Journal of Clinical Microbiology

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J. Clin. Microbiol. **2005, 43(9):4558. DOI: 10.1128/JCM.43.9.4558-4566.2005.**

JOURNAL OF CLINICAL MICROBIOLOGY, Sept. 2005, p. 4558–4566 Vol. 43, No. 9
0095-1137/05/$08.00+0 doi:10.1128/JCM.43.9.4558–4566.2005

Evaluation of tRNA Gene PCR for Identification of *Mollicutes*

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Received 15 December 2004/Returned for modification 14 February 2005/Accepted 2 June 2005

We evaluated the applicability of tRNA gene PCR in combination with fluorescent capillary electrophoresis with an ABI310 genetic analyzer (Applied Biosystems, Calif.) for the identification of different mollicute species. A total of 103 strains and DNA extracts of 30 different species belonging to the genera *Acholeplasma*, *Mycoplasma*, and *Ureaplasma* were studied. Reproducible peak profiles were generated for all samples, except for one *M. genitalium* isolate, the three *M. gallisepticum* isolates, and 8 of the 24 *Ureaplasma* cultures, where no amplification could be obtained. Clustering revealed numerous discrepancies compared to the identifications that had been previously obtained by means of biochemical and serological tests. Final identification was obtained by 16S rRNA gene amplification followed by sequence analysis and/or restriction digestion. This confirmed the identification obtained by tRNA gene PCR in all cases. Seven samples yielded an unexpected tRNA gene PCR profile. Sequence analysis of the 16S rRNA genes showed that six of these samples were mixed and that one had a unique sequence that did not match any of the published sequences, pointing to the existence of a not-yet-described species. In conclusion, we found tRNA gene PCR to be a rapid and discriminatory method to correctly identify a large collection of different species of the class of *Mollicutes* and to recognize not-yet-described groups.

Having no cell wall, *Mollicutes* form a special class of bacteria. Their small, compact genomes evolved from AT-rich, gram-positive bacteria by means of genome reduction. At the same time, they developed innovative mechanisms to survive as parasitic organisms in a wide variety of host environments. To date, eight genera belonging to the class *Mollicutes* have been described, and within these genera, up to 200 species, mostly of the genus *Mycoplasma*, are acknowledged. This variety of species is associated with several taxonomic ambiguities (15, 17, 18), and a correct identification may be very difficult for numerous reasons. First, a number of *Mollicutes*, especially the plant-pathogenic spiroplasmas, have not been cultivated, while others require very complex media. As a result, for some species, only a limited number of isolates exist, and these are often not easily accessible. Second, as more sequences and better isolation media become available, more species are continuously being discovered. Finally, for some species, limited data and very few reports are published, especially for less-virulent and nonvirulent species.

To correctly differentiate all these species, a universal and fast identification technique would be extremely useful. Some promising methods have already been described (see, e.g., reference 25), but they do not yield digitized data, making exchange between laboratories difficult. An optimized tRNA gene PCR technique, originally described by Welsh and McClelland (27, 48), has been shown to be useful for correct and reproducible identification of very diverse bacterial species when combined with high-resolution electrophoresis (3–5, 10, 11, 23, 45). The technique is based on the amplification of spacer regions between tRNA genes using consensus tRNA gene primers. The amplified products are separated by electrophoresis for exact sizing, and the resulting species-specific peak profiles are subsequently archived in a database. Profiles obtained from an unknown sample can be compared with this data set while not-yet-included and/or newly described species can be added to expand the database further. We investigated the potential of this technique to correctly identify a large number of *Acholeplasma*, *Mycoplasma*, and *Ureaplasma* species.

MATERIALS AND METHODS

Strains. A total of 103 strains and DNA extracts were used during this study and are listed in Table 1. Purified genomic DNA of isolates belonging to the *Mycoplasma mycoides* cluster was kindly supplied by L. Manso-Silivan (Agricultural Research Centre for International Development, Montpellier, France), and purified genomic DNA of *M. hyosynoviae* isolates was supplied by B. Kokotovic (Danish Institute for Food and Veterinary Research, Copenhagen, Denmark). The DNA extracts from clinical samples that were positive for *M. genitalium* were received from the Institute of Tropical Diseases (Antwerp, Belgium) and had been extracted directly from vaginal swabs of five Asian women. Apart from the reference strains included, all isolates were obtained over the years during routine diagnostics. The origins of these strains were not always clear, since some strains were retrieved from old collections.

Culture media and DNA extraction. F broth (7), A7 differential agar (37), modified Hayflick broth (MHB) (44), SP-4 broth (44), SP-4 broth supplemented with L-arginine (SP4A), HS broth (16), or Friis' broth with ampicillin instead of methicillin (NHS20) (21) was used to cultivate the different species, as listed in

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TABLE 1. Isolates used in this study[a]

