

Comparison of Different PCR Approaches for Typing of *Francisella tularensis* Strains

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In this study, we evaluated three PCR methods for epidemiological typing of *Francisella tularensis*: repetitive extragenic palindromic element PCR (REP-PCR), enterobacterial repetitive intergenic consensus sequence PCR (ERIC-PCR), and random amplified polymorphic DNA (RAPD) assay with both M13 and T3-T7 primers. The analysis was performed with 40 strains of *F. tularensis* isolated from hares, humans, ticks, and a vole. On the basis of the combination of REP, ERIC, and RAPD fingerprints, *F. tularensis* strains were divided into 17 genetic groups (designated A to Q), and one *Francisella novicida* strain was classified in group R. The *F. novicida* strain is of special concern, since previous genetic methods have been unable to clearly distinguish between *F. tularensis* and *F. novicida*. The *F. tularensis* isolates recovered from hares were included in groups A to J, M, and P; those recovered from humans were included in groups A, D, G, J, L, O, and N; those isolated from ticks were included in groups B and Q; and that recovered from a vole was in group K. The diversities calculated for the 40 *F. tularensis* isolates, according to Simpson's index, were 0.14 for REP-PCR, 0.52 for ERIC-PCR, 0.39 for RAPD assay with the M13 primer (RAPD/M13-PCR), and 0.65 for RAPD/T3-T7-PCR, and the diversity increased up to 0.90 when ERIC-PCR, RAPD/M13-PCR, and RAPD/T3-T7-PCR were combined. Our results suggest that although limited genetic heterogeneity among *F. tularensis* strains was observed, this small variation is enough to validate the PCR methods used in this study and their combinations, because they can provide safe, useful, and rapid tools for the typing of *F. tularensis*.

Tularemia is a clinical syndrome caused by the gram-negative, facultative intracellular bacterium *Francisella tularensis*. The agent is extremely infectious, and it is thought that as few as 10 organisms can cause infection in humans (2). A severe epidemic tularemia outbreak occurred in 1998 in northwestern Spain (where the disease had never been reported before), with ulceroganglionic, typhoidal, ganglionic, pneumonic, oculoganglionic, and atypical clinical forms diagnosed in humans (1, 5, 11).

There are four recognized biotypes of the bacterium. *F. tularensis* subsp. *tularensis* (*nearctica*, type A) is the common causal agent of tularemia in North America, and it has been recently observed for the first time in Europe (6). This subspecies is highly virulent, causes severe illness in mammals, and is associated mainly with tick-borne tularemia in rabbits. *F. tularensis* subsp. *palaeartica* (*holarctica*, type B) is more widely distributed in nature and is found in Europe, Asia, and to a minor extent in North America. This subspecies is linked to waterborne disease of rodents and hares, and it is considered to be less pathogenic for mammals than *F. tularensis* subsp. *tularensis* (15). *F. tularensis* subsp. *mediaasiatica* has been found only in central Asia in a part of the former Soviet Union, while *F. tularensis* subsp. *palaeartica japonica* has been isolated only in Japan (14).

The correct identification of strains of *F. tularensis* can be verified by agglutination with specific antisera. However, since strains of *Francisella* have similar antigenic compositions (to date all of the isolates belong to a unique antigenic group), it is not possible to distinguish the different subspecies or types

from each other by this method (12). Subspecies of *F. tularensis* have been differentiated by biochemical tests (10) and by using probes specific to the 16S rRNAs of each of the two main subspecies (4, 14).

Epidemiological analysis of the 1998 Spanish outbreak was impeded by the lack of a safe, rapid, and easy method to type the isolates. Therefore, there is a need to develop new typing methods for this bacterial species. Repetitive element sequence-based PCR (rep-PCR) is a group of methods which generate DNA fingerprints that allow discrimination between bacterial strains. Two main sets of repetitive elements are used for typing purposes. The repetitive extragenic palindromic (REP) elements are 38-bp sequences consisting of six degenerate positions and a 5-bp variable loop between each side of a conserved palindromic stem. The enterobacterial repetitive intergenic consensus (ERIC) sequences are a second set of DNA sequences which have been successfully used for DNA typing. ERIC sequences are 126-bp elements which contain a highly conserved central inverted repeat and are located in extragenic regions of the bacterial genome (13). On the other hand, the random amplified polymorphic DNA (RAPD) assay is based on the use of short random sequence primers, about 10 to 20 bases in length, which hybridize with sufficient affinity to chromosomal DNA sequences at low annealing temperatures such that they can be used to initiate amplification of regions of the bacterial genome (13).

