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Evaluation of PCR-Based Methods for Discrimination of *Francisella* Species and Subspecies and Development of a Specific PCR That Distinguishes the Two Major Subspecies of *Francisella tularensis*

ANDERS JOHANSSON,[1,2,3] ASHRAF IBRAHIM,[4] INGELA GÖRANSSON,[2] ULLA ERIKSSON,[2] D. GURYCOVA,[5] JILL E. CLARRIDGE III,[6] AND ANDERS SJÖSTEDT[2,3]*

Infectious Diseases[1] and Clinical Bacteriology,[3] Department of Clinical Microbiology, Umeå University, and Department of Microbiology, Defence Research Establishment,[2] Umeå, Sweden; Department of Biotechnology, Technical University of Denmark, Lyngby, Denmark[4]; Department of Epidemiology, Komensky University, Bratislava, Slovak Republic[5]; and Microbiology, Serology and Molecular Biology Section, PALMS, Veterans Affairs Medical Center, Houston, Texas[6]

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Previous studies have demonstrated that the four subspecies of the human pathogen *Francisella tularensis*, despite showing marked variations in their virulence for mammals and originating from different regions in the Northern Hemisphere, display a very close phylogenetic relationship. This property has hampered the development of generally applicable typing methods. To overcome this problem, we evaluated the use of PCR for discrimination of the subspecies using various forms of long arbitrary primers or primers specific for repetitive extragenic palindromic sequences (REP) or enterobacterial repetitive intragenic consensus (ERIC) sequences. Patterns generated by use of REP, ERIC, or long arbitrary primers allowed differentiation at the species level and of the four subspecies of *F. tularensis*. With each of these three methods, similar or identical clustering of strains was found, and groups of strains of different geographical origins or differing in virulence showed distinct patterns. The discriminatory indices of the methods varied from 0.57 to 0.65; thus, the patterns were not sufficiently discriminatory to distinguish individual strains. The sequence of a fragment generated by amplification with an arbitrary primer was determined, and a region showing interstrain heterogeneity was identified. Specific primers were designed, and a PCR was developed that distinguished strains of *F. tularensis* subsp. *holarctica* from strains of other *F. tularensis* subspecies, including strains of the highly virulent *F. tularensis* subsp. *tularensis*. Notably, one European isolate showed the genetic pattern typical of the highly virulent *F. tularensis* subsp. *tularensis*, generally believed to exist only in North America. It is proposed that a combination of the specific PCR together with one method generating subspecies-specific patterns is suitable as a rapid and relatively simple strategy for discrimination of *Francisella* species and subspecies.

Francisella tularensis is one of two recognized species of the genus *Francisella*, and almost all knowledge of the genus originates from work on this species. It is a virulent, facultative intracellular bacterium and the etiological agent of tularemia, a disease found in rodents, lagomorphs, and humans. The bacterium is widely distributed in nature and has been isolated from about 250 wildlife species (20), many of which can transmit disease to humans. Tularemia is acquired by direct contact with infected animals, through contaminated water or food, or from vectors such as biting insects or ticks. Airborne transmission also occurs, especially during processing of agricultural products. The disease is often epidemic, both in humans and in animals, and clinical manifestations depend on the type of reservoir involved and the means of transmission. *F. tularensis* is found throughout the Northern Hemisphere, and large outbreaks have been reported in parts of the continental United States, the southern part of the former USSR, and northern Scandinavia.

F. philomiragia, the other species of the genus, is a rarely isolated, opportunistic pathogen closely linked to water (9). The genus also comprises endosymbionts of ticks, although their exact phylogenetic positions are uncertain (15, 16, 24).