Initial ID	Final ID (technique[b])	tRNA gene PCR-based ID	Strain ID	Origin[c]	Date of isolation (mm/yy)	Strain name	Culture medium
Avian mollicutes							
M. columbinasale	*M. columbinasale* (A)	*M. columbinasale*	MYP030	CODA	04/89	397	HS
M. columbinum	*M. columbinum* (S)	*M. columbinum*	MYP031	CODA	12/87	446	HS
M. columbinum	*M. columbinum* (S)	*M. columbinum*	MYP032	CODA	11/85	423VD	HS
M. columbinum	*M. columbinum* (S)	*M. columbinum*	MYP033	CODA	12/87	447	HS
M. gallinaceum	*M. gallinarum* (S)	*M. gallinarum*	MYP038	CODA	NA[d]	CODA 18A	HS
M. gallisepticum	*M. gallinarum* (A)	*M. gallinarum*	MYP041	CODA	01/89	D63P	F
M. gallisepticum	*M. gallinarum* (A)	*M. gallinarum*	MYP042	CODA	02/89	CODA 19E	F
M. gallisepticum	*M. gallisepticum* (A, S)	No peaks	MYP013	CODA	NA	ATCC 19610	MHB
M. gallisepticum	Mixed profile (S)	Mixed profile	MYP039	CODA	04/89	X95	HS
M. gallisepticum	*M. gallisepticum* (S)	No peaks	MYP040	CODA	04/89	A5969	F
M. gallisepticum	*M. gallisepticum* (A)	No peaks	MYP071	CODA	NA	2000Myc58	F
M. glycophilum	*M. glycophilum* (A)	*M. glycophilum*	MYP043	CODA	02/89	CODA 20A	MHB
M. lipofaciens	*M. lipofaciens* (A)	*M. lipofaciens*	MYP049	CODA	01/88	R171	MHB
M. pullorum	*M. glycophilum* (A)	*M. glycophilum*	MYP052	CODA	12/84	412VD	F
M. pullorum	*M. columborale* (S)	*M. columborate*	MYP053	CODA	12/86	Pul46	MHB
M. synoviae	*M. neurolyticum* (A)	*M. neurolyticum*	MYP058	CODA	02/69	WVU1853	HS
Bovine, caprine, and ovine mollicutes							
M. agalactiae	*M. arginini* (A)	*M. arginini*	MYP016	CODA	06/84	884/200	HS
M. agalactiae	*M. agalactiae* (A)	*M. bovis-M. agalactiae*	MYP017	CODA	NA	NCTC 10123 (PG2)	HS
M. agalactiae	*M. bovis* (A)	*M. bovis-M. agalactiae*	MYP018	CODA	04/97	83/61	HS
M. agalactiae	*M. agalactiae* (A)	*M. bovis-M. agalactiae*	MYP019	CODA	NA	5725	HS
M. bovigenitalium	*M. bovigenitalium* (S)	*M. bovigenitalium*	MYP020	CODA	06/89	MN120	MHB
M. bovirhinis	*M. bovirhinis* (A)	*M. bovirhinis*	MYP066	CODA	NA	ATCC 27748	NHS20
M. bovis	*M. bovis* (A)	*M. bovis-M. agalactiae*	MYP022	CODA	06/83	295VD	F
M. bovis	*M. bovis* (A)	*M. bovis-M. agalactiae*	MYP023	CODA	NA	Widanka309	F
M. bovis	*M. bovis* (S)	*M. bovis-M. agalactiae*	MYP067	CODA	NA	O422	MHB
M. bovis	*M. bovirhinis* (A)	*M. bovirhinis*	MYP068	CODA	NA	O475	MHB
M. dispar	*M. bovis* (A)	*M. bovis-M. agalactiae*	MYP034	CODA	11/83	CODA 17A	SP-4
M. dispar	*M. dispar* (A)	*M. dispar*	MYP035	CODA	12/82	CODA 17B	SP-4
M. dispar	*M. dispar* (A)	*M. dispar*	MYP036	CODA	NA	ATCC 27140	SP-4
M. dispar	*M. bovis* (A, S)	*M. bovis-M. agalactiae*	MYP037	CODA	11/83	CODA 17E	SP-4
M. capricolum subsp. *capricolum*	*M. capricolum* subsp. *capricolum* (A)	*M. capricolum*	MYP080	CIRAD	NA	ATCC 27343 (California Kid)	NA
M. capricolum subsp. *capripneumoniae*	*M. capricolum* subsp. *capripneumoniae* (S)	*M. capricolum*	MYP076	CIRAD	NA	NCTC 10192 (F38)	NA
M. mycoides subsp. *capri*	*M. mycoides* subsp. *capri* (A)	*M. mycoides*	MYP078	CIRAD	NA	Pg3	NA
M. mycoides subsp. *mycoides* LC	*M. mycoides* subsp. *mycoides* LC (A)	*M. mycoides*	MYP079	CIRAD	NA	YG	NA
M. mycoides subsp. *mycoides* SC	*M. mycoides* subsp. *mycoides* SC (A)	*M. mycoides* subsp. *mycoides* SC	MYP075	CODA	NA	Pg1	NA
Mycoplasma sp. bovine group 7	*Mycoplasma* sp. bovine group 7 (A)	*M.* sp. bovine group 7	MYP077	CIRAD	NA	Pg50	NA
M. ovipneumoniae	*M. bovis* (S)	*M. bovis-M. agalactiae*	MYP051	CODA	08/83	CODA 29C	F
M. putrefaciens	*M. putrefaciens* (A)	*M. putrefaciens*	MYP054	CODA	11/85	Put85	F
M. putrefaciens	*M. putrefaciens* (A)	*M. putrefaciens*	MYP055	CODA	03/87	B387	F
M. putrefaciens	*M. putrefaciens* (A)	*M. putrefaciens*	MYP056	CODA	03/87	B791	F
M. putrefaciens	*M. putrefaciens* (A)	*M. putrefaciens*	MYP057	CODA	02/98	7578.95	F
Human mollicutes							
M. genitalium	*M. genitalium* (P)	*M. genitalium* A	MYP106	ITG	NA	MSE 0883	NA
M. genitalium	*M. genitalium* (P)	No peaks	MYP107	ITG	NA	MSE 0896	NA
M. genitalium	*M. genitalium* (P)	*M. genitalium* B	MYP108	ITG	NA	MSE 1028	NA
M. genitalium	*M. genitalium* (P)	*M. genitalium* A	MYP109	ITG	NA	MSE 1209	NA
M. genitalium	*M. genitalium* (P)	*M. genitalium* B	MYP110	ITG	NA	MSE 1318	NA
M. hominis	Mixed profile (NT)	Mixed profile	MYP081	VUB	03/04	040319/5	A7
M. hominis	*M. hominis* (S)	*M. hominis*	MYP111	GUH	02/02	020211 2245	A7

