR-RFLP analysis of the spacer 16S–23S rDNA was developed to characterize *S. suis* strains. This approach had previously been used to differentiate species of streptococci (25), including *S. suis*, but not to characterize *S. suis* strains. Under the conditions described in this paper, this technique was discriminatory with *Rsa*I and with the combination of *Rsa*I and *Mbo*II. The ISR-RFLP method is also reproducible: a similar pattern was shown for each strain after the 2 independent DNA extractions and for some strains after 1 to 32 passages on artificial media. The location of the ISR, flanked by the highly conserved 16S rRNA and 23S rRNA genes, allows a specific amplification of the spacer region of *S. suis* and good reproducibility.

Using the ISR-RFLP method with *Rsa*I and *Mbo*II to analyze a collection of 138 independent *S. suis* strains (86 isolated from diseased pigs, 22 from healthy pigs, and 29 from humans, most of whom had meningitis, as well as a reference strain from a pig), we confirmed that strains isolated from humans are genetically more homogeneous than strains isolated from pigs (21). Most strains isolated from humans are clustered in group C (*Rsa*I), subgroup c (*Mbo*II), and have the *Rsa*I patterns R6, R36, R11, and R38 and the *Mbo*II patterns M10, M2, and M9. A 700-bp fragment is present more frequently in these strains than in strains isolated from pigs. As observed by PFGE (21) and in this study, some *S. suis* strains isolated from humans and pigs show the same pattern.

We found that the *S. suis* serotype 2 strains were significantly associated with the same clusters (subgroup a and group C with *Rsa*I and subgroup c with *Mbo*II) and with 2 molecular patterns (R6 and M9). A marker of serotype 2 was described, a fragment sextuplet of 140 to 224 bp, observed after restriction with *Rsa*I. This marker seems to be a good indicator of strain virulence, because among the 98 strains of serotype 2, 88% were isolated from cases of septicemia, meningitis, or arthritis; serotype 2 has been considered the most virulent and prevalent type isolated from diseased pigs (7–9). Two patterns were significantly associated with strains isolated from healthy pigs (R17 and M8). However, the *S. suis* strains corresponding to these patterns cannot be considered potentially avirulent, because these patterns were also observed in strains isolated from cases of septicemia, meningitis, or arthritis. Moreover, it would be necessary to confirm this hypothesis by analyzing a new collection of *S. suis* strains isolated from healthy pigs and to test them in a standardized virulence model.

In conclusion, this genetic tool could be valuable in distinguishing individual isolates of *S. suis* to establish the origin of the infection in a herd and to monitor the kinetics of an outbreak.

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