All of the four recognized subspecies of *F. tularensis* (9, 18, 20) have been associated with human tularemia, although they differ drastically in virulence for humans and rabbits. Until recently, isolates of *F. tularensis* subsp. *tularensis* were isolated only in North America, but a mite-derived isolate from Slovakia showed the phenotypic characteristics of the subspecies (8). *F. tularensis* subsp. *tularensis* isolates in North America are often associated with tick-borne tularemia in lagomorphs and are highly virulent for many mammalian species, including primates. Before the advent of effective antibiotics, mortality in humans ranged from 5 to 30% (3, 4). *F. tularensis* subsp. *holarctica*, found in Europe, North America, and Japan, is often associated with lagomorphs in Scandinavia, continental Europe, and Japan, ground voles in the former USSR, and beavers and muskrats in North America. The subspecies causes a less severe form of human illness, often a localized ulceroglandular disease. *F. tularensis* subsp. *mediaasiatica* is moderately virulent for rabbits and humans and has been isolated only in the Central Asian republics of the former USSR (18). In 1950, the type strain of *F. tularensis* subsp. *novicida* was isolated from a water sample in Utah, and isolates of the subspecies known as "*novicida*-like" isolates have been linked to human disease on a few occasions (1, 9). Isolates of this subspecies have less

* Corresponding author. Mailing address: Department of Clinical Microbiology, Clinical Bacteriology, Umeå University, SE-901 85 Umeå, Sweden. Phone: 46 90 7851120. Fax: 46 90 7852225. E-mail: anders.sjostedt@climi.umu.se.

TABLE 1. Strains included in the study

Species or subspecies	Source	Strain	Other strain designation(s)	Pattern in:			
				REP	ERIC	RAPD	Specific PCR
F. tularensis subsp. *holarctica*	Human lymph node, 1926, Japan	FSC017	Jap S2	A	a	1	I
	Japan	FSC024	Yerma	A	a	1	I
	City water supply, Helena, Mont.	FSC044	Helena	B	b	1	I
	North America	FSC056	Eigelsbach	B	b	1	I
	Norway	FSC091	NO 9/15/51	B	b	1	I
	Sweden	FSC093	Sv 43	B	b	1	I
	Sweden	FSC094	Sv 121	B	b	1	I
	Sweden	FSC095	Sv 219	B	b	1	I
	Human ulcer, 1981, Sweden	FSC108	SBL R45/81	B	b	1	I
	Human ulcer, 1981, Sweden	FSC109	SBL R74/81	B	b	1	I
	Norway rat, 1988, Russia	FSC150	250	B	b	1	I
	Russia	FSC155	ATCC 29684, LVS	B	b	1	I
	Human blood, 1994, Sweden	FSC157	CCUG 33270	B	b	1	I
	Water, 1995, Sweden	FSC170		B	b	1	I
	Human ulcer, 1995, Sweden	FSC171		B	b	1	I
	Human ulcer, 1995, Sweden	FSC172	SMI R8/95	B	b	1	I
	Ticks, 1995, Czech Republic	FSC180	T-17	B	b	1	I
	Human ulcer, 1996, Sweden	FSC188		B	b	1	I
	Mites, 1988, Slovakia	FSC196	SE-210/37	B	b	1	I
F. tularensis subsp. *tularensis*	Squirrel, Georgia	FSC033	SnMF	C	c	2	II
	Tick, 1935, British Columbia, Canada	FSC041	Vavenby	C	c	2	II
	Canada	FSC042	Utter	C	c	2	II
	Human ulcer, 1941, Ohio	FSC043	Schu	C	c	2	II
	Hare, 1954, Nevada	FSC054	Nevada 14	C	c	2	II
	Human lymph node, 1920, Utah	FSC138	ATCC 6223	D	d	3	II
	Mites, 1988, Slovakia	FSC198	SE-219/38	C	c	2	II
F. tularensis subsp. *mediaasiatica*	Hare, 1965, Central Asia	FSC149	120	E	e	4	II
F. tularensis subsp. *novicida*	Water, 1950, Utah	FSC040	ATCC 15482	F	f	5	II
	Human blood, 1991, Texas	FSC156	Fx1	G	g	6	II
	Human blood, 1995, Texas	FSC159	Fx2	H	h	7	II[a]
F. philomiragia	Water, 1960, Utah	FSC038	ATCC 25017	I	i	8	None
	Muskrat, 1959, Utah	FSC144	ATCC 25015	J	j	9	None
	Human abscess, 1982, Sweden	FSC145	CCUG 12603	K	k	10	None
	United States	FSC153		L	l	11	None
	Human blood, 1979, Switzerland	FSC154	CCUG 13404	M	m	12	None

[a] The strain displayed a slightly smaller 0.4-kb amplicon due to a gene truncation.

stringent growth requirements than representatives of the other *F. tularensis* subspecies and can therefore be misidentified as belonging to other genera (1).