Continued on following page

TABLE 1—*Continued*

Initial ID	Final ID (technique[b])	tRNA gene PCR-based ID	Strain ID	Origin[c]	Date of isolation (mm/yy)	Strain name	Culture medium
M. hominis	*M. hominis* (S)	*M. hominis*	MYP112	GUH	NA	BVS058A4	A7
M. orale	*M. orale* (S)	*M. orale*	MYP115	NCTC	NA	ATCC 23714	SP4A
M. pneumoniae	*M. pneumoniae* (A)	*M. pneumoniae*	MYP072	CODA	06/96	CODA 38B	SP-4
M. pneumoniae	*M. pneumoniae* (A)	*M. pneumoniae*	MYP073	CODA	12/85	CODA 38C	SP-4
M. pneumoniae	*M. pneumoniae* (A)	*M. pneumoniae*	MYP074	CODA	12/84	CODA 38D	SP-4
M. salivarium	*M. salivarium* (S)	*M. salivarium*	MYP113	GUH	NA	ED135	SP4A
M. salivarium	*M. salivarium* (S)	*M. salivarium*	MYP114	GUH	NA	TC010	SP4A
U. urealyticum and *M. hominis*	Mixed profile (NT)	Mixed profile	MYP082	VUB	NA	040324/3	A7
U. urealyticum and *M. hominis*	Mixed profile (NT)	Mixed profile	MYP083	VUB	10/03	031002/9	A7
U. urealyticum and *M. hominis*	NA	No peaks	MYP084	VUB	04/04	040413/1	A7
U. parvum	NA	*U. parvum*	MYP085	VUB	NA	Serotype 01	A7
U. urealyticum	NA	No peaks	MYP086	VUB	NA	Serotype 02	A7
U. parvum	NA	No peaks	MYP087	VUB	NA	Serotype 03	A7
U. urealyticum	NA	*U. urealyticum*	MYP088	VUB	NA	Serotype 04	A7
U. urealyticum	NA	*U. urealyticum*	MYP089	VUB	NA	Serotype 05	A7
U. parvum	NA	*U. parvum*	MYP090	VUB	NA	Serotype 06	A7
U. urealyticum	NA	*U. urealyticum*	MYP091	VUB	NA	Serotype 07	A7
U. urealyticum	NA	No peaks	MYP092	VUB	NA	Serotype 08	A7
U. urealyticum	NA	*U. urealyticum*	MYP093	VUB	NA	Serotype 09	A7
U. urealyticum	NA	*U. urealyticum*	MYP094	VUB	NA	Serotype 10	A7
U. urealyticum	NA	*U. urealyticum*	MYP095	VUB	NA	Serotype 11	A7
U. urealyticum	NA	*U. urealyticum*	MYP096	VUB	NA	Serotype 12	A7
U. urealyticum	NA	No peaks	MYP097	VUB	NA	Serotype 13	A7
U. parvum	NA	*U. parvum*	MYP098	VUB	NA	Serotype 14	A7
U. urealyticum	NA	No peaks	MYP099	VUB	03/04	040327/1	A7
U. urealyticum	NA	*U. urealyticum*	MYP100	VUB	03/04	040323/1	A7
U. urealyticum	NA	No peaks	MYP101	VUB	03/04	040330/8	A7
U. urealyticum	NA	No peaks	MYP102	VUB	03/04	040330/7	A7
U. urealyticum	NA	*U. parvum*	MYP103	VUB	03/04	040329/7	A7
U. urealyticum and *M. hominis*	NA	Mixed profile	MYP104	VUB	10/03	031001/3	A7
U. urealyticum and *M. hominis*	NA	Mixed profile	MYP105	VUB	03/04	040324/3	A7
Murine mollicutes							
M. neurolyticum	*M. neurolyticum* (A)	*M. neurolyticum*	MYP050	CODA	01/75	CODA 28A	HS
Porcine mollicutes							
A. granularum	*A. granularum* (A)	*A. granularum*	MYP015	CODA	02/77	CODA 2D	MHB
M. flocculare	*M. flocculare* (P)	*M. flocculare*	MYP001	CODA	NA	MP102	NHS20
M. flocculare	*M. flocculare* (P)	*M. flocculare*	MYP002	CODA	NA	ATCC 27399 (Ms42)	NHS20
M. flocculare	*M. flocculare* (P)	*M. flocculare*	MYP003	CODA	07/00	MflocF6A	NHS20
M. hyopneumoniae	*M. hyopneumoniae* (A, P)	*M. hyopneumoniae*	MYP007	CODA	NA	ATCC 25934 (J)	NHS20
M. hyopneumoniae	*M. hyopneumoniae* (A, P)	*M. hyopneumoniae*	MYP008	CODA	12/99	MhF5C	NHS20
M. hyopneumoniae	*M. hyopneumoniae* (A, P)	*M. hyopneumoniae*	MYP009	CODA	07/00	MhF6A	NHS20
M. hyopneumoniae	*M. hyorhinis* (P)	*M. hyorhinis*	MYP044	CODA	12/83	Pf	NHS20
M. hyopneumoniae	*A. laidlawii* (A)	*A. laidlawii* A	MYP045	CODA	03/83	RotaRA	NHS20
M. hyorhinis	*M. hyorhinis* (P)	*M. hyorhinis*	MYP004	CODA	07/00	MhyorF7A	NHS20
M. hyorhinis	*M. hyorhinis* (P)	*M. hyorhinis*	MYP005	CODA	08/00	MhyorF6A	NHS20
M. hyorhinis	*M. hyorhinis* (P)	*M. hyorhinis*	MYP006	CODA	01/01	MhyorF9A	NHS20
M. hyosynoviae	*M. hyosynoviae* (A)	*M. hyosynoviae*	MYP059	DFVF	NA	Mp6	NA
M. hyosynoviae	*M. hyosynoviae* (A)	*M. hyosynoviae*	MYP060	DFVF	NA	Mp96	NA
M. hyosynoviae	*M. hyosynoviae* (A)	*M. hyosynoviae*	MYP061	DFVF	NA	Mp178	NA
M. hyosynoviae	*M. hyosynoviae* (A)	*M. hyosynoviae*	MYP062	DFVF	NA	Mp356	NA
M. hyosynoviae	*M. hyosynoviae* (A)	*M. hyosynoviae*	MYP063	DFVF	NA	Mp1023	NA
M. hyosynoviae	*M. hyosynoviae* (A)	*M. hyosynoviae*	MYP064	CODA	NA	ATCC 25591 (S16)	NA
Other mollicutes							
A. laidlawii	*A. laidlawii* (A)	*A. laidlawii* A	MYP010	CODA	03/84	84/DAW	MHB
A. laidlawii	*A. laidlawii* (A)	*A. laidlawii* B	MYP011	CODA	08/87	87/328VD	MHB
A. laidlawii	*A. laidlawii* (A)	*A. laidlawii* B	MYP012	CODA	07/82	CODA 1E	MHB
A. laidlawii	*Acholeplasma* sp. nov. (S)	*Acholeplasma* sp. nov.	MYP014	CODA	08/85	CODA 1G	MHB
A. laidlawii	*A. laidlawii* (A)	*A. laidlawii* B	MYP065	CODA	NA	ATCC 23206 (PG8)	NHS20

