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Evaluation of Intraspecies Genetic Variation within the 16S rRNA Gene of *Mycoplasma hominis* and Detection by Polymerase Chain Reaction

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***Mycoplasma hominis* is a heterogeneous species with DNA-DNA hybridization values ranging from 51 to 100%. We report here the sequencing of the 16S rRNA gene of a strain (183) that greatly differs from the type strain (PG21) of this species. Comparison of 16S rDNA sequences from these two strains showed limited differences, indicating that the two strains belong to the same rRNA species complex. Using these nucleotide sequence data, we established a rapid method for the detection of *M. hominis* by using polymerase chain reaction. This method was shown to be sensitive and specific when tested with reference strains and clinical isolates.**

Mycoplasma hominis can be isolated from the urogenital tracts of up to 40% of asymptomatic males and females. However, it is a proven cause of pelvic inflammatory disease, postpartum septicemia and endometritis, clinical amnionitis, and pyelonephritis (4). It is also a major cause of pneumonia and central nervous system infections in newborn infants (for a review, see reference 5). Furthermore, *M. hominis* is increasingly being recognized as a common cause of septicemia, arthritis, surgical wound infections, and peritonitis in immunocompromised patients (16, 17).

Identification of clinical isolates and serodiagnosis of *M. hominis* infections are complicated by the heterogeneity within the species. Remarkable differences between strains of *M. hominis* have been documented by serology (3, 14), DNA-DNA hybridization (7), comparison of protein patterns (1), analysis of DNA restriction enzyme cleavage patterns (7, 8), and genome mapping (13).

Heterogeneity has also been documented in DNA hybridization studies using rRNA genes as probes to detect restriction fragment length polymorphisms (9). Although most of the restriction fragment length polymorphisms could be explained by variations in restriction enzyme cleavage sites outside the rRNA cistrons, some were also observed within these cistrons. The 16S rRNA gene sequences of more than 40 different mycoplasma species, including the type strain, PG21, of *M. hominis*, have been determined, providing the basis for a phylogenetic study of these organisms (24). In addition, this information allows the identification of specific sequences that can be used to design primers for DNA amplification by polymerase chain reaction (PCR) for improved detection of mycoplasmas in clinical specimens. However, there are no data concerning possible variations within this gene between strains of a single heterogeneous species such as *M. hominis*.

The first goal of this study was to sequence the 16S rRNA gene of *M. hominis* 183, which is known to differ antigenically, phenotypically, and genetically from the type strain, PG21. The second goal was to compare the sequences not only to detect the extent of variation, if any, within this gene but also to detect constant regions. The results indicated limited differences and allowed the design of primers shown to be species specific and able to reliably detect diverse *M. hominis* isolates by PCR.

The mycoplasmas that were used to assess the specificity of PCR detection included the following species: the 7 reference strains of *M. hominis*, PG21^T, 10, 93, 132, 183, 4195, and W2; *Mycoplasma genitalium* JB, G37^T, and VTMB; *Mycoplasma fermentans* PG18^T, MT2, incognitus, and K7; *Mycoplasma pneumoniae* Eaton^T, 142-48, POLT, and TW1-6; *Acholeplasma laidlawii* PG8^T; *Acholeplasma oculi* 19-L^T; *Mycoplasma buccale* CH20247^T; *Mycoplasma faucium* DC333^T; *Mycoplasma orale* CH19299^T; *Mycoplasma pirum* BER; *Mycoplasma primatum* HRC292^T; *Mycoplasma salivarium* PG20^T; the newly isolated *Mycoplasma penetrans* GTU-54 (15); and all 14 serotype strains of *Ureaplasma urealyticum*. The type strains of *M. hominis*, *M. fermentans*, *M. orale*, and *M. pneumoniae* were obtained from the Research Resources Branch of the National Institute of Allergy and Infectious Diseases (Bethesda, Md.). The six additional strains of *M. hominis* were obtained from J. S. Lin (Channing Laboratory, Brigham and Women's Hospital, Boston, Mass.) and have been previously described (14). The source of the serotype strains of *U. urealyticum* was previously described (12), and the other strains of mycoplasmas were kindly provided by J. G. Tully (National Institute of Allergy and Infectious Diseases, Frederick, Md.).