Isolates of the genus *Francisella* are readily distinguishable based on a unique set of phenotypic characteristics, including coccoidal morphology, gram negativity, acid but no gas production from a limited number of carbohydrates, a growth requirement for cysteine, and a unique fatty acid composition (20). However, within the genus and particularly within the species *F. tularensis*, discrimination of strains is not performed conveniently. Due to the contagiousness of *Francisella* isolates, only limited work has been performed to develop typing methods based on cultivation. Such work is further restricted by the nonfermentative nature of *F. tularensis*, limiting the number of biochemical tests available for biotyping. To this end, the development of techniques based on analyses of genomic variations is of special interest, since they can be done with killed preparations of the bacterium.

Several molecular methods have been successfully used to discriminate the *Francisella* species but not the subspecies (5, 11). Repetitive extragenic palindromic sequence (REP)-PCR has been applied to specifically identifying strains of *F. tularensis* subsp. *novicida*, but patterns from *F. tularensis* subsp. *holarctica* and *F. tularensis* subsp. *tularensis* strains were found to be similar (1). A one-base variability in the 16S rRNA sequences of *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *holarctica* has been demonstrated, and on this basis, a PCR that differentiated the two subspecies was developed (5). However, there is not sufficient sequence information available to corroborate that there is a consistency at this position among all isolates of each subspecies (5). A recent study investigated PCR methods for the typing of *F. tularensis* subsp. *holarctica* isolates, the majority of which originated from Spain (2). In the present study, we evaluated if PCR-based methods are suitable as generally applicable tools for discrimination of species and subspecies within the genus *Francisella.*

MATERIALS AND METHODS

Bacteria, media, and growth conditions. The bacterial strains used in this study are listed in Table 1. The strains belong to the *Francisella* Strain Collection, which contains more than 200 *Francisella* strains (5). Each strain has been given a strain collection number, indicated in Table 1. Strains were grown for 3 days at 37°C on modified Thayer-Martin agar plates (19) in 5% CO_2 (GasPak; Becton Dickinson, Paramus, N.J.). Cell suspensions of virulent *Francisella* strains in saline, at a concentration of $\sim 10^9$ cells/ml, were heat killed at 65°C for 2 h. Debris was removed by centrifugation at $12,000 \times g$ for 5 min. Supernatants were collected and then stored at −20°C until used.

PCR template preparation. In an initial evaluation, DNA from bacteria was extracted using a modification (12) of a technique based on the binding of DNA to uniform glass beads (Bio 101, La Jolla, Calif.) in the presence of the chaotropic nuclease inhibitor guanidine isothiocyanate. This method has been previously shown to be superior to three other methods for the extraction of DNA from *F. tularensis* (21). In an initial evaluation of the REP-PCR and enterobacterial repetitive intragenic consensus sequence (ERIC)-PCR protocols, the usefulness of these DNA preparations as DNA templates was compared to that of

heat-killed whole-cell preparations. No significant differences were noted. In subsequent experiments, a supernatant of a heat-killed *Francisella* suspension was therefore used as a template.

Primer sequences. The previously described primer pairs REP1R-I–REP2-I and ERIC1R-ERIC2 were used (28). REP consensus primers contained inosine at ambiguous positions. A number of 20-mer oligonucleotides were designed at random, and their usefulness alone or in combination as PCR primers was evaluated using a modified protocol for long-primer randomly amplified polymorphic DNA (RAPD) analysis (LP-RAPD) (6). The primer yielding the pattern with the highest resolution was selected: 5′-GGTAATCGATGAATAAA TGA (LP-RAPD2). Oligonucleotides were purchased from Pharmacia Biotech, Uppsala, Sweden.