[a] The samples are listed according their hosts and in alphabetical order as received (and discussed in Results). ID, identification.
[b] A, ARDRA; P, species-specific PCR; S, 16S rRNA gene sequence analysis; NT, not tested.
[c] CODA, Veterinary and Agrochemical Research Centre (Brussels, Belgium); ITG, Institute of Tropical Diseases (Antwerp, Belgium); GUH, Ghent University Hospital (Belgium); VUB, Free University of Brussels (Belgium); DFVF, Danish Institute for Food and Veterinary Research (Copenhagen, Denmark); CIRAD, Agricultural Research Centre for International Development (Montpellier, France); NCTC, National Collection of Type Cultures (London, United Kingdom).
[d] NA, not applicable/not available.

Table 1. Genomic DNA was prepared from the cultivated strains and from the vaginal swabs by means of phenol-chloroform extraction as described previously (5a), except for the *Ureaplasma urealyticum*, *U. parvum*, *M. hominis*, and *M. salivarium* strains, for which DNA was extracted by alkaline lysis (46).

Identification of strains. Most strains (i.e., MYP10 to MYP58 and MYP65 to MYP74) had been identified previously by means of phenotypical characteristics and the growth precipitation test using absorbed rabbit antisera (13, 36). After identification, the strains had been lyophilized in the presence of 20% sterile milk and stored at 4°C. *U. urealyticum*, *U. parvum*, and some of the *M. hominis* isolates were previously identified by their characteristic growth on A7 differential agar medium and by their ability to hydrolyze urea and arginine, respectively. Due to the numerous discrepancies with the results obtained in this study by means of tRNA gene PCR, most of the strains were reidentified. The identity of *M. hyopneumoniae*, *M. hyorhinis*, and *M. flocculare* was confirmed by a specific PCR (40). Also, the *M. genitalium* samples were identified by two specific PCR tests as described previously by Jensen et al. (19, 20). Most other isolates were reidentified using amplified rRNA gene restriction analysis (ARDRA) (unpublished data) and/or sequence analysis of the 16S rRNA genes (12, 46) (Table 1).

tRNA gene PCR and cluster analysis. tRNA gene PCR was performed as described previously using primers T5A (5′-AGTCCGGTGCTCTAACCAACT GAG) and T3B (5′-AGGTCGCGGGTTCGAATCC) (4, 27). Cluster analysis of the obtained tRNA gene PCR fingerprints was carried out by calculating a distance matrix using the differential base pair (DBP) algorithm (3) with a tolerance of 1.2 bp and including all peaks (i.e., no noise subtraction) from 50 to 500 base pairs in length. The DBP clustering algorithm was used to calculate similarity by taking the average of the two results that are obtained by dividing the number of tRNA gene-intergenic spacers in common between two strains by the total number of spacers of one strain, respectively, of the other strain. A similarity tree was constructed using the unweighted-pair group method using average linkages (PHYLIP, V3.6; J. Felsenstein, Department of Genome Sciences, University of Washington, Seattle, Wash.) and visualized using Treeview V1.6.6 (29).

Construction of a digital library. A digital library composed of consensus library entries was constructed. Each consensus library entry contained only peaks (amplified tRNA gene-intergenic spacers) present in all sample files of a particular species. Identification was carried out by comparing the fingerprint of a strain with all entries of the constructed library using the DBP algorithm. For identification, the DBP algorithm takes only peaks that are mutually present in the sample file and in the library entry into account, discarding the peaks only present in the sample file. For example, comparison of an unknown strain with 15 peaks, of which 10 are identical to all 10 peaks of a library entry, gives a similarity of 100%, as would have been the case for an unknown strain with 10 peaks, all identical to the 10 peaks of the library entry. An unknown strain with 8 peaks, of which all 8 are identical to 8 of the 10 peaks of the library entry, gives a value of only 80%. An unknown with 15 peaks, of which 8 are identical to an entry with 10 peaks, gives an identity of 80% as well. This method is (by experience) better suited for identification of unknown patterns, since intraspecific variability of the peaks with low intensity is better compensated for. To differentiate between resembling, but distinct, tRNA gene PCR patterns, the algorithm allows us to take absent peaks into account by adding a minus in front of the absent peak. The software counts the absence of the peak as a positive match, increasing the similarity score and increasing the reliability of the identification result.

Nucleotide sequence accession number. The 16S rRNA gene sequence for *Acholeplasma* sp. strain MYP14 was deposited in GenBank under accession number AY785356.

RESULTS

Cluster analysis of tRNA gene PCR fingerprints. tRNA gene PCR fingerprints could be obtained from 91 of the 103 strains and DNA extracts in total. In general, strains showed fingerprints with more than 10 amplified DNA fragments, i.e., intergenic tRNA spacer regions. In most cases, the obtained tRNA gene PCR patterns were species specific and highly identical for all the strains tested from a single species. The consensus library entries for each species are listed in Table 2. Below, we present the clustering results (shown in Fig. 1) obtained for the different species, grouped according to their host.

Avian mollicutes. One strain (MYP30), received as *M. columbinasale*, of which the identification was confirmed by ARDRA, clustered separately, most closely to the *M. gallinarum* cluster.

The three strains MYP31 to MYP33, received as *M. columbinum*, had identical patterns, clustering separately. One strain received as *M. gallinaceum* (MYP38) and two of the six strains received as *M. gallisepticum* (MYP41 and MYP42) had identical, unique tRNA gene PCR patterns and were identified by ARDRA as *M. gallinarum*.

