J Vet Diagn Invest 9:186-190 (1997)

Polymerase chain reaction and restriction endonuclease digestion for selected members of the "*Mycoplasma mycoides* cluster" and *Mycoplasma putrefaciens*

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Abstract. A specific diagnostic method using the polymerase chain reaction, together with restriction endonuclease digestion patterns, was developed for members of the "*Mycoplasma mycoides* cluster" that normally occur in the United States (i.e., *Mycoplasma mycoides* subsp. *mycoides* Large Colony and *Mycoplasma capricolum* subsp. *capricolum* in addition to "cluster" mycoplasma, bovine serogroup 7, and *Mycoplasma putrefaciens.* The digestion of "cluster" polymerase chain reaction DNA (1,225 bp) amplification products with restriction enzymes AseI and SspI gave mycoplasma species-specific patterns for all strains of *M. mycoides* subsp. *mycoides* Large Colony, *M. capricolum* subsp. *capricolum,* and bovine group 7 tested. Moreover, we found a nonspecific amplification product for *M. putrefaciens* that occurred with the oligonucleotide primers used for the "*M . mycoides* cluster" reaction. However, the restriction endonuclease digestion patterns observed with the restriction enzymes AluI, AseI, and SspI for *M. putrefaciens* were different than the digestion patterns obtained for the other "cluster" mycoplasmas. This report confirms the usefulness of polymerase chain reaction DNA amplification allied with restriction enzyme digestion profile analysis for the rapid and specific identification of mycoplasmas belonging to the "*M. mycoides* cluster" and *M. putrefaciens.*

Mycoplasma infections in ruminants are frequently observed, particularly in goats. However, it is extremely important to distinguish between mycoplasmas causing severe and contagious diseases with serious economic consequences and mycoplasmas that may cause sporadic disease with minor economic impact. For example, worldwide, the most important mycoplasmas economically are the related ruminant pathogens classified as the "*Mycoplasma mycoides* cluster." This "cluster" [4] consists of six mycoplasma species: *Mycoplasma mycoides* subsp. *mycoides* Small Colony (MmSC), *Mycoplasma mycoides* subsp. *mycoides* Large Colony (MmLC), *Mycoplasma mycoides* subsp. *capri* (Mmc), *Mycoplasma capricolum* subsp. *capricolum* (MC), *Mycoplasma capricolum* subsp. *capripneumoniae* (Mcc), and an unspeciated bovine mycoplasma designated "bovine serogroup 7."

In the United States, the "cluster" mycoplasmas causing disease, principally in goats, are MmLC, which is widespread, and MC, which is reported sporadically. Moreover, *Mycoplasma putrefaciens* (Mp) is often reported in disease processes and *Mycoplasma agalactiae* has been described on three occasions, including one serious outbreak/ For the purpose of this report, the "*M.* mycoides cluster" shall infer only MmLC, MC, and IM. sp. serogroup 7. Other members of the "cluster" were not analyzed due to their "exotic" status in the United States.

In addition to the basic questions of taxonomy within the "cluster," differential diagnostic tests are often difficult to develop due to shared common antigens among these mycoplasma.[10] Cross-reactive antibodies can impede the identification of the pathogenic agents thereby complicating the interpretation of serological tests.[6] Moreover, biochemical differentiation may be time-consuming and ambiguous. Recently, DNA probes have been used to develop more specific diagnostic procedures.[7,14,15] These tests may be complicated by nonspecific binding of probes to nontarget areas with poor reproducibility and difficult interpretation. Similarly, the comparison of restriction endonuclease digestion patterns is an additional molecular classification criterion, but this procedure often requires sophisticated digitizing scanning to remove subjectivity and obtain precise data comparisons.[3,12,13,7]

Recently, tests based on the polymerase chain reaction (PCR) have proven to be highly specific and inexpensive for the identification of MmSC.[2,8] Thus, the objective of this study was to develop a rapid and specific diagnostic method using the PCR for DNA amplification of the "*M. mycoides* cluster." This reaction was coupled with restriction endonuclease digestion patterns to distinguish the ruminant myco-

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Received for publication April 13, 1995.

Table 1. Mycoplasmas used in this study.