REP, ERIC, and LP-RAPD amplification. A 15-μl mixture containing 1 μl of bacterial supernatant, 3 mM $MgCl_2$, and buffer 4 (Advanced Biotechnologies Inc., London, United Kingdom) was heated to 94°C for 10 min. A 10-μl mixture containing (all concentrations per final volume) 2 μM each REP primer, 0.8 μM each ERIC primer, or 0.8 μM LP-RAPD2 primer, 200 μM each deoxynucleoside triphosphate (dNTP) (Pharmacia Biotech), and 1 U of *Taq* DNA polymerase (Advanced Biotechnologies) was added to the 15-μl mixture. After an additional denaturation at 94°C (REP and ERIC, 10 min; LP-RAPD, 7 min), the reaction mixtures were subjected to 40 cycles of amplification. Each cycle consisted of denaturation at 94°C for 60 s, annealing (REP, 42°C, 120 s; ERIC, 52°C, 60 s; LP-RAPD, 42°C, 60 s), and extension at 72°C for 5 min. The PCR amplification was terminated after the samples were incubated at 72°C for 10 min (REP and ERIC) or 7 min (LP-RAPD). Three microliters of each reaction mixture was loaded onto a 1.5% agarose gel and subjected to electrophoresis, and the amplified products were visualized with UV light after ethidium bromide staining.

Pattern analysis. PCR patterns were analyzed by visual examination by two individuals. Patterns were regarded as distinct if they repeatedly showed a one-band difference.

Specific PCR amplification. The degenerate primer

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5′-GCCTTAATAGTATGCATACGATT
   T  G  T  G     C TG
      T           T
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amplified a distinct PCR product from each *F. tularensis* strain. The PCR product, approximately 0.7 kb, showed one of two distinct sizes depending on the strain used as the DNA template, with FSC043 exhibiting the larger fragment. The fragment was isolated from a 1.5% SeaKem GTG agarose gel (FMC BioProducts, Rockland, Maine) and was purified by use of a GenElute agarose spin column (Supelco, Bellafonte, Pa.). The fragment was ligated into vector pGEM-T according to the instructions of the manufacturer (Promega Corp., Madison, Wis.) After transformation of *Escherichia coli* DH5α, recombinant clones were identified by blue-white color screening; plasmid DNA was isolated by the Wizard Plus miniprep procedure (Promega). Inserts were sequenced using pUC/M13 forward and reverse primers and the Big Dye terminator cycle sequencing ready reaction kit (PE Applied Biosystems, Stockholm, Sweden). The sequence was analyzed using an ABI 377 sequencer (PE Applied Biosystems).

Based on the sequence of the 0.7-kb amplicon derived from FSC043, specific primers were designed. A 0.4-kb fragment was amplified from seven strains representing the various *Francisella* species (FSC024, FSC033, FSC040, FSC056, FSC144, FSC149, and FSC155), and the sequence was determined. Based on the sequence, primers C1 (5′TCCGGTTGGATAGGTGTTGGATT) and C4 (5′G CGCGGATAATTTAAATTTCTCATA) were designed. These primers, yielding an amplicon of approximately 0.3 kb that included the variable region, were used in a multiplex PCR together with the *F. tularensis*-specific primers, TUL4-435 and TUL4-863. The latter primers generate a 0.4-kb fragment of the gene encoding a 17-kDa lipoprotein shown previously to be useful for the identification of *F. tularensis* (13, 21, 22).

The multiplex PCR was performed with a reaction mixture containing 1 μl of bacterial supernatant, 0.8 μM each primer, 1 U of DyNAzyme polymerase (Finnzymes OY, Espoo, Finland), and 200 μM each dNTP (Finnzymes OY) in a final volume of 25 μl. After denaturation at 94°C for 5 min, 30 cycles of amplification were performed according to the following protocol: denaturation at 94°C for 30 s, primer annealing at 66°C for 30 s, and primer extension at 72°C for 30 s. After a final extension at 72°C for 5 min, each reaction mixture was subjected to electrophoresis with 3% NuSieve 3:1 agarose gel (FMC BioProducts). After ethidium bromide staining, the DNA products were visualized with UV light.

Nucleotide sequence accession numbers. The 0.7-kb sequence from strain FSC043 (Schu) has been assigned GenBank accession number AF240631. The GenBank accession numbers of the 0.4-kb fragments are AF247690, (FSC024), AF247689 (FSC033), AF247688 (FSC040), AF247687 (FSC056), AF247686 (FSC144), AF247685 (FSC149), and AF247642 (FSC155).