Six strains (MYP13, MYP39 to MYP42, and MYP71) were received as *M. gallisepticum*. As explained, MYP41 and MYP42 were shown to be *M. gallinarum*. No amplification products were obtained from strains MYP13, MYP40, and MYP71, identified as *M. gallisepticum* according to ARDRA or sequence analysis. The tRNA gene PCR pattern of strain MYP39 showed a high similarity to members of the *M. bovis-M. agalactiae* cluster, although the tRNA gene pattern profile clearly had many additional peaks. Sequence analysis showed a mixed profile, confirming the contamination of this sample, which was therefore left out for further analysis.

The one *M. glycophilum* strain, MYP43, of which the identification was confirmed by ARDRA, was indistinguishable from strain MYP52, received as *M. pullorum*, which was identified by ARDRA as *M. glycophilum*.

The one *M. lipofaciens* strain (MYP49), confirmed as such by ARDRA, had a very specific tRNA gene PCR pattern. Two strains (MYP52 and MYP53) were received as *M. pullorum*. One (MYP52) was identified as *M. glycophilum* by ARDRA as discussed above. The other one (MYP53) was identified by sequence analysis as *M. columborale*, a species that was not included initially, which is consistent with the fact that this strain had a unique tRNA gene PCR pattern.

The one strain received as *M. synoviae* (MYP58) had a tRNA gene PCR pattern that strongly resembled that of the sole *M. neurolyticum* strain (MYP50), an identification that was confirmed by ARDRA.

Bovine, caprine, and ovine mollicutes. Four strains (MYP16 to MYP19) were received as *M. agalactiae*. The DNA extract obtained from *M. agalactiae* strain MYP16 was found to yield a separate tRNA gene PCR fingerprint (Fig. 1). This strain was later identified with ARDRA as *M. arginini*. The two genuine *M. agalactiae* strains (MYP17 and MYP19) had a very similar tRNA gene profile and clustered together with strain MYP18, which was later identified as *M. bovis* by means of ARDRA.

M. bovigenitalium strain MYP20 had a unique tRNA gene PCR pattern, and its identification could be confirmed by 16S rRNA gene sequence analysis.

Strains MYP22 to MYP23 and MYP67 to MYP68 had previously been identified as *M. bovis*. MYP68 clustered together with MYP66, which was received as *M. bovirhinis* and was indeed shown by ARDRA to be *M. bovirhinis*. The remaining three *M. bovis* strains clustered together with *M. agalactiae* strains MYP17 and MYP19.

Four strains were received as *M. dispar* (MYP34 to MYP37). MYP34 and MYP37 were clustered by tRNA gene PCR in the *M. bovis-M. agalactiae* group, a finding substantiated by ARDRA, which identified both strains as *M. bovis*. This identification was confirmed for MYP37 by sequencing. The remaining *M. dispar* strains, MYP35 and MYP36, clustered sep-

TABLE 2. Consensus tRNA gene PCR profiles of tested *Mollicutes* species

Species[a]	No. of strains tested	Consensus tRNA gene PCR fragments (bp)[b]
A. laidlawii A	2	58.6; 62.2; 66.7; 69.1; 70.9; 156.6; 189.3; 281.1; 389.6
A. laidlawii B	3	58.6; 62.2; 66.7; 70.9; 72.3; 191.3; 281.1; 391.6
A. granularum	1	57.5; 61.4; 70; 178.2; 270; 385.2; 463.5
Acheloplasma sp. nov.	1	57.6; 62.4; 66.5; 68.7; 70.9; 283.5; 303.2; 352.8; 433.3
M. bovis-M. agalactiae	7, 2	57.4; 61.5; 67.3; 70; 78.6; 131.4; 144.2; 151.8; 159.5; 257.4
M. arginini	1	56.9; 64.4; 86.4; 132.3; 144.1; 152.6; 161.1; 197.5; 224.2; 227.7
M. bovigenitalium	1	59.9; 67.6; 76.6; 88.8; 132.6; 146.4; 153.2; 161.1; 358.1
M. bovirhinis	2	55.3; 61.3; 63.6; 67.7; 68.7; 69.7; 76.9; 79.2; 135.6; 140.1; 141.8; 147.2; 150; 157.3
M. capricolum subsp. *capricolum* and *M. capricolum* subsp. *capripneumoniae*	2	55.3; 70; 124.5; 132.1; 135.5; 138.3; 140.6; 142.3; 143.9; 145; 147.1; 156.7; 158.1; 212.9; 225.6; 226.3; 228.6; 235.4; 241.3; 245.5
M. columbinasale	1	57.4; 59.9; 65.2; 67.7; 76.6; 85.9; 91.8; 132; 146.1; 152.8; 160.3; 256.6; 349.9
M. columbinum	3	57.3; 60.6; 66.2; 69; 77.7; 130.5; 143.6; 148.1; 150.4; 158.3; 222.5; 257.2; 350.3
M. columborale	1	57.2; 59.4; 67.6; 74.9; 77.1; 133.4; 146.9; 154.2; 247.6
M. dispar	2	69.7; 131; 150.6; 287.6
M. flocculare	3	128.7; 148.3; 158.2; 291.3
M. gallinarum	3	57; 64.5; 66.4; 75.9; 85.8; 113.1; 131; 144.7; 151.5; 160.1; 256.6; 347; 349.9
M. genitalium A	2	56.4; 66.2; 145.6; 181.2
M. genitalium B	2	58.8; 64.4; 66.5; 76.6; 88.3; 125.6; 143.6; 154.5; 177.2; 198.5; 217.6; 228.5
M. glycophilum	2	58.5; 66.7; 74.3; 76.5; 133.2; 147; 154.2; 358.2
M. hominis	2	151.9; 226.7
M. hyopneumoniae	3	69.2; 132.6; 153.8; 311.1
M. hyorhinis	4	59.3; 67.3; 144.6; 150; 230; 245.3
M. hyosynoviae	6	57.1; 66.1; 129.5; 149.2; 153.1; 198.2; 228.7; 244.9; 262.3; 322
M. lipofaciens	1	56.4; 57.7; 62.2; 64.2; 65.8; 74.9; 85.3; 127.1; 139.8; 146.3; 152.8
M. mycoides subsp. *mycoides* LC and *M. mycoides* subsp. *capri*	2	55.3; 72.1; 134.8; 155.4; 211.5; 223.8; 226.8; 233.4; 237.7; 239.2; 243.5
M. mycoides subsp. *mycoides* SC	1	55.3; 69.4; 72.4; 156.4; 159.9; 212; 216.2; 218.6; 224.5; 232.4; 234.4; 236.3; 238.7; 240.3; 244.6
M. neurolyticum	2	63.5; 73.2; 126.7; 139.9; 145.7; 168.4; 230.5
M. orale	1	56.5; 59.4; 65.6; 66.7; 67.6; 89.8; 118; 131.3; 134.3; 135.6; 137.5; 139.6; 147.7; 150; 151.4; 153; 161.1; 173.1; 199.1; 221.8; 225.8; 229.7; 238.3; 241.6; 256.7; 323.7
M. pneumoniae	3	55.3; 59.1; 61.4; 65.8; 67.2; 70.8; 73.6; 75.1; 77.2
M. putrefaciens	4	60.3; 68.8; 134; 149.5; 154.8; 165; 210.3; 225.2; 241.6; 249.4
M. salivarium	2	64.6; 66.4; 131.9; 152.1; 229.3; 258.2; 320.9
Mycoplasma sp. bovine group 7	1	55.3; 72.2; 73.2; 106.2; 121.6; 124.5; 131.1; 132.1; 135.5; 138.3; 139.4; 140.6; 141.7; 142.6; 144.1; 145; 147.2; 156.7; 158; 212.2; 215.8; 216.5; 224.8; 227.6; 232.7; 234.6; 236.2; 238.8; 240.2; 244.5
Ureaplasma urealyticum	8	56; 144.2; −279.5; 280.7
Ureaplasma parvum	4	55.4; 144.2; 279.5; −280.7