Species	Strain	Species of origin*	Source*
M. mycoides subsp. *mycoides* (LC)**	GM12	Goat, M, P	AJD
	GM975	Goat, M	AJD
	GM1013A	Goat, P	AJD
	GM1043	Goat, P	AJD
M. capricolum subsp. *capricolum***	CAL.KID	Goat, P	ATCC
	CAL.KID	Goat, P	J. G. Tully
	GM13	Goat, P	AJD
	GM209	Goat, E	AJD
	GM262-G	Goat, E	AJD
	GM630-A	Lamb, P	AJD
Bovine serogroup 7**	PG50	Bovine, A	J. G. Tully
M. putrefaciens	KS-1	Goat, ?	ATCC
	GM1	Goat, M	AJD
	GM499	Goat, M	AJD
	GM1032	Goat, E	AJD
M. agalactiae	GM139	Goat, M	AJD
M. arginini	G230	Mouse	ATCC
M. cottewii	VIS	Goat, E	AJD
M. yeatsii	GIH	Goat, E	AJD
M. auris	UIA	Goat, E	AJD
M. gallinarum	PG16	Chicken, RS	ATCC
M. bovis	PG45	Bovine, M	ATCC
M. sp. G145	G145	Goat, foot	ATCC

* M = mastitis; P = polyarthritis; AJD = A. J. DaMassa; ATCC = American Type Culture Collection; E = external ear canal; A = arthritis; RS = respiratory tract.

** "*M. mycoides* cluster" members used in this study.

plasma "cluster" pathogens commonly described in the United States.

Materials and methods

Mycoplasma. Table 1 lists the mycoplasma species and sources included in this study along with three recently described caprine mycoplasma species: *Mycoplasma auris, Mycoplasma cottewii,* and *Mycoplasma yeatsii.*[5]

Mycoplasma media. Liquid and solid mycoplasma medium "B," as described elsewhere, were used in this study.[9]

DNA extraction. Template DNA was extracted by two methods. In the first method, a proteinase K digestion was followed by a phenol-chloroform extraction (1 ml of broth culture with about 10^7-10^9 colony-forming units of the mycoplasma) and centrifuged for 10 minutes at 14,000 x g. The pellet was resuspended in 600 µl of TE buffer[a] (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) containing 0.5% sodium dodecyl sulphate to which was added 3 µl of proteinase K[a] (20 mg/ml). After incubation for 3 hr at 37 C, the lysate was extracted twice with 500 µl of phenol-chloroform isoamyl alcohol[a] (25:24:1). The DNA was precipitated with 50 µl of 3 M sodium acetate[a] and 1 ml of cold 95% ethanol and left overnight at -20 C. After 30 min centrifugation, the DNA was washed with 70% ethanol, dried, and resuspended in 100 µl of deionized water. One microliter of extracted DNA template was used in the PCR amplifications.

The second DNA extraction method was with "Genereleaser,"[b] which is a proprietary reagent that releases DNA from whole blood, cell cultures, and bacterial colonies, etc. One microliter of broth culture and 5 µl of "Genereleaser" were mixed, and the extractions were made within 10 min according to the manufacturer's instructions.

Oligonucleotide primer design. Primers were designed from the published nucleic acid sequences of the six "*M. mycoides* cluster" species that are complementary to the gene probe CAP-21.[15] Briefly, two PCR primers were designed for the mycoplasmas belonging to the "cluster" (Table 2). These primers (1 and 2) were selected in DNA regions where complete homology was present between the six sequences, giving an amplified DNA segment of about 403 bp, depending on the "cluster" species involved. In addition, two sets of 5' and 3' oligonucleotide primers were designed to give specific amplification products for MmLC and Mc (Table 2). The primers 3, 4, and 7 were specific for MmLC, while primers 5 and 6 were specific for Mc. These primers were selected from regions in which there were differences between the "cluster" species.

One microliter of extracted DNA and 10 µl of each primer[c] (2 µM concentration) were added to 30 µl of the PCR mixture[d] that consisted of 5 µl thermophilic buffer 10 x (500 mM KCl, 100 mM Tris-HCl, 1% Triton X-100) 3 µl 25 mM $MgCl_2$, 2 µl 1.25 mM (each) dATP, dGTP, dTTP, and dCTP, and 1.5 units *Taq* polymerase.[d] The mixture was amplified in a thermal cycler for 30-40 cycles with each cycle consisting of melting at 94 C for 1 min, annealing at 50-60 C for 1 min, and extension at 72 C for 2 min. The concentrations of MgCl, and the 5' and 3' primers were varied to improve the spec-

Table 2. Primers used in PCR amplification.