RESULTS

ERIC-PCR and REP-PCR. Amplification using REP-PCR revealed similar but distinguishable patterns from strains of each of the three subspecies, *F. tularensis* subsp. *holarctica* (19 representatives), *F. tularensis* subsp. *mediaasiatica* (1 representative), and *F. tularensis* subsp. *tularensis* (7 representatives), whereas patterns from strains of *F. tularensis* subsp. *novicida* (3 representatives) and strains of the species *F. philomiragia* (5 representatives) were unique for each strain. A sample of strains representing each of the species and subspecies is shown in Fig. 1. Clearly visible bands ranged from approximately 0.2 to 4.0 kb. Patterns from the seven isolates of *F. tularensis* subsp. *tularensis* were discernible from patterns of the other subspecies and were characterized by prominent bands of approximately 4.0 and 1.0 kb. Notably, FSC198, a Slovakian isolate derived from a mite and reported to show the biochemical characteristics and virulence typical of *F. tularensis* subsp. *tularensis* strains (8), also showed this pattern, thereby confirming the first isolation of this subspecies outside North America. The attenuated type strain of the subspecies, ATCC 6223 (FSC138), also demonstrated the typical pattern but with a minor variation in the sizes of the 0.9- and 3.0-kb bands. PCR patterns from *F. tularensis* subsp. *holarctica* strains were distinguished by a prominent 1.6-kb band and the absence of a 1.2-kb band. Patterns from the *F. tularensis* subsp. *holarctica* strains were identical, with the exception of those from the Japanese strains. The pattern from the *F. tularensis* subsp. *mediaasiatica* strain was distinct from those of the other strains but was similar to patterns from *F. tularensis* subsp. *tularensis* strains. A summary of the various REP patterns is given in Table 1.

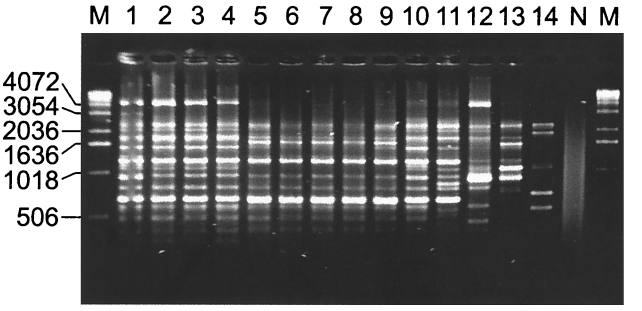

FIG. 1. PCR amplification of DNA from various *Francisella* strains using the REP1R-I and REP2-I primers. Samples represent *F. tularensis* subsp. *tularensis* strains (FSC138, FSC043, FSC041, and FSC198) (lanes 1 to 4), *F. tularensis* subsp. *holarctica* strains (FSC196, FSC155, FSC150, FSC108, and FSC157) (lanes 5 to 9), an *F. tularensis* subsp. *mediaasiatica* strain (FSC149) (lane 10), an *F. tularensis* subsp. *holarctica* strain from Japan (FSC024) (lane 11), *F. tularensis* subsp. *novicida* strains (FSC040 and FSC156) (lanes 12 and 13), and an *F. philomiragia* strain (FSC 144) (lane 14). Strain designations are indicated in Table 1. Lane N, water used as a negative control. Lane M, molecular markers (sizes in base pairs).

PCR amplification by use of ERIC primers corroborated the clustering of strains observed with REP-PCR, although the patterns were slightly less complex (data not shown). The visualized bands ranged in size from approximately 0.2 to 3.0 kb. A summary of the various ERIC patterns is shown in Table 1. A calculation of the discriminatory power (10) of REP-PCR or ERIC-PCR for typing of the species *F. tularensis* revealed a discriminatory index of 0.65.

Although the *F. tularensis* strains displayed very similar patterns after ERIC-PCR or REP-PCR, the patterns were distinct from each of 15 patterns resulting from the amplification of DNA of other clinically relevant species using the same primers (data not shown). Thus, the analyses indicated that patterns generated by the *F. tularensis* isolates were unique and readily distinguished from those of other clinically relevant species.