REP, ERIC, and RAPD sequences have been used as primer binding sites to amplify the genomes of a variety of bacteria by PCR. In this report, rep-PCR and RAPD were used to generate DNA fingerprints of *F. tularensis* strains isolated from hares, humans, voles, and ticks in Spain and to evaluate the potential of PCR methods for typing *F. tularensis* isolates of various origins.

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TABLE 1. Characteristics of *F. tularensis* strains and the *F. novicida* strain used in this study and ERIC-PCR, REP-PCR, and RAPD types

Strain designation		Host and geographic origin	REP-PCR type	ERIC-PCR type	RAPD type (M13 primer)	RAPD type (T3-T7 primers)	Global type
This study	Source ^a						
1	ALG1	Hare; Valladolid, Spain	1	1	1	1	A (1111)
2	ALG2	Hare; Valladolid, Spain	1	2	1	1	B (1211)
3	ALG3	Hare; Valladolid, Spain	1	2	1	1	B (1211)
4	ALG4	Hare; León, Spain	1	1	1	2	C (1112)
5	ALG5	Hare; León, Spain	1	1	1	1	A (1111)
6	ALG6	Hare; Palencia, Spain	1	2	1	1	B (1211)
7	ALG7	Hare; León, Spain	1	2	1	1	B (1211)
8	ALG8	Hare; Palencia, Spain	1	2	1	1	D (1212)
9	ALG9	Hare; Palencia, Spain	1	2	1	1	B (1211)
10	ALG10	Hare; Valladolid, Spain	1	1	1	2	C (1112)
11	ALG11	Hare; Valladolid, Spain	1	1	1	3	E (1113)
12	ALG12	Hare; Zamora, Spain	1	2	1	1	B (1211)
13	ALG13	Hare; Palencia, Spain	1	1	1	1	A (1111)
14	ALG14	Hare; Valladolid, Spain	1	2	1	1	B (1211)
15	ALG15	Hare; Valladolid, Spain	2	1	2	1	F (2121)
16	ALG16	Hare; Segovia, Spain	1	1	1	2	A (1111)
17	ALG17	Hare; Palencia, Spain	1	2	1	4	G (1214)
18	ALG18	Hare; Palencia, Spain	1	1	1	4	H (1114)
19	ALG19	Hare; Palencia, Spain	2	1	2	3	I (2123)
20	ALG20	Hare; Palencia, Spain	1	1	1	3	E (1113)
21	ALG21	Vole (<i>Microtus arvalis</i>); Zamora, Spain	1	1	1	5	K (1115)
22	HLE1	Human; León, Spain	1	2	1	3	J (1213)
23	HZA1	Human; Zamora, Spain	1	2	1	4	G (1214)
24	HZA2	Human; Zamora, Spain	1	2	2	3	L (1223)
25	HZA3	Human; Zamora, Spain	1	2	1	2	D (1212)
26	CΔPM1	Hare; strain 130 (= CAPM5536), Czech Republic	1	1	3	1	M (1131)
27	CΔMP2	Hare; strain 2713 (= CAPM5537), Czech Republic	1	1	3	1	M (1131)
28	CAMP3	Human; strain SCHU (= CAPM 5600), United States	3	3	1	6	N (3316)
29	ALG24	Human; Valladolid, Spain	1	1	4	1	O (1141)
30	ALG25	Human; Valladolid, Spain	1	1	4	1	O (1141)
31	ALG26	Human; Valladolid, Spain	1	1	1	1	A (1111)
32	ALG27	Human; Palencia, Spain	1	1	1	1	A (1111)
33	CAPM4	Hare; strain T-1/59 (= CAPM5151), Czech Republic	1	2	3	3	P (1233)
34	RLAH1	Hare; Palencia, Spain	1	2	1	2	D (1212)
35	RLAH2	Hare; Valladolid, Spain	1	2	1	2	D (1212)
36	RLAH3	Hare; Soria, Spain	1	2	1	3	J (1213)
37	RLAH4	Hare; Zamora, Spain	1	2	1	1	B (1211)
38	RLAH5	Hare; Zamora, Spain	1	2	1	1	B (1211)
39	RLAH6	Tick; Zamora, Spain	1	2	1	1	B (1211)
40	CΔPM5	Tick; strain 503, former Soviet Union	1	2	3	1	Q (1231)
41	CΔMP6	<i>F. novicida</i> reference strain ATCC 15482, France	4	4	5	7	R (4457)

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MATERIALS AND METHODS

Bacterial strains and culture conditions. Thirty-five *F. tularensis* isolates recovered from an epidemic tularemia outbreak that occurred in Castilla y León (northwestern Spain) in 1998, as well as some *F. tularensis* strains (26, 27, 28, 33, and 40) and *Francisella novicida* (strain 41) reference strains, were used in this study (Table 1). Of these isolates, 25 were from hares, 8 were from humans, 1 was from a vole, and 1 was from a tick. The geographical origins of these isolates are shown in Table 1. All *F. tularensis* strains except no. 40 were grown at 37°C for 3 days on modified Thayer-Martin agar plates containing GC medium base (36 g/liter), hemoglobin (10 g/liter), and Vitox supplement (2 vials/liter; Oxoid Ltd., Hampshire, England). *F. tularensis* no. 40 and *F. novicida* were grown on Bacto Cystine heart agar (Difco) supplemented with 10% rabbit blood at 37°C for 2 days.