[a] Species that were indistinguishable are listed together.
[b] A minus (−) preceding a number indicates the absence of a peak with that length in base pairs.

arately (Fig. 1) and were shown to be genuine *M. dispar* by ARDRA.

The so-called *M. mycoides* cluster comprises six species or subspecies of closely related mycoplasmas. The type strains of these ruminant mycoplasmas were included for analysis (MYP75 to MYP80). *M. capricolum* subsp. *capricolum* (MYP80) and in particular *M. capricolum* subsp. *capripneumoniae* (MYP76) clustered most closely to a *Mycoplasma* sp. bovine group 7 strain (MYP77). These profiles showed up to 30 peaks, with over 20 peaks in common. Likewise, the tRNA gene profiles of the three *M. mycoides* isolates showed several small peaks and clustered together, with *M. mycoides* subsp. *capri* (MYP78) and *M. mycoides* subsp. *mycoides* LC (large colony) (MYP79) most closely related to each other.

The one strain received as *M. ovipneumoniae* (MYP51) clustered within the *M. bovis-M. agalactiae* group (Fig. 1) and was identified as *M. bovis* by 16S rRNA gene sequence analysis.

The four *M. putrefaciens* strains (MYP54 to MYP57), confirmed as such by ARDRA, had very identical and characteristic tRNA gene PCR patterns.

Human mollicutes. Five samples were received as *M. genitalium* (MYP106 to MYP110). Strain MYP107 did not yield a fingerprint. Strikingly, there were two different groups observed within *M. genitalium* (Table 2) which did cluster separately but close to each other.

Three *M. hominis* cultures were included in this study. Two pure *M. hominis* isolates (MYP111 and MYP112), identified by means of 16S rRNA gene sequencing, had identical and specific tRNA gene PCR patterns composed of only two peaks (of 151.9 and 226.6 bp). One culture (MYP081) that was positive for *M. hominis* on A7 agar plates was clearly contaminated, since additional peaks of 56.0, 144.2, and 280.7 bp, shown in this study to be characteristic for *U. urealyticum*, were present. Only the tRNA gene PCR fingerprints of the pure isolates MYP111 and MYP112 were included in the cluster analysis.