ERIC and REP sequences have been reported to be present in a multitude of bacterial species (28), but there is no information confirming their presence in *Francisella* genomes. To

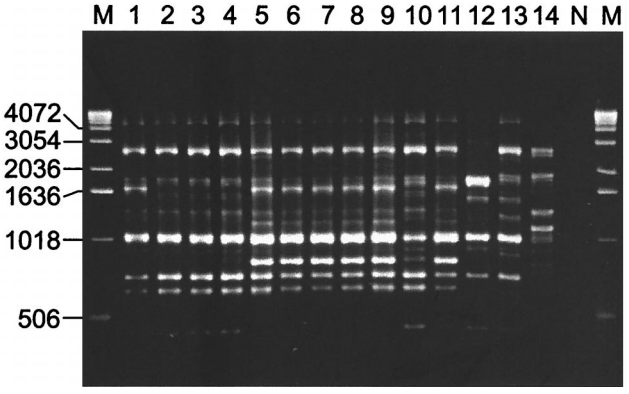

FIG. 2. PCR amplification of DNA from various *Francisella* strains using the LP-RAPD2 primer. Samples represent *F. tularensis* subsp. *tularensis* strains (FSC138, FSC043, FSC041, and FSC198) (lanes 1 to 4), *F. tularensis* subsp. *holarctica* strains (FSC196, FSC155, FSC150, FSC108, and FSC157) (lanes 5 to 9), an *F. tularensis* subsp. *mediaasiatica* strain (FSC149) (lane 10), an *F. tularensis* subsp. *holarctica* strain from Japan (FSC024) (lane 11), *F. tularensis* subsp. *novicida* strains (FSC040 and FSC156) (lanes 12 and 13), and an *F. philomiragia* strain (FSC144) (lane 14). Strain designations are indicated in Table 1. Lane N, water used as a negative control. Lane M, molecular markers (sizes in base pairs).

this end, we searched for such sequences in the genome of strain Schu S4 (unpublished data). More than 98% of the genome has been sequenced. No sequences with similarities to REP or ERIC sequences could be identified. Thus, it is likely that the banding patterns obtained from PCR amplification with primers complementary to consensus motifs of REP or ERIC sequences were based on annealing to other types of sequences present in the *Francisella* genome. It has been reported that ERIC primers can generate relatively complex patterns from eukaryotic and prokaryotic genomes despite a lack of the specific target sequences (7).

PCR amplification with random primers. Previously, it was reported that the use of random primers containing 18 to 24 nucleotides results in patterns after PCR-based amplification that are more reproducible than those seen after amplification with shorter random primers (6, 7). PCR amplification based on different combinations of six such random primers was assessed. Only one of the primers gave an amplification pattern of sufficient complexity. The numbers of amplicons observed were similar to the numbers obtained by use of ERIC or REP primers (Fig. 2). The sizes of the amplicons ranged from 0.4 to 5.0 kb. Strains of *F. tularensis* subsp. *tularensis* displayed two distinct patterns, one for the avirulent type strain of the subspecies, ATCC 6223 (FSC138), and one for the other six strains. Again, the recently isolated representative from Europe (FSC198) also showed the pattern typical of *F. tularensis* subsp. *tularensis.* Patterns from *F. tularensis* subsp. *holarctica* strains, including the Japanese ones, were all identical and clearly discernible from those of other subspecies. A distinct pattern was observed for the representative of *F. tularensis* subsp. *mediaasiatica*. Patterns from strains of *F. philomiragia* (five representatives) and *F. tularensis* subsp. *novicida* (three representatives) were each unique (Fig. 2; not all strains are shown). Clustering of the strains was thus very similar with LP-RAPD–PCR, ERIC-PCR, and REP-PCR (Table 1). The discriminatory index of LP-RAPD–PCR was 0.57. A summary of the various LP-RAPD patterns is given in Table 1.

All the banding patterns produced by REP, ERIC, or LP-RAPD from each of the studied strains were reproducible, but variations in the relative intensities of the bands were observed (data not shown). REP, ERIC, and LP-RAPD reactions were performed at least four times on each strain using templates prepared on three different occasions.

Amplification with *F. tularensis*-specific primers. Sequencing of a fragment obtained after amplification with a random primer revealed that a 30-bp sequence was found only in some *F. tularensis* genomes. By designing *F. tularensis*-specific primers specific for the region adjacent to this sequence, amplicons of variable length were obtained after PCR. Amplification using these primers was combined with a previously described PCR specific for a gene encoding a 17-kDa lipoprotein conserved in all investigated strains of *F. tularensis* (22).