Preparation of genomic DNA. Chromosomal DNAs from the strains listed in Table 1 were obtained by suspension of log-phase cells in 10 mM Tris-1 mM EDTA and were incubated at 37°C for 2 h in 0.5% sodium dodecyl sulfate and 100 µg of proteinase K per ml. The DNA was then purified by repeated phenol-

chloroform extraction, precipitated from the aqueous phase by adding 0.6 volume of isopropanol and 70% ethanol, dried, and resuspended in sterile water.

rep primers and rep-PCR conditions. For REP-PCR, the primers REP 1R-I (5'-IIICGICGICATCIGGC-3') and REP 2-I (5'-ICGICTTATCIGGCCTAC-3') were used, and for ERIC-PCR, the primers ERIC IR (5'-ATGTAAGCTCCTGGGGATTCA-3') and ERIC II (5'-AAGTAAGTGACTGGGGTGAGC-3') were used. The REP 1R-I and REP 2-I primers contain the nucleotide inosine (I) at ambiguous positions in the REP consensus sequence. Inosine can form Watson-Crick base pairs with A, T, G, or C. PCRs were carried out with 5 µl of template DNA per reaction for REP-PCR and ERIC-PCR. Amplification reactions were performed in 50 µl of a solution containing a 1 µM concentration of each of the two opposing primers (Boehringer Mannheim OligoSynthesis, Mannheim, Germany), 0.2 mM each deoxynucleoside triphosphate (Bioline, London, United Kingdom), 2.5 mM MgCl₂, 5 µl of 10× amplification buffer [160 mM (NH₄)₂SO₄, 670 mM Tris-HCl, pH 8.8 at 25°C], and 4 U of *Taq* DNA polymerase (Bioline). Amplifications were performed with a DNA thermocycler (GeneAmp PCR System 2400, Perkin-Elmer, Berlin, Germany). For REP-PCR,