A total of 51 clinical isolates from the urethras of 10 males with nongonococcal urethritis and from the cervixes of 41 females with nongonococcal cervicitis were evaluated. Identification of the isolates as *M. hominis* was based on growth and metabolic characteristics and on positive identification by immunoblotting with monoclonal antibodies which were shown to react with *M. hominis* but with no other mycoplasma species known to occur in humans. The specificity of these immunological reagents was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

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TABLE 1. Specificity of anti-*M. hominis* monoclonal antibodies as determined by SDS-PAGE immunoblot

Organism	Reaction to antibody:	
	8B1.20[a]	10A4.11[b]
M. hominis reference strains		
PG18	±[c]	+
W2	+	+
93	+	+
10	+	+
132	+	+
183	+	+
4195	+	+
Other species[d]		
M. pneumoniae	−	−
M. genitalium	−	−
M. fermentans	−	−
A. laidlawii	−	−
M. salivarium	−	−
A. oculi	−	−
M. faucium	−	−
M. primatum	−	−
M. orale	−	−
M. buccale	−	−
M. pirum	−	−
M. penetrans	−	−
Mycoplasma spermatophilum	ND[e]	ND
Mycoplasma lipophilum	ND	ND
U. urealyticum	−	−

[a] Isotype immunoglobulin G2a.
[b] Isotype immunoglobulin G2b.
[c] ±, low titer (weak reaction) on Western blot.
[d] Evaluation was done with only one reference strain.
[e] ND, not done.

immunoblots (Table 1) using methods described previously (22).

Near-full-length 16S rDNA of *M. hominis* 183 was PCR amplified with the primers fD1 and rP1, which were previously described by other investigators (23). These primers have restriction sites added onto the 5′ ends of the gene-specific sequences (*Sal*I for fD1 and *Bam*HI for rP1). The amplification was initiated under low stringency and involved a thermal profile of four cycles of denaturation at 94°C for 60 s, primer annealing at 37°C for 60 s, and extension at 72°C for 60 s, and then it was continued for 35 cycles with an increased annealing temperature (50°C for 60 s). The PCR product was purified by phenol-chloroform and chloroform extractions and ethanol precipitated. After digestion with *Bam*HI and *Sal*I, this DNA fragment was ligated to the plasmid vector pUC18, which was also digested with the same enzymes. The ligation mixture was used to transform competent *Escherichia coli* JM103 cells. After selection, one recombinant plasmid (pRHO) containing most of the 16S rDNA gene of *M. hominis* 183 was purified by CsCl gradient centrifugation following standard procedures (19). The DNA inserted was sequenced by using the dideoxy reactions of Sanger et al. (20) with modified T7 DNA polymerase (Sequenase; U.S. Biochemical Corp., Cleveland, Ohio).

Stringent precautions to prevent PCR product carryover were in routine use, including the use of three different rooms for the processing of clinical samples, the setup of the PCR assay, and the analysis of the amplified products. Mycoplasmal lysates were prepared by a neutral-detergent–proteinase K procedure (2) with an increased concentration of proteinase K (0.5 mg/ml in the lysis solution), and PCR