The multiplex PCR was designed to generate bands of approximately 0.4 and 0.3 kb (Fig. 3). From all *F. tularensis* strains, except for the *F. tularensis* subsp. *novicida*-like strain FSC159, a fragment of the expected size, 0.4 kb, was amplified from the 17-kDa lipoprotein gene. From strain FSC159, a slightly smaller fragment resulted due to a gene truncation (data not shown). No amplicons were amplified from *F. philomiragia* strains by use of the primers specific for the 17-kDa lipoprotein gene. From all *F. tularensis* subsp. *holarctica* strains, including the two strains from Japan, a 300-bp amplicon was amplified, whereas amplification from all strains of *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *mediaasiatica* and the reference strain of *F. tularensis* subsp. *novicida* (FSC 040; ATCC 15482) generated a 330-bp band. The two *F. tularensis* subsp. *novicida*-like strains (FSC156 and FSC159) both showed very faint 330-bp bands. A summary of the two patterns for the strains is shown in Table 1.

DISCUSSION

The family *Francisellaceae*, a member of the γ-subclass of *Proteobacteria*, comprises closely related organisms within the single genus *Francisella* (20). Previous studies have revealed that the two recognized species, *F. tularensis* and *F. philomiragia*, show a 16S rRNA sequence similarity of ≥98% (5). The close relationship has hampered the development of generally applicable methods for discrimination of *F. tularensis* subspecies that differ in virulence or geographical origins. PCR amplification of REP and ERIC sequences often enables discrimination of bacterial isolates (17, 26), and it has even been proposed that PCR fingerprinting can be used to elucidate the genetic basis of phenotypic variability for certain species (26).

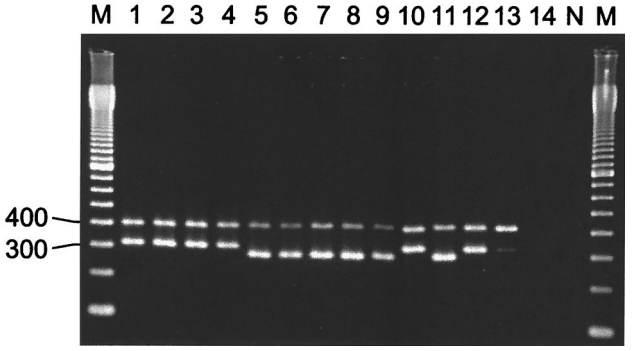

FIG. 3. Multiplex PCR amplification using *F. tularensis*-specific primers. Samples represent *F. tularensis* subsp. *tularensis* strains (FSC138, FSC043, FSC041, and FSC198) (lanes 1 to 4), *F. tularensis* subsp. *holarctica* strains (FSC196, FSC155, FSC150, FSC108, and FSC157) (lanes 5 to 9), an *F. tularensis* subsp. *mediaasiatica* strain (FSC149) (lane 10), an *F. tularensis* subsp. *holarctica* strain from Japan (FSC024) (lane 11), *F. tularensis* subsp. *novicida* strains (FSC040 and FSC156) (lanes 12 and 13), and an *F. philomiragia* strain (FSC144) (lane 14). Strain designations are indicated in Table 1. Lane N, water used as a negative control. Lane M, molecular markers (sizes in base pairs).

In addition, amplification of bacterial genomes using arbitrary primers has been successfully used for the same purpose.

In the present study, all the protocols based on PCR amplification using specific or arbitrary primers allowed differentiation of strains at the species level. Moreover, the PCR analyses based on the use of ERIC, REP, or long arbitrary primers yielded reproducible banding patterns of similar complexity and allowed the differentiation of strains at the subspecies level. We believe that the significance of this clustering was strengthened by the facts that the fingerprints were reproduced for a rather large number of strains, that each of the three methods supported the same clustering of strains, and that the clusters correlated with the geographical origins of the strains. It was not totally unexpected that the patterns of the subspecies were rather similar, considering that previous analyses of 16S rRNA sequences (5) and recent analyses of three complete 23S rRNA gene sequences, representing three out of the four subspecies of *F. tularensis*, and seven partial sequences of gyrase B genes (unpublished data) showed them to be very similar and not suitable as a basis for the development of molecular typing methods discriminatory at the subspecies level.