Primer number	Nucleotide number*	Primer sequence (5′–3′)**	Direction
1	837	CTC AAG AGC AAG AAC GTG GGA	Forward
2	1,240	TTC CAT CAA ACT CAT AAG C	Reverse
3	1,136	GAT TAG GTG CTA AAG CTG CT	Forward
4	1,440	GCT AAT ACT GGG AAG TCG	Reverse
5	621	CAA ACA AAG CAT TTG CAC AC	Forward
6	893	TTT GTG AAT TTT TCC AGT G	Reverse
7	99	ATA AAT CAA ATT AAA TAA GTT	Forward
8	15	GGT ACT TTA GAT ACT CAA GG	Forward

* The numbers correspond to the nucleotide positions of the first base of the primer relative to the total sequence described for CAP-21 insert.

** Oligonucleotides were commercially synthesized.[c] The purity is approximately equivalent to cartridge-purified DNA.

ificity of the PCR for each "cluster" species. The amplified products were analyzed by gel electrophoresis using 2% agarose, and the DNA fragments were visualized directly by using ethidium bromide staining and ultraviolet transillumination.

Restriction emzymes. DNA templates were amplified by PCR with primers 2 and 8 (Table 2). The 5' primer (primer 8) was designed at the beginning of the 1.5 Kb sequence as established by Taylor.[14] The 1,225 bp DNA products were amplified as described above for 30 cycles at 94 C for 1 min, 50 C for 1 min, and 72 C for 2 min. The DNA PCR products were digested with AseI, AluI, and SspI restriction enzymes.[e] The digestion mixtures were prepared according to the manufacturer's instructions for each enzyme, and the samples were digested for 2 hr. The digestion products were analyzed by gel electrophoresis as described above.

Results

The mycoplasma species-specific set of primers gave distinct and intensive bands for MmLC and Mc. However, the procedure was not diagnostic as a single comprehensive test due to the presence of nonspecific bands. To produce discrete bands specific for each mycoplasma required different times for annealing, different temperatures of annealing, variation in the numbers of cycles, different concentrations of $MgCl_2$, and different concentrations of 5' and 3' primers. Thus, with parameters unique for each set of primers, specific bands for the individual mycoplasma were obtained. Conversely, the amplified DNA fragments with primers 1 and 2 always resulted in a single band of about 403 bp for all the "cluster" mycoplasmas. No extra bands were observed in the PCR amplifications with this set of primers. Moreover, DNA amplification products were not observed with caprine genomic DNA or with negative controls.

The digestion of DNA PCR (1,225 bp) products (amplified with primers 2 and 8) with restriction enzymes

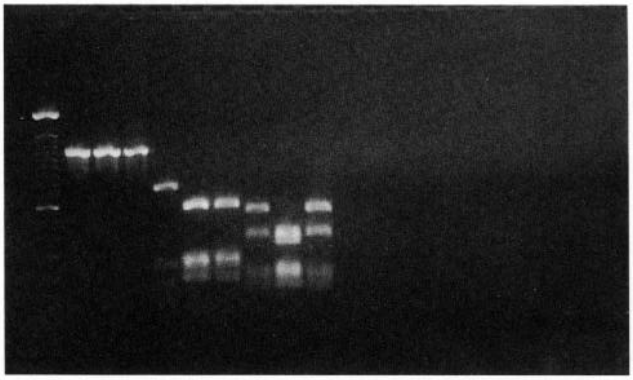

Figure 1. DNA amplification products of the "Mycoplasma *mycoides* cluster" on an agarose gel stained with ethidium bromide. Lanes 1, 2, and 3 are, respectively: 1, *M. Mycoides* subsp. *mycoides* Large Colony (MmLC); 2, *M. capricolum* (Mc), and 3, *bovine serogroup* 7 without digestion with restriction enzymes. Lanes 4, 5, and 6 are, respectively: 4, MmLC (750, 230, and 168 bp); 5, MC; and 6, bovine serogroup 7 (571, 250, 230, and 181 bp) digested with AseI. Lanes 7, 8, and 9 are, respectively: 7, MmLC (529, 370, 186, and 141 bp); 8, MC (373, 331, 329, and 180 bp); and 9, bovine serogroup 7 (511, 370, 186, and 145 bp) digested with enzyme SspI.