One *M. orale* strain (MYP115) was obtained from a culture

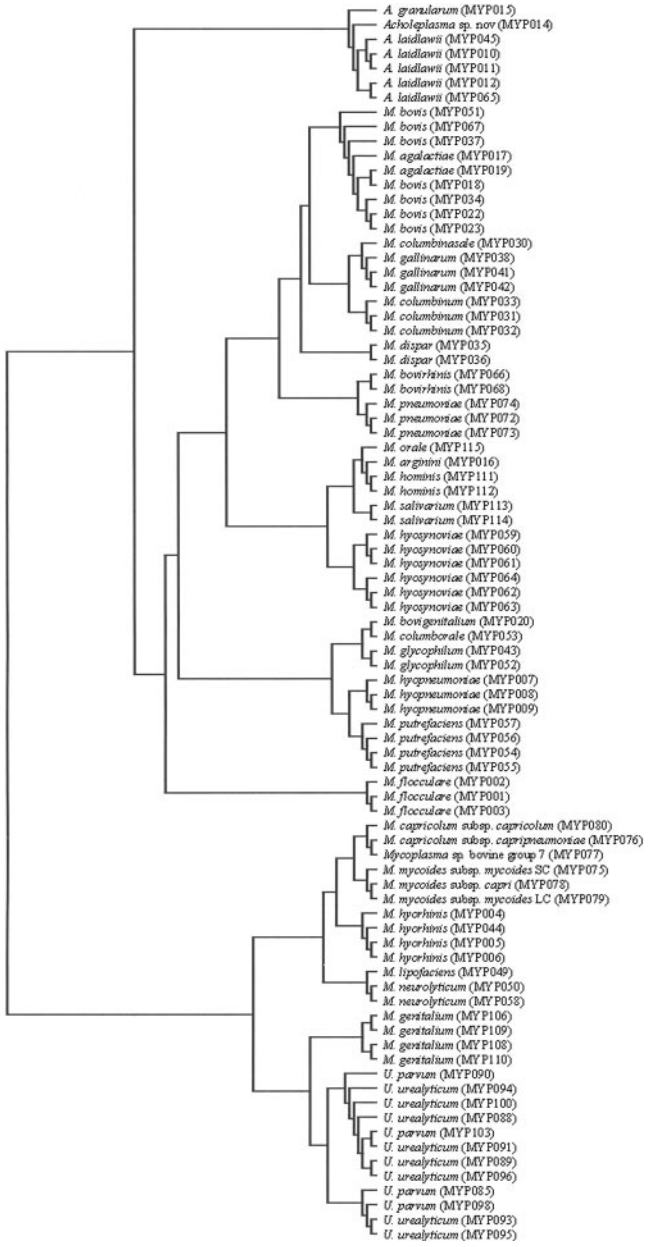

FIG. 1. Dendrogram of tRNA gene PCR fingerprints obtained after cluster analysis with unweighted-pair group method using average linkages of DBP-based similarity coefficients. Strains are listed with the final identifications obtained.

collection (National Collection of Type Cultures, United Kingdom) and had a very specific tRNA gene PCR profile, with over 25 characteristic peaks.

The tRNA gene PCR patterns of the three strains received as *M. pneumoniae* (MYP72 to MYP74), confirmed as such by ARDRA, were almost identical and were characterized by very short spacers (usually no longer than 77 bp).

The two *M. salivarium* strains (MYP113 and MYP114) had been isolated during studies of the complex microflora of tonsils and teeth, and their identity had been established by 16S rRNA sequencing. Their nearly identical and highly characteristic tRNA gene patterns clustered together.

A total of 24 *Ureaplasma* strains, received as *U. urealyticum* or *U. parvum* (MYP82 to MYP105), were included. For eight strains, no amplification could be obtained. Of the remaining samples, four (MYP82, MYP83, MYP104, and MYP105) had been received as contaminated with *M. hominis*, which was also apparent (as explained above when the results for *M. hominis* were presented) from the mixed tRNA gene PCR pattern that was obtained and which contained peaks characteristic to both species. These tRNA gene PCR fingerprints were not included in the cluster analysis. All other strains of both *Ureaplasma* species gave similar tRNA gene PCR patterns and clustered together.

Murine mollicutes. The one strain (MYP50) that was received as *M. neurolyticum* and confirmed as such by ARDRA had a tRNA gene PCR pattern that clustered together with that of strain MYP58, received as *M. synoviae*, but was shown to be *M. neurolyticum*, as described above.

Porcine mollicutes. The one strain that was received as *Acholeplasma granularum* (MYP15) was confirmed as such by ARDRA and could be identified easily by tRNA gene PCR, since its pattern was highly characteristic.

The three *M. flocculare* strains (MYP1 to MYP3) had a very similar pattern that made it possible to differentiate this species from all other species.

Five strains (MYP7 to MYP9, MYP44, and MYP45) were received as *M. hyopneumoniae*. A specific PCR identified MYP44 as *M. hyorhinis*, while MYP45 was identified as *A. laidlawii* as described below. MYP7, MYP8, and MYP9 had identical and characteristic tRNA gene PCR fingerprints, and their identity as *M. hyopneumoniae* was confirmed by ARDRA and by a specific PCR.

Strains MYP4 to MYP6, received as *M. hyorhinis*, were very much alike and had a typical pattern. The genuine *M. hyorhinis* strains clustered together with MYP44, which had been shown to be *M. hyorhinis* as well (see above).

Six strains (MYP59 to MYP64), received as *M. hyosynoviae* and confirmed as such by ARDRA, were very much alike with regard to their characteristic tRNA gene PCR patterns.

Other mollicutes. Five strains (MYP10 to MYP12, MYP14, and MYP65) were received as *A. laidlawii*. MYP14 had a unique tRNA gene PCR pattern and clustered separately. Sequence analysis showed significant differences with other known *Acholeplasma* spp., and the 16S rRNA gene sequence was submitted to GenBank. The other four strains clustered together with strain MYP45, which was received as *M. hyopneumoniae* but was also identified as *A. laidlawii* by means of ARDRA.

tRNA gene PCR-based identification. A digital library was constructed as described above. For *A. laidlawii* and *M. genitalium*, different tRNA gene PCR profiles were apparent, and for these species, two different consensus patterns were included in the library. All individual fingerprints (sample files including all peaks) were compared with this library using the similarity calculation designated DBP. The two *M. agalactiae* strains were indistinguishable from the eight *M. bovis* strains, having tRNA gene spacers with lengths of 57.4, 61.5, 67.3, 70.0, 78.6, 131.4, 144.2, 151.8, 159.5, and 257.4 bp in common. All other samples were identified correctly. Although the *U. urealyticum* and *U. parvum* strains grouped together during cluster analysis, they could clearly be distinguished on the basis of

their specific tRNA gene PCR pattern. The strains MYP85 (serovar 1), MYP90 (serovar 6), and MYP98 (serovar 14) belong to *U. parvum*. These three stains, together with MYP103, for which serovar determination had not been carried out, had a peak of 279.5 bp (standard deviation, 0.1 bp) in common, whereas the *U. urealyticum* strains MYP88 (serovar 4), MYP89 (serovar 5), MYP91 (serovar 7), MYP93 (serovar 9), MYP94 (serovar 10), MYP95 (serovar 11), MYP96 (serovar 12), and MYP100 (serovar not determined) had a peak of 280.7 bp (standard deviation, 0.03 bp) in common.

DISCUSSION

The combination of biochemical and serological results has always been a valuable tool for the identification of mollicutes. However, biochemical data often lack discriminatory power, while the problem of serological cross-reaction has also been described (6, 34, 35, 41). The problems with serological identification are exemplified in this study by the fact that 14 out of 53 serologically characterized strains had been misidentified. In this study, we evaluated whether a genotypic identification method, like tRNA gene PCR, might increase the efficiency of identification.