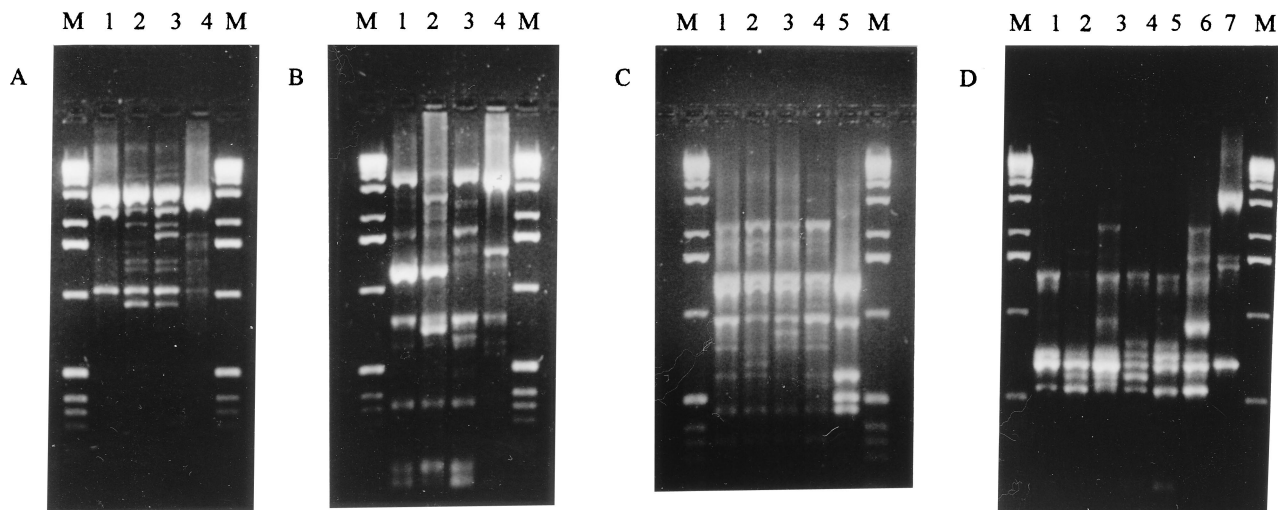


FIG. 1. (A) ERIC-PCR fingerprints of type 1 (lane 1), type 2 (lane 2), type 3 (lane 3), and type 4 (lane 4). Lanes M, molecular size marker X (Roche Diagnostics). (B) REP-PCR fingerprints of type 1 (lane 2), type 2 (lane 1), type 3 (lane 3), and type 4 (lane 4). Lanes M, molecular size marker X (Roche Diagnostics). (C) RAPD fingerprints, with the M13 primer, of type 1 (lane 1), type 2 (lane 2), type 3 (lane 3), type 4 (lane 4), and type 5 (lane 5). Lanes M, molecular size marker X (Roche Diagnostics). (D) RAPD fingerprints, with T3-T7 primers, of type 1 (lane 1), type 2 (lane 2), type 3 (lane 3), type 4 (lane 4), type 5 (lane 5), type 6 (lane 6), and type 7 (lane 7). Lanes M, molecular size marker X (Roche Diagnostics).

the temperature profile was 1 cycle at 95°C for 3 min; 30 cycles at 90°C for 30 s, 50°C for 30 s, 45°C for 1 min, and 65°C for 8 min; and 1 cycle at 65°C for 16 min. For ERIC-PCR, the temperature profile was 1 cycle at 95°C for 5 min; 30 cycles at 90°C for 30 s, 50°C for 30 s, 52°C for 1 min, and 72°C for 1 min; and 1 cycle at 72°C for 8 min.

RAPD primers and RAPD-PCR conditions. For RAPD assay, the primers Universal M13 (5'-TTATGTAAAACGACGGCCAGT-3'), T3 (5'-GCAATTAACCTCACTAAAG-3'), and T7 (5'-GTAATACGACGCACTATAG-3') were used. PCRs were carried out as described above. Amplifications were performed under the following conditions: 1 cycle at 94°C for 5 min; 2 cycles at 94°C for 5 min, 40°C for 5 min, and 72°C for 5 min; 35 cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min; and 1 cycle at 72°C for 5 min.

Reproducibility. The day-to-day reproducibilities of the above-described REP-PCR and ERIC-PCR were examined by comparing patterns provided by the same strain and method on four different days. One strain was randomly selected from each of the two most significant profiles obtained by every PCR method and then subjected to the whole typing process for four consecutive days. The RAPD assay (with M13 and T3-T7 primers) was further evaluated for reproducibility by testing one randomly selected strain from each of the profiles obtained.

Analysis of PCR products. rep-PCR (9 μ l) and RAPD assay (10 μ l) products were analyzed by agarose gel electrophoresis (with 1.1% agarose gels run in 0.5 \times Tris-borate-EDTA [TBE] buffer). The DNA molecular weight marker X from Boehringer was used as a size standard.

First, DNA fingerprints of the strains were compared for similarity by visual inspection of the band patterns. Two fingerprints were considered different if the presence or absence of at least one band differed in one of the patterns. If the variation was only one band, this result was confirmed by repeating the PCR two more times. Variations in band intensity were not considered to be differences. Bands that were too faint to be interpreted when reproduced were not considered.

Subsequently, Simpson's index of diversity, which is based on the probability that two unrelated strain samples from the test population will be placed into different typing groups, was calculated. This index (D) is given by the following equation:

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^s n_j(n_j - 1)$$

where N is the total number of strains in the sample population, s is the total number of types described, and n_j is the number of strains belonging to the j th type. Simpson's index of diversity ranges from 0.0 to 1.0, where 1.0 indicates that a typing method is able to distinguish each member of a strain population from all other members of that population and, conversely, 0.0 indicates that all members of a strain population are of an identical type. An index of 0.50 means that if one strain was chosen at random from a strain population, then there would be a 50% probability that the next strain chosen at random was indistinguishable from the first (7, 8).

RESULTS

Molecular typing of *Francisella* strains by REP-PCR generated from four to eight major amplification products ranging in size from 4 to 0.35 kb. Visual comparison of banding patterns revealed four distinct REP profiles for the 41 strains tested (Fig. 1B). Most of the *F. tularensis* strains were REP type 1, with six visible bands and an intense band of approximately 1.25 kb in size. Only two of the isolates (strains 15 and 19), recovered from hares, were REP type 2. This profile and type 1 shared REP-PCR amplification bands of approximately 1.25, 0.75, and 0.35 kb in size, but they differed in bands of about 2.5, 1.75, and 0.6 kb. The only reference strain belonging to *F. tularensis* subsp. *tularensis* (type A) showed a unique pattern (REP type 3), in which the intense band of approximately 1.25 kb was not present. The *F. novicida* profile (REP type 4) shared only two bands, approximately 4 and 3.8 kb in size, with *F. tularensis* profiles. It also had a unique and characteristic band of approximately 1.5 kb in size, but it lacked the band of about 1.25 kb in size (Table 1).