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AGAGTTTGATCCTGGCTCAGGATGAACGCTGGCTGTGTGCCTAATACATGCATGTCGAGC60
GAGGTTAGCAATAA(C/.)CTAGCGGCGAATGGGTGAGTAAC(AC/TN)GTGCTTAATCT111
ACCTTTTAGATTGGAATACCCATTGGAAACAATGGCTAATGCCGGATACGCATGGAACCG171
CATGGTTCCGTTGTGAAA(G/N)GCGCTGTAAGGCGC(C/N)ACTAAAAGATGAGGGTGC223
GGAACATTAGTTAGTTGGTGAGGTAATGGCCCACCAAGACTATGATGTTTAGCCGGGTCG283
AGAGACTGAACGGCCACATTGGGACTGAGATACGGCC(CA/NN)AACTCCTACGGGAGGC338
AGCAGTAGGGAATATTCCACAATGAGCGAAAGCTTGATGGAGCGACACAGCGTGCACGAT398
GAAGGTCTTCGGATTGTAAAGTGC(T/N)GTTATAAGGGAAGAACATTTGCAATAGGAAA454
TGATTGCAGACTGACGGTACCTTGTCAGAAAGCGATGGCTAACTATGTGCCAGCAGC(CG513
/NN)CGGTAATACATAGGTCGCAAGCGTTATCCG(G/N)AATTATTGGGCGTAAAGCGTT565
C(G/N)TAGGC(TGT/NNN)TTGTTAAGTCTGGAGTTAAATCCCGGGGCTCAACCCC(G/613
GG)CTCGCTTTGGATACTAGCAAACTAGAGTTAGATAGAGGTAAGCGGAATTCCATGTGA670
AGCGGTGAAATGCGTAGATATATGGAAGAACACCAAAGGCGAAGGCAGCTTACTGGGTCT730
ATACTGACGCTGGGACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCAC790
GCCGTAAACGATGATCATTAGTCGGTGGAGAATCACTGACGCAGCTAACGCATTAAATGA850
TCCGCCTGAGTAGTATGC(T/N)CGCAAGAGTGAAACTTAAAGGAATTGACGGGNNACGC906
ACAAGNGGAGCATGTGGTTTAATTTGAAGATACACGGAAAACCTTACCCACTCTTGACAT966
CCTTCGCAAAGCTATAGAGATATAGTGGAGGTTATCGGAGTGACAGATGGTGCATGGTTG1026
TCGTCAGCTCGTGTCGTGAGATGTTTGGTCAAGTCCTGCAACGAGCGCAACCCCTATC(T1085
/N)TTA(G/N)TTACTAACATTAAGTTGAG(G/C)ACTCTAGAGATACTGCCTGGGTAAC1134
TGGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCTCTTACGAGTGGGGCCACACACG1194
TGCTACAATGGTCGGTACAAAGAGAAGCAATATGGCGACACTGAGCAAATCTCAAAAAGC1254
CGATCTCAGTTCGGATTGGAGTCTGCAATTCGACTCCATGAAGTCGGAATCGCTAGTAAT1314
CGCAGATCAGCTATGCTGCGGTGAATACGTTCTCGGGTCTTGTACACACCGCCCGTCACA1374
CCATGGGAGCTGGTAATACCCAAAGTCGGTTTGCTAACC(T/NNN)CGGAGGCGACCGCC1418
TAAGGTAGGACTGGTGACTGGG1440
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FIG. 1. Nucleotide sequence of the *M. hominis* 183 16S rRNA gene. The location of the primer, fD1, used for PCR amplification of the 16S rDNA sequence is underlined; the region homologous to the other primer, rP1, was not sequenced. Arrows show locations of primer sequences used for PCR detection of *M. hominis*. The internal probe (RNAH3) used to confirm the specificity of the amplification is double underlined. Differences between the 16S rDNA sequences of *M. hominis* 183 and PG21^T are indicated in parentheses, with the 183 sequence given first. The sequence of the region that is heavily underlined was not determined for strain PG21^T. The source for the *M. hominis* PG21^T sequence is reference 24.

amplification was performed in a total volume of 50 μl, as described by Saiki et al. (18). Lysates to be analyzed (5 μl) were always added last. A positive control (diluted lysate of *M. hominis* corresponding to 100 color-changing units [CCU]) and a negative control (sterile water) were included in each PCR experiment. The thermal profile involved 40 cycles of denaturation at 95°C for 25 s, primer annealing at 62°C for 60 s, and extension at 72°C for 60 s. Following PCR amplification, aliquots (20 μl) were analyzed by electrophoresis through a 2% agarose gel, and DNA was visualized by UV fluorescence after ethidium bromide staining. Southern transfer of DNA onto nylon membrane and 5′-end labeling of hybridization probes with T4 polynucleotide kinase were done as previously described (2).

For evaluation of the sensitivity of PCR detection, exponential cultures of *M. hominis* 10, PG21^T, 93, and 183 were centrifuged (12,000 × *g*, 20 min, 4°C). The supernatant was discarded, and the bacterial pellet was lysed as described above. Lysates were subsequently serially diluted in the lysis solution without proteinase K, and the dilutions were evaluated by PCR. The concentration of *M. hominis* organisms in the cultures was determined by establishing the number of CFU present on SP-4 agar plates and the number of CCU in SP-4 broth (21). The sensitivity was also evaluated with purified DNA from *M. hominis*, and the concentrations of DNA stock solutions were determined by the spectrophotometric method (19). DNA stock solutions were serially diluted in sterile water prior to PCR analysis.