AseI and SspI gave mycoplasma species-specific patterns for the reference strains of MmLC, MC, and bovine group 7. After digestion with AseI, the digestion fragment sizes were respectively 750, 230, and 168 bp for MmLC and 571, 250, 230, and 181 bp for Mc and bovine group 7 (Fig. 1). The restriction patterns with SspI were characterized by four digestion bands of 529, 370, 186, and 141 bp for MmLC, 373, 331, 329, and 180 bp for MC, and 511, 370, 186, and 145 bp for bovine group 7. All members of the "*M. mycoides* cluster" tested (Table 1) gave similar digestion patterns to the mycoplasma reference strains except for strain GM 13 of MC. This strain gave a normal digestion pattern with enzyme AseI and a multiple pattern with enzyme SspI. As expected, the restriction digestion pattern with enzyme AluI was the same for MmLC, MC, and bovine group 7 with two bands representing fragments of approximately 615 and 360 bp and multiple bands less than 70 bp (Fig. 2).

All of the mycoplasmas that did not belong to the "cluster" were negative in the PCR except for four strains of *M. putrefaciens* (Mp). The Mp strains (which include the type strain of the species [KS-l] showed PCR amplification products (primer sets 1 and 2 and 2 and 8) with sizes similar to all of the "cluster" mycoplasmas used in this study. For this reason, we used the restriction enzymes AluI, AseI, and SspI to compare the digestion patterns of the mycoplasma that belong to the "cluster" and Mp. The digestion of Mp DNA PCR products (primers 2 and 8) resulted in the restriction patterns shown in Fig. 2. Digestion with enzyme AluI resulted in different bands with the largest fragment about 200 bp and multiple smaller fragment

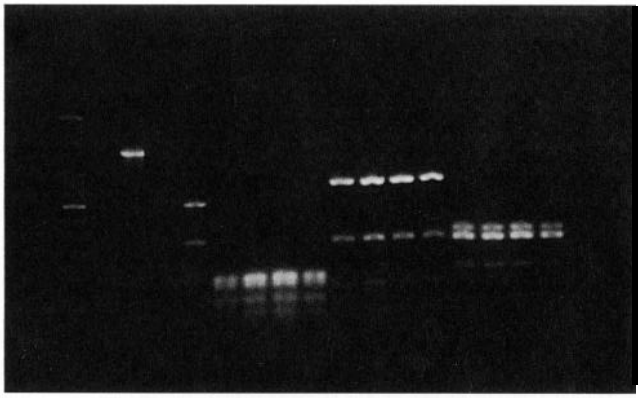

Figure 2. DNA amplification products of *Mycoplasma putrefaciens* (Mp) strains KS1, GM1, GM499, GM1032, and "*Mycoplasma mycoides* cluster" on an agarose gel stained with ethidium bromide. Lanes are: 1, negative control without template DNA, 2, Mp template DNA without digestion with restriction enzymes; 3, negative control (DNA from blood of noninfected goat); 4, MmLC template DNA digested with enzyme AluI (615 and 360 bp); 5, 6, 7, and 8, Mp template DNA strains KS1, GM1, GM499, and GM1032, respectively, digested with AluI; 9, 10, 11, and 12, Mp template DNA strains KS1, GM1, GM499, and GM1031, respectively, digested with AseI; 13, 14, 15, and 16, Mp template DNA strains KS1, GM1, GM499, and GM1031, respectively, digested with SspI.

bands (Fig. 2). This digestion pattern was different than the AluI pattern observed for MmLC, MC, and bovine serogroup 7. The restriction pattern with enzyme AseI was characterized by three bands of about 750, 350, and 150 bp. With enzyme SspI, the size of the bands were similar to those detected with MC (380, 330, and 180 bp).