With the ERIC-PCR method, profiles of *Francisella* strains revealed amplified bands ranging from 3 to 0.9 kb, with various intensities (Fig. 1A). The 41 strains were grouped into four distinct patterns of fingerprints. All of the types shared a very intense band of approximately 3 kb in size. *F. novicida* (ERIC type 4) did not share any other band, while the American human strain (ERIC type 3) differed from the other *F. tularensis* strains in one unique band of approximately 1.75 kb in size. In addition, two major common bands of about 2.25 and 1.1 kb were present in all *F. tularensis* strains tested, and two other bands of approximately 2 and 0.9 kb and two more faint bands of between 1 and 1.6 kb were seen in ERIC types 2 and 3. Of the 25 Spanish isolates recovered from hares, 15 belonged to ERIC type 2 and 10 belonged to ERIC type 1. The distribution of the Spanish hare isolates into two different groups was also observed for the other hare strains isolated in the Czech Republic. The vole and tick isolates were also ERIC type 2. The Spanish human isolates belonged to ERIC profile

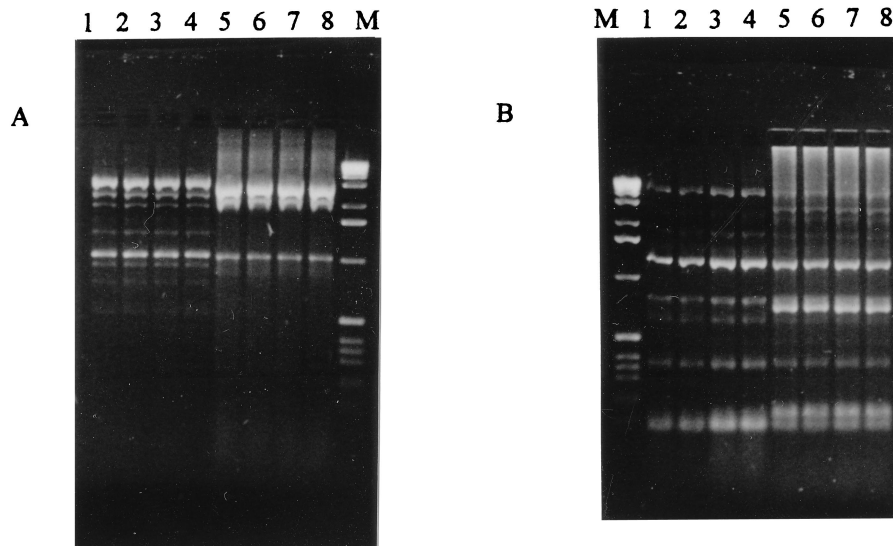


FIG. 2. Reproducibility studies. The day-to-day reproducibilities of the PCR assays were examined by comparing patterns provided by the same strain and method on four different days. Lanes M, molecular size marker X (Roche Diagnostics). (A) ERIC-PCR fingerprints of strains 27 (type 1) (lanes 1, 2, 3, and 4) and 3 (type 2) (lanes 5, 6, 7, and 8). (B) REP-PCR fingerprints of strains 33 (type 1) (lanes 1, 2, 3, and 4) and 19 (type 2) (lanes 5, 6, 7, and 8).

1 or 2, while the American human strain was ERIC type 3 (Table 1).

RAPD-PCR produced five distinguishable patterns for the 41 *Francisella* strains when the M13 primer was used (RAPD/M13). The fragments ranged from 2.2 to 0.35 kb, with various band intensities (Fig. 1C). All strains of *F. tularensis* (RAPD/M13 types 1 to 4) have common bands of approximately 2.2, 1.6, 1.4, 1.3, 0.9, 0.75, 0.65, 0.45, and 0.35 kb in size. However, *F. novicida* (RAPD/M13 type 5) shared only bands of approximately 1.4, 1.3, 0.65, and 0.45 kb but showed two unique bands of about 0.67 and 0.5 kb. Additional bands were present only in RAPD/M13 types 2 (0.7 kb), 3 (0.8 kb), and 4 (0.6 kb). RAPD/M13 type 1 included 31 *F. tularensis* strains and, among them, most of the strains isolated from humans and hares. RAPD/M13 type 2 included the two same hare isolates that were REP type 2 and also human isolate no. 24. RAPD/M13 type 3 included the three hare strains and the tick strain recovered in eastern Europe; RAPD/M13 type 4 was observed for two Spanish human isolates, and RAPD/M13 type 5 was observed for the *F. novicida* strain (Table 1).