PCR amplification of DNA from *M. hominis* 183 with primers fD1 and rP1 yielded a product of approximately 1.4 kbp, which was cloned and sequenced. Excluding positions 892 to 936 and 17 additional nucleotides (/N in Fig. 1) that were not previously determined for strain PG21^T, the 16S

rRNA genes from strains PG21^T and 183 were identical in all but five positions (Fig. 1). These differences were located at positions 75, 114, 613, 1118, and 1405. Three of them (75, 1118, and 1405) are typically variable among mycoplasmas, while the other two are located in invariable positions and may represent errors in the reported sequence of DNA from strain PG21^T.

PCR primers for the specific amplification of a 334-bp DNA fragment of the 16S rRNA gene of *M. hominis* were chosen after the two *M. hominis* sequences were aligned with the published homologous sequences from different mycoplasmas. They were chosen in regions that are variable among other mycoplasma species (and a fortiori among other bacteria) but identical for the two strains of *M. hominis*. The sequences of the oligonucleotides were 5′-CAATGGCTAAT GCCGGATACGC-3′ (RNAH1; sense), 5′-GGTACCGTCA GTCTGCAAT-3′ (RNAH2; antisense), and 5′-CGCTGTAA GGCGCACTAAA-3′ (RNAH3; internal probe). They are homologous to nucleotides 149 to 170 (RNAH1), 465 to 483 (RNAH2), and 200 to 219 (RNAH3) in the 16S rRNA sequence of *M. hominis* PG21^T (24) (accession no. M24473 in GenBank), and their positions are indicated in Fig. 1. DNA hybridization with the internal probe RNAH3 was used to evaluate the specificity of the amplification after electrophoresis.

PCR specificity was evaluated by using multiple strains of the 13 mycoplasma species most commonly isolated from humans and DNAs from human epithelial and peripheral blood mononuclear cells. PCR detection with primers RNAH1 and RNAH2 was highly specific, as it consistently allowed the amplification of a product with the expected length (334 bp) with the seven reference strains of *M. hominis* (Fig. 2) but not with heterologous species or with DNA from humans. Nonspecific amplification was obtained with strains of *M. salivarium*. However, the amplified DNA fragment was larger than that obtained with *M. hominis* (433 bp versus 334 bp). Analysis of the restriction pattern of this DNA fragment after digestion with *Hin*dIII, *Rsa*I, and *Taq*I indicated that this amplicon is part of the 16S rRNA gene of *M. salivarium*, although only a weak homology between RNAH2 and the published sequence for this gene was found. However, only the specific amplified product obtained with *M. hominis* was shown to be homologous to the internal probe RNAH3 by Southern hybridization (Fig. 2B).

The specificity of PCR detection was also evaluated by using 51 clinical isolates of *M. hominis* which had been artificially passaged only a few times and which were from diverse geographic locations. All of them were positive by this assay.

The sensitivity of the PCR assay was determined with lysates of four different reference strains of *M. hominis*: for strain 10, 0.07 CFU or 0.05 CCU; for strain PG21^T, 0.4 CFU or 5 CCU; for strain 93, 0.2 CFU or 0.5 CCU; and for strain 183, 0.13 CFU or 0.05 CCU. The sensitivity of the PCR assay was also evaluated with purified DNAs from three *M. hominis* strains: 10, 93, and 132. The limit of detection of the PCR assay was between 10 and 15 fg of *M. hominis* DNA; results obtained with strain 93 are shown in Fig. 3. Since the genome size of this mycoplasma is about 800 kbp (13), 10 fg is equivalent to the amount of DNA derived from about 12 organisms.

On the basis of DNA-DNA hybridization, genomic homology between strains PG21^T and 183 of *M. hominis* was found to be only 55% (7); however, the differences between their 16S rDNA sequences are limited. This confirms the high degree of intraspecies conservation within this gene. However, 16S rDNA sequence identity can be found between two close but distinct species, as was recently described for the genus *Bacillus* (10). Clearly, the two strains of *M. hominis* that were compared in this study belong to what has been called "the same rRNA species complex" (10), but the DNA-DNA hybridization data suggest that they could be relegated to different subspecies.