Within all of the four subspecies, with the exception of *F. tularensis* subsp. *novicida*, very similar or identical patterns were generated. One notable exception was that *F. tularensis* subsp. *holarctica* isolates from Japan were distinguished from other isolates of the subspecies after amplification with REP or ERIC primers. Japanese isolates are also distinguished by lower virulence in experimental animals than *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *holarctica* isolates from Europe and North America, and they cause a relatively mild form of human disease. On this basis, a more detailed analysis of the phenotypic and genotypic properties of Japanese isolates may be warranted to determine whether they should comprise a separate subspecies. Isolates of *F. tularensis* subsp. *mediaasiatica* are found only in some areas of the Central Asian republics of the former USSR, and only limited information is available regarding their characteristics. The isolates have been considered to belong to a separate subspecies on the bases of their ability to produce acid from glycerol and degrade ornithine and their low virulence in experimental models of tularemia. This subspecies differentiation was supported by the observation that the isolates showed distinct patterns in each of the three PCR-based methods.

In contrast to isolates of the other subspecies of *F. tularensis, F. tularensis* subsp. *tularensis* isolates cause a life-threatening disease, and this high virulence necessitates the availability of methods for their rapid identification. It has long been accepted that isolates of the subspecies exist only in North America (8, 20). Notably, we found that the pattern obtained from a Slovakian strain derived from a mite was identical to those obtained from North American *F. tularensis* subsp. *tularensis* strains, thus supporting the recently reported finding that, based on biochemical characteristics and virulence for rabbits, this strain should be classified as a member of *F. tularensis* subsp. *tularensis*. This situation is of concern for European reference laboratories, since the highly contagious *F. tularensis* strains always pose a risk that laboratory staff may acquire tularemia. Although the presented specific PCR does not distinguish among *F. tularensis* subsp. *tularensis, F. tularensis* subsp. *novicida*, and *F. tularensis* subsp. *mediaasiatica* strains, it nevertheless could provide useful clinical information by rapidly identifying the highly virulent strains of *F. tularensis* subsp. *tularensis*, especially in North America, where these strains, in contrast to strains of the other two subspecies, are relatively common. Moreover, when the specific PCR is combined with one of the described arbitrary-primer PCR methods, isolates of *F. tularensis* subsp. *tularensis* are readily distinguished from those of the other subspecies.

A recently published study (2) aimed to identify methods that allowed discrimination of isolates of *F. tularensis* subsp. *holarctica*, the majority of which originated from Spain. The study evaluated PCR methods based on REP, ERIC, M13, or T3-T7 primers. It was reported that the discriminatory indices (10) of the methods ranged from 0.14 (REP) to 0.65 (T3-T7), below the recommended value of >0.95 for a method considered suitable for routine typing of individual isolates (23). Our finding of discriminatory indices of ≤ 0.65 correlates well with those of the Spanish study and shows that the investigated methods do not meet the required minimum criterion for typing of individual isolates. The test population is also an important factor to consider when assessing discriminatory power; the population should reflect the diversity of the particular species as much as possible (23). We believe that our collection of strains, which included strains from the various regions of the Northern Hemisphere where tularemia is endemic and all four subspecies of *F. tularensis*, is more representative than the Spanish collection for an analysis of the discriminatory indices of the PCR-based methods. It should be noted that even when the results of the four methods analyzed by de la Puente-Redondo et al. (2) were combined, the discriminatory index was 0.90, still below the recommended value of >0.95 (23). Moreover, considering that REP and ERIC sequences do not exist in the *Francisella* genome, probably all of the methods used are variants of RAPD-PCR, a technique with moderate or low reproducibility (14, 25–27). Although we consider that the investigated methods have discriminatory powers too low to be useful for typing of strains, this conclusion does not exclude, however, the use of one or several of the methods by reference laboratories because a positive finding, i.e., strains showing distinct patterns, may still be of epidemiological interest.

In summary, the relative simplicity and discriminatory power of several of the investigated methods make them useful for clinical laboratories as tools for rapidly identifying and discriminating *Francisella* species and subspecies but not for discriminating individual strains.

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