Amplification of genomic DNAs from *F. tularensis* strains with the T3-T7 primers resulted in six different patterns consisting of four common fragments of approximately 1.5, 0.75, 0.70, and 0.55 kb in size (Fig. 1D). Their differences were in amplification products ranging from 0.95 to 0.55 kb. A seventh profile was observed for the *F. novicida* strain, which shared with *F. tularensis* strains only the band of about 0.70 kb but exhibited characteristic main bands of approximately 1.6 and 1.5 kb in size. Hare strains were assigned to RAPD/T3-T7 types 1 to 4 (type 1 included 57.1% of hare strains); human strains were assigned to RAPD/T3-T7 types 1 to 4 and 6 (type 1 included 44.4% of human strains); the two tick strains were assigned to RAPD/T3-T7 type 1; the *F. tularensis* isolate from a vole was assigned to RAPD/T3-T7 type 5; and the *F. novicida* strain was assigned to RAPD/T3-T7 type 7 (Table 1).

The day-to-day reproducibilities of the PCRs were examined by comparing patterns amplified on four different days. Excellent reproducibilities were obtained with REP-PCR, ERIC-PCR, and RAPD/M13 assays (Fig. 2 and 3). Although sometimes the major bands produced were less intense and the

minor bands were difficult to visualize, their overall positions and whether they were present or absent were highly consistent. For the RAPD assay using the T3-T7 primers, the reproducibility was also high for six of the seven profiles obtained; the exception was type 5 (the *F. tularensis* strain isolated from a vole), for which one of the bands was not always present (Fig. 4).

When the four assays compared were taken together, the 41 strains listed in Table 1 could be divided into 18 distinct groups, designated A to R. Spanish hare *F. tularensis* isolates belonged to 10 groups (A to J); Czech hare isolates belonged to groups M and P; Spanish human isolates belonged to groups A, D, G, J, L, O, and N; human strain SCHU belonged to group N; tick isolates belonged to groups B and Q; the vole

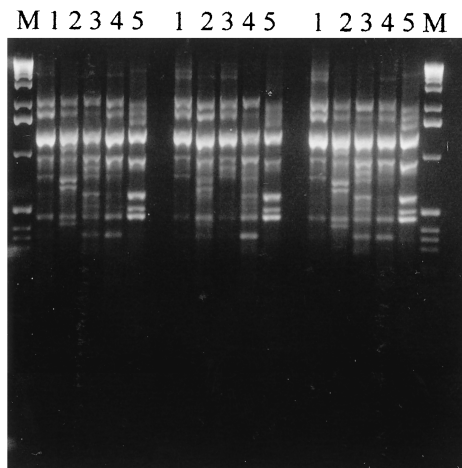


FIG. 3. Reproducibility studies. The day-to-day reproducibilities of the RAPD/M13 assay were examined by comparing the patterns provided by the five different fingerprints (strain 6, type 1 [lane 1]; strain 19, type 5 [lane 2]; strain 33, type 3 [lane 3]; strain 30, type 4 [lane 4]; and strain 41, type 5 [lane 5]) on four different days (only three days are shown). Lanes M, molecular size marker X (Roche Diagnostics).