Discussion

The existence of both Small and Large Colony types of *M. mycoides* subsp. *mycoides* has presented problems with serologic testing for many years.[6,15] In countries such as the USA, which is reported to be free of the SC type of the species, specific diagnostic tests are required to differentiate it from the prevalent caprine LC type.[7] Likewise, the occurrence of MC and possibly bovine serogroup 7 makes the diagnostic testing and distinction of these "cluster" mycoplasmas very important. The data in this study identify a set of fast, sensitive, and reliable tests for the differentiation between all of the "cluster" mycoplasmas and Mps that have been described in the USA.

Our initial trials, which were directed toward the development of a one-step MmLC- and MC-specific PCR assay, did not provide definitive and specific bands for a reliable diagnostic test. These results were most likely due to the high degree of sequence similarity between the different mycoplasmas within the "cluster" group. For example, the differences between the specific mycoplasma primers and the DNA of other "cluster" mycoplasmas were in two or three nucleotides, which is not enough to establish a specific diagnostic DNA amplification test. The poor specificity could also be due to the high proportion of adenine and thymidine in our primers that prevented the use of high annealing temperatures to improve the specificity of the primers.

In subsequent experiments, the use of the PCR assay followed by restriction endonuclease digestions gave specific results confirming the amplified DNA fragments as MmLC, MC, or bovine serogroup 7. The unique DNA amplification band of about 1,225 bp with primers 2 and 8 confirmed the specificity of these primers as previously reported.[14] Moreover, the clear multiple bands observed after digestion with the restriction enzymes AseI and SspI for the reference strains of each mycoplasma confirmed the presence of species-specific internal restriction sites. Four strains of MmLC and six strains of MC showed bands similar to those produced by the reference strains. An extra band of about 170 bp was observed with the enzyme AseI in all of the MmLC strains and may indicate one extra restriction site around nucleotide 64 of the MmLC sequence. The PCR amplification with primers 2 and 8 followed by digestion with restriction enzymes AseI and SspI provided excellent differential identification for the USA strains within the "cluster."

Interestingly, the digestion patterns for strain GM13 of MC with enzyme SspI indicated some potential minor sequence differences from the reference strain of the species. However, with the addition of enzyme AseI the digestion pattern allowed the classification of this strain as MC. The restriction patterns of MmSC with enzyme AsnI were different from the other "cluster" mycoplasmas.[2,15] With our oligonucleotide primers, enzymes AseI and AsnI cut the same nucleotides, producing restriction digestion fragments that were 750, 400, and 86 bp. Thus, this restriction digestion test can be used diagnostically to distinguish MmSC from all the other "cluster" mycoplasmas.

The nonspecific reaction observed with Mp interferes with the specific diagnosis of other "cluster" mycoplasmas by PCR amplification followed by restriction enzyme digestion when only one enzyme is used.[2] However, the combination of three enzymes was diagnostic for Mp, providing a restriction pattern that was different from the "cluster" mycoplasmas. Presently, this series of tests will be very useful for the diagnostic identification of Mp.

The apparent similarity between the PCR amplification products obtained with Mp and the other "cluster" mycoplasmas indicates a high degree of homology. Other investigators have sequenced 16S rRNAs from Mp and, based on sequence homology, have suggested that Mp should be included in the "*M. mycoides* cluster."[1,16] Our results support this suggestion.

In summary, this report confirms the usefulness of the PCR and restriction endonuclease digestion for the rapid and specific identification of the ruminant mycoplasmas listed in Table 1. Our experiments have indicated that a set of tests including PCR DNA amplifications followed by restriction endonuclease digestions can be used to make a diagnosis in less than 24 hours. This set of reliable, sensitive, and quickly performed diagnostic tests has great economic potential and could be substituted for other time-consuming DNA techniques.[1,2,7,8,17] Studies with "exotic" strains of the "*M. mycoides* cluster" from other countries should be conducted to further evaluate the specificity of these procedures.

Acknowledgement

We would like to express our gratitude to the University Foundation of the University of Las Palmas de Gran Canaria for the grant provided to J. L. Rodriguez that allowed him to pursue these studies at the University of California, Davis.

Sources and manufacturers

a. Sigma Chemical Company, St. Louis, MO.
b. Bioventure Inc., Murfreesboro, TN.
c. The Midland Certified Reagent Company, Midland, TX.
d. Promega, Madison, WI.
e. Pharmacia Biotech Inc., Piscataway, NJ.

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