In general, over 10 different peaks were visible in the tRNA gene PCR fingerprints of different *Mollicutes* species, which is a high number compared to those of most other bacteria. This is a somewhat unexpected finding, since mollicutes have only a limited number of tRNA genes. In view of the fact that the tRNA gene PCR technique applied with the ABI310 genetic analyzer (Applied Biosystems, Calif.) only takes into account small PCR fragments of less than 500 bp, the close proximity of tRNA genes or the possibly high rate of tRNA gene-like sequences (14) may partly explain these results. The presence of this high number of peaks makes the technique well applicable for the identification of a complex and diverse class of bacteria like the *Mollicutes*.

In addition, the tRNA gene PCR technique has been shown to be very reproducible (4). This is also apparent from our results, since nearly identical fingerprints were obtained even for strains that were received from different laboratories and isolated on different dates. Thanks to interlaboratory reproducibility (4) and digitized output data, the tRNA gene PCR fingerprints of more species and subspecies can be collected from different laboratories and published in a shared online database.

No amplified PCR fragments were observed in 12 cases. The reason for failure is unclear, but in the case of the one *M. genitalium* sample and the eight *Ureaplasma* samples, this is probably due to poor DNA quality or the possible presence of PCR inhibitors (1), since other strains of the same species were amplified without problems. For the three *M. gallisepticum* strains, the reason is less clear, since none of the strains yielded a tRNA gene PCR pattern despite the high quality of the DNA samples used, as can be concluded from the efficient amplification of the 16S rRNA gene. Alignment and comparison of all tRNA gene sequences of the fully sequenced *M. gallisepticum* strain R with those of other known *Mycoplasma* tRNA gene sequences did not reveal any exceptional differences (30). Also, the arrangement of tRNA gene clusters in the *M. gallisepticum* genome was very much like that of other mollicute species (38, 43).

For all other cases, correct identification was obtained, except for the indistinguishable tRNA gene patterns of *M. bovis* and *M. agalactiae*. The high similarity of the 16S rRNA gene sequences of both species has already been reported (24), and the close relatedness of both species is also reflected by the fact that *M. bovis* was first considered as a subspecies of *M. agalactiae*. Later studies involving DNA homology and serology led to the proposal that *M. bovis* should not be regarded as a subspecies of *M. agalactiae* but should be regarded as a distinct species (2). In contrast with the common peaks observed for both species, several minor differences were noted between individual tRNA gene PCR patterns. Importantly, genetic variability between *M. bovis* isolates was already demonstrated by McAuliffe et al. using several other molecular DNA techniques (26). Those authors observed two distinct groups of *M. bovis* isolates, and an earlier report also showed the existence of two distinct groups of *M. agalactiae* isolates based on antigenic profiles (39). Whether the presence of the minor peaks coincides with these subgroups is not yet known.

Still, tRNA gene PCR enabled differentiation between subgroups of a number of other species, as was the case for the strains of *A. laidlawii*. The two distinct tRNA gene PCR profiles may indicate the existence of two different genomic groups for this species. This has also been indicated by earlier pulsed-field gel electrophoresis results showing different genome sizes for two *A. laidlawii* strains (32) and especially by nucleic acid hybridization studies that demonstrated extensive genomic variation between different strains (42).

Based on tRNA gene PCR, we also observed genotypic diversity within *M. genitalium*. A recent report demonstrated the presence of a number of different *M. genitalium* genotypes (22), but possible correspondence with the different tRNA gene PCR groups established in this study remains to be studied.

In the dendrogram obtained (Fig. 1), some groups, like the *M. hominis* taxon, clustered close together, similar to phylogenetic data based on 16S rRNA gene sequence analyses, while other groups, like the *M. neurolyticum* taxon, were scattered throughout the dendrogram (31, 47). Therefore, although the use of tRNA gene PCR for phylogenetic studies of divergent species is limited, it can be a helpful tool in resolving taxonomic ambiguities for very related species with almost identical tRNA gene PCR patterns. For example, the patterns of the strains belonging to the species of the mycoides cluster closely resembled each other and were clearly different from all other species. The finding that *Mycoplasma* sp. bovine group 7 strain PG50 (MYP77) showed close resemblance to the *M. capricolum* strains is in accordance with suggestions to place these species together in an *M. capricolum* taxon (8, 9). Our results are also in agreement with the advice of the subcommittee on the taxonomy of *Mollicutes*, which favors the combination of *M. mycoides* subsp. *capri* and *M. mycoides* subsp. *mycoides* LC strains into one taxon (8) and a separate position for *M. mycoides* subsp. *mycoides* SC (small colony). Accordingly, tRNA gene PCR also supports the recent differentiation of *Ureaplasma urealyticum* into two distinct species (33). The clustering results indicate the close relationship between these spe-

cies, but one of the intergenic tRNA spacers differs by 1 bp in length between both species.

The power of the technique was further demonstrated by the fact that tRNA gene PCR enabled detection of mixed cultures. For the mixed cultures of *U. urealyticum* and *M. hominis*, the presence of both species could be recognized because their specific patterns were included in the constructed consensus library.

In conclusion, tRNA gene PCR proved very useful for the identification of mollicute species. The unexpected high number of peaks provides the technique with a high discriminatory power. Although tRNA gene PCR is especially suited for the identification of unknown isolates, the technique can be a helpful tool to confirm current and future phylogenetic insights concerning the subdivision or merging of closely related species. Finally, we show that tRNA gene PCR can also be used to resolve mixed samples and to point to the existence of additional species.

ACKNOWLEDGMENTS

This work was supported by a grant of the Belgian Federal Agency of Health, Food Chain Security, and Environment (grant number S-6136).

We kindly thank Branko Kokotovic (Danish Veterinary Institute, Copenhagen, Denmark), Lúcia Manso-Sillivan (CIRAD, Montpellier, France), and Jozef Bogaerts (Federal Agency of Health, Food Chain Security, and Environment, Brussels, Belgium) for supplying DNA of *M. hyosynoviae* isolates, the strains of the mycoides cluster, and the *M. genitalium* samples, respectively.

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