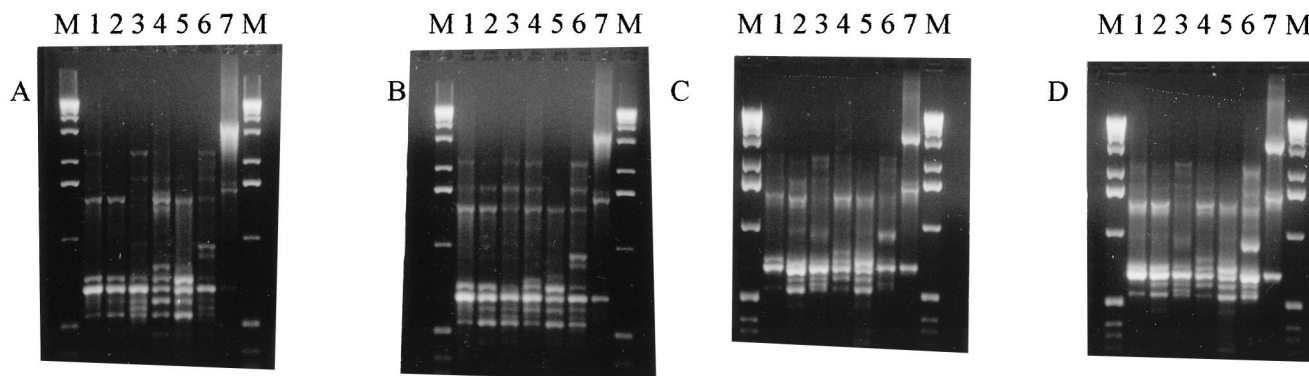


FIG. 4. Reproducibility studies. The day-to-day reproducibilities of the RAPD/T3-T7 assay were examined by comparing the patterns provided by the seven different fingerprints (strain 7, type 1 [lane 1]; strain 10, type 2 [lane 2]; strain 20, type 3 [lane 3]; strain 23, type 4 [lane 4]; strain 21, type 5 [lane 5]; strain 28, type 6 [lane 6]; and strain 41, type 7 [lane 7]) on four different days (panels A to D). Lane M, molecular size marker X (Roche Diagnostics).

strain belonged to group K; and the *F. novicida* strain belonged to group R (Table 1).

Table 2 shows Simpson's index of diversity for the different PCR typing methods used in this study. When the methods were taken separately, RAPD/T3-T7 exhibited the highest discriminating power, with diversity indices (DI) of 0.65 for both the Spanish and the total group of *F. tularensis* strains. In contrast, the REP-PCR method provided the lowest degree of discrimination (below 0.15). Globally, the highest DI were obtained when ERIC-PCR, RAPD/M13, and RAPD/T3-T7 were combined and when the four methods were taken together (with DI of 0.87 for Spanish strains and of 0.90 for total strains). With the combination of just two methods, ERIC and RAPD/T3-T7, the DI increased to 0.83. The DI for the total *F. tularensis* strains tested were quite similar to those for the Spanish strains for the different methods compared, with the exception of those obtained for RAPD/M13 and the combination of REP-PCR and RAPD/M13, which were almost twice as high for the total number of strains as for the 35 Spanish isolates.

DISCUSSION

To our knowledge, molecular epidemiological typing for *F. tularensis* has never been reported before. During the last decade, approaches at the molecular level, such as pulsed-field

gel electrophoresis and ribotyping, have been used to assess the relatedness of organisms other than *F. tularensis*. Despite the broad applicability of these techniques, their use in clinical microbiology laboratories has been limited because they are both time-consuming and labor-intensive. However, the PCR techniques described here have recently been considered easy to perform and to interpret, with high discrimination power, a time of only 1 day to result, moderate cost of equipment, low cost per test, and good (rep) or moderate (RAPD) reproducibility (13).

In addition, molecular typing methods are of special concern when working with pathogenic bacteria, because direct manipulation with live organisms (which takes place when traditional biochemical typing techniques are used) is avoided. On the other hand, although biochemical tests can be used to discriminate among subspecies of *F. tularensis* strains, such tests have obvious drawbacks for unambiguous classification (14).

In this study, rep-PCR and RAPD assays were used as tools to characterize *F. tularensis* strains isolated from hares, humans, ticks, and a vole. Both rep-PCR methods, REP- and ERIC-PCR, as well as RAPD assay with the two sets of primers used, showed great possibilities for the identification of *F. tularensis* isolates. Thus, the banding patterns of *F. novicida*, an organism closely related to *F. tularensis*, were completely different from those produced by the four PCR assays compared

TABLE 2. Simpson's index of diversity for the *F. tularensis* strains used in this study

Method	No. of different types	No. of strains belonging to the most numerous type	Simpson's index of diversity for:	
			Total strains (n = 40)	Spanish strains (n = 35)
REP	3	37	0.14	0.11
ERIC	3	23	0.52	0.49
RAPD (M13 primer)	4	31	0.39	0.21
RAPD (T3-T7 primers)	6	22	0.65	0.65
REP-ERIC	5	23	0.55	0.51
REP-RAPD (M13 primer)	6	30	0.44	0.26
REP-RAPD (T3-T7 primers)	8	21	0.68	0.69
ERIC-RAPD (M13 primer)	8	20	0.70	0.60
ERIC-RAPD (T3-T7 primers)	10	11	0.83	0.83
RAPD (M13 primer)-RAPD (T3-T7 primers)	8	16	0.81	0.75
REP-ERIC-RAPD (M13 primer)	8	20	0.70	0.60
REP-ERIC-RAPD (T3-T7 primers)	11	11	0.84	0.85
REP-RAPD (M13 primer)-RAPD (T3-T7 primers)	9	16	0.81	0.75
ERIC-RAPD (M13 primer)-RAPD (T3-T7 primers)	17	10	0.90	0.87
REP-ERIC-RAPD (M13 primer)-RAPD (T3-T7 primers)	17	10	0.90	0.87

for the 40 *F. tularensis* isolates tested in this study. This is of special interest, since previous studies have been unable to clearly discriminate between *F. tularensis* and *F. novicida*. Genetic probes have failed so far in this aim (4). Similarly, rep-PCR and RAPD methods were efficient and sensitive molecular typing tools for *F. tularensis* strains. The lower degree of reproducibility of RAPD compared with rep-PCR has been previously reported for other organisms (9), and it is confirmed in this study for *F. tularensis* when the T3-T7 primers are used, although the result was only ambiguous for one of the seven profiles obtained. On the other hand, the reproducibilities of both PCR methods were comparable when the M13 primer was used in the RAPD assay.