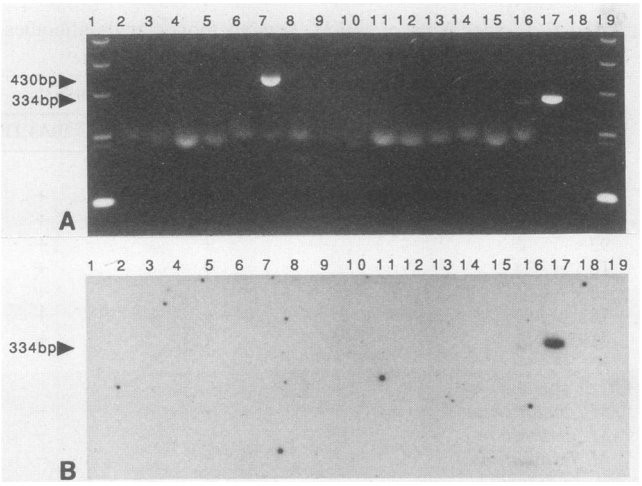

FIG. 2. Specificity of the PCR detection assay. Strains of mycoplasmas were PCR amplified by using the primers RNAH1 and RNAH2 and analyzed by electrophoresis in a 2% agarose gel (A) and by DNA hybridization following transfer of the DNA to a nylon membrane (B). Lysates from the following mycoplasmas were subjected to amplification: *A. laidlawii* PG8^T (lane 2), *A. oculi* 19-L^T (lane 3), *M. buccale* CH20247^T (lane 4), *M. faucium* DC333^T (lane 5), *M. orale* CH19299^T (lane 6), *M. salivarium* PG20^T (lane 7), *U. urealyticum* serotype 4 (lane 8), *M. fermentans* E-10 (lane 9), *M. fermentans incognitus* (lane 10), *M. genitalium* G37^T (lane 11), *M. pneumoniae* Eaton^T (lane 12), *M. pneumoniae* POLT (lane 13), *M. pneumoniae* 142-48 (lane 14), *Mycoplasma pulmonis* CT (lane 15), a clinical isolate of *M. hominis* (lane 16) (a limited amount, close to the sensitivity of the assay, was added), *M. hominis* PG21^T (lane 17), and a negative control (lane 18). The DNA marker (lanes 1 and 19) was a 123-bp DNA ladder (GIBCO BRL, Gaithersburg, Md.). RNAH3 was the probe used in panel B.

We have established a PCR assay for the detection of *M. hominis* using part of the 16S rDNA as a target. The assay was shown to be specific after evaluation of the 7 reference strains and 51 clinical isolates from different geographic sites. The cross-reaction obtained with *M. salivarium* can be

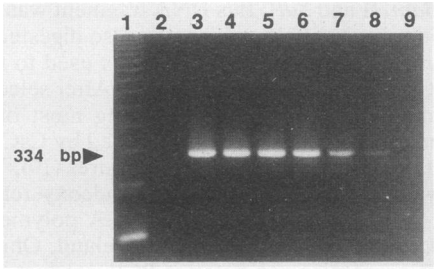

FIG. 3. Sensitivity of the PCR detection assay. A DNA stock solution of *M. hominis* 93 was serially diluted in sterile water, and aliquots of these dilutions were analyzed by PCR. The amounts of DNA that were added to the PCR reaction were 0 ng (negative control) (lane 2), 1.35 ng (lane 3), 135 pg (lane 4), 13.5 pg (lane 5), 1.35 pg (lane 6), 135 fg (lane 7), 13.5 fg (lane 8), and 1.35 fg (lane 9). The DNA marker (lane 1) was a 123-bp DNA ladder (GIBCO BRL).

easily differentiated because the size of the amplified fragment is different from that of the fragment obtained with *M. hominis* and does not hybridize with the internal probe (RNAH3). Under optimal conditions, the PCR assay consistently allowed the detection of less than 1 CFU or CCU from a culture of the four reference strains of *M. hominis* that were tested. This result is not surprising because DNA of both viable and nonviable mycoplasmas can be amplified, whereas only viable organisms will produce colonies on agar or grow in broth. In addition, *M. hominis* in culture can form clumps of cells that will also contribute to the higher sensitivity of PCR compared with culture. The limits of detection of this PCR assay (about 12 organisms) as determined with purified DNA are comparable to what was reported with other systems (11). Because microbiological detection of *M. hominis* is costly, requires special expertise, and does not yield results until 2 to 5 days later (6), this rapid PCR detection method should prove to be of considerable value in directly detecting this organism in clinical samples without prior cultivation.

Nucleotide sequence accession number. The 16S rDNA sequence of *M. hominis* (Fig. 1) will appear in the EMBL/GenBank/DDBJ nucleotide sequence data libraries under accession no. M96660.

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