However, RAPD with the T3-T7 primers provided a higher degree of discrimination by visual analysis within strains than ERIC-PCR and especially than REP-PCR: a DI of 0.65 for RAPD/T3-T7 versus DI of 0.49 and 0.52 for ERIC-PCR and of 0.11 and 0.14 for REP-PCR for Spanish and total strains, respectively, were obtained (Table 2). Some of these values are low, but the combination of some of the PCR methods allow us to achieve DI of 0.8 to 0.9. These results can be considered excellent, especially if both the small area from which the Spanish isolates were recovered and previous information about typing tools for *Francisella* strains are taken into account (the DI observed for serologic methods is 0, as only one antigenic group has been described so far [3, 12], and DNA methods have been unable to discriminate between *F. novicida* and *F. tularensis* [5]). On the other hand, the results in this study were clearly different from those reported by Vila et al. (16), who found that the RAPD assay was less discriminating than rep-PCR methods for typing of the *Acinetobacter calcoaceticus*-*A. baumannii* complex.

The existence among the 17 global PCR types of four *F. tularensis* groups (A, D, G, and J) containing human and hare isolates clearly indicates a common origin for them and confirms that hare *F. tularensis* strains are infectious for humans. In this respect, it must be pointed out that most of the human infections occurred during the hunting season (hunting is a usual practice among people in Spain). However, it should be noted that the most common profile (group B, containing 25% of the tested strains) was isolated only from hares and a tick, and never from humans. Although the number of isolates in this study is not enough to make epidemiological conclusions, this information should be considered carefully in the future, since it seems to suggest the existence of an *F. tularensis* group that has a low prevalence in humans but is present in hares and ticks.

Strain no. 39 was recovered from a tick located during the necropsy in the skin of the hare from which strain no. 38 was isolated. The two strains showed identical profiles in the four PCR tests and were included in group B. This finding clearly indicates the existence of tick-borne tularemia in hares. On the other hand, the isolate recovered from a vole was the only strain assigned to group K, and this seems to suggest a completely different origin for this isolate. In any case, as only one strain could be isolated in the outbreak that occurred in Spain, this observation should be validated by the examination of a larger set of vole isolates.

On the other hand, the hare strains were divided into twelve groups (A to J, M, and P) (Table 1). This fact clearly indicates the existence of genetic diversity among *F. tularensis* strains isolated from this animal. This diversity degree can be considered greater if it is taken into account that 25 of the hare isolates used in this study, recovered from Spain during 1998 in a geographical area of about 11,000 km², were assigned to 10 different genetic groups (A to J). In the same way, the eight

human isolates recovered in Spain were classified into six groups (A, D, G, J, L, and O), while the American human strain showed completely different fingerprints and was assigned to group N. This fact can be easily explained because strain SCHU was the only reference strain used in this study belonging to *F. tularensis* subsp. *tularensis* (type A).

Although a high number of groups was obtained, the differences were not more than two or three bands by every PCR method, and, what is more important, many common bands were seen for all of the *F. tularensis* strains tested. Even without a dendrogram (which was not the purpose of this work), visual inspection of Fig. 1 outlines the possibility of a high clonality among the isolates compared in this study, with several shared bands that would allow, if needed, identification of the genus *Francisella* or species *F. tularensis*. Therefore, we can conclude that there is a limited genetic diversity among strains, which is enough for distinguishing subspecies and even different types within subspecies by PCR methods. Our results need further validation by the study of larger amounts of strains isolated in other geographical regions or for longer periods of time. However, because of the high risk assumed when transporting *F. tularensis* strains and because of the implications of this organism as a biological war agent, we were not able to obtain more isolates from other countries.

In conclusion, all these PCR-based approaches represent useful tools for the epidemiological typing of *F. tularensis* because of their simplicity and speed. RAPD with T3-T7 primers has the advantage of being more discriminative than rep-PCR. In addition, the results of this study add further evidence for the idea that rep-PCR may be broadly applicable for fingerprinting bacteria which possess repetitive elements such as REP or ERIC sequences.

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