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# Comparison of Multiplex PCR Assay with Culture for Detection of Genital Mycoplasmas

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***Ureaplasma*, spp. *Mycoplasma genitalium*, and *Mycoplasma hominis* are associated with infection of the genitourinary tract, reproductive failure, and neonatal morbidity and mortality. We have developed a multiplex PCR for the detection of these *Mycoplasmatales* in a single amplification reaction. The analytical sensitivities of this assay were 10.8, 10.8, and 8.8 CFU for each organism, respectively. This multiplex PCR was compared to culture on 26 cervical swabs, 2 vaginal swabs, 4 female urine specimens, 49 semen samples, 2 male urine specimens, and 1 nonspecified sample. A total of 21 specimens were culture positive (25%); 17 of these were PCR positive. An additional 11 specimens were PCR positive but culture negative. Of the 21 culture-positive specimens, 17 (81%) grew *Ureaplasma* spp. and 4 (19%) grew *Mycoplasma* spp. Of the 28 PCR-positive specimens, *Ureaplasma* spp. was detected in 23 (82%), *M. hominis* was detected in 3 (11%), and both were detected in 2 (7%). In a confirmatory analysis, all samples were tested by amplification of a second target of the ureaplasma genome. True-positive cases were defined as a positive result by culture or by both amplification assays. The multiplex PCR detected organisms in 26 of the 30 true-positive specimens, as well as in 2 other specimens. Based on a 36% prevalence of infection, the sensitivity, specificity, and positive and negative predictive values of multiplex PCR analyses were 87, 96, 94, and 93%, respectively. Multiplex PCR offers a rapid, sensitive, and easy method to detect genital mycoplasmas.**

*Mycoplasmatales* are associated with infection of the genitourinary tract, reproductive failure, and neonatal morbidity and mortality. Infection with genital mycoplasmas has been linked with infertility (12, 17, 21, 24, 37). *Ureaplasma* spp. are the main cause of nonchlamydial, nongonococcal urethritis and acute prostatitis (6). In pregnancy, ureaplasma can cause chorioamnionitis and preterm delivery (7). *Mycoplasma genitalium* has also been associated with urethritis (16, 18, 19, 39) and acute endometritis (10). *M. hominis* has been associated with pyelonephritis, pelvic inflammatory disease, and postpartum septicemia (6). In a clinical study, ca. 40% of infants born to infected mothers became infected with these bacteria (9), and colonization of the respiratory tract of infants has been associated with pneumonia and meningitis (3, 7, 8, 30, 42).

Genital mycoplasma infections are commonly diagnosed by culture. However, culture is costly in that it requires special media and expertise. It can take 2 to 5 days to culture *Ureaplasma* spp. and *M. hominis* and up to 8 weeks to culture *M. genitalium*. Infectious agents can be detected in <8 h by nucleic acid amplification techniques. PCR methods have been developed for the detection of each of these bacteria (4, 5, 36). We developed a multiplex PCR assay for the detection of all three genital mollicutes from a single amplification reaction (N. G. Mishrik, K. A. Stellrecht, and R. A. Venezia, Abstr. 100th Gen. Meet. Am. Soc. Microbiol. 2000, abstr. C-185, p. 172, 2000; A. M. Woron, N. G. Mishrik, and K. A. Stellrecht, 17th Annu. Clin. Virol. Symp. Clearwater, Fla., poster S53, 2001). The purpose of the present study was to determine the sensitivity, specificity, and predictive values of this assay versus culture.

(This study was presented in part in May 2000 at the 100th General Meeting of the American Society for Microbiology in Los Angeles, Calif., and in April 2001 at the 17th Annual Clinical Virology Symposium in Clearwater, Fla.)

## MATERIALS AND METHODS

***Mycoplasma* isolates and other bacterial strains.** The following organisms were purchased from the American Type Culture Collection (ATCC): *Ureaplasma urealyticum* (ATCC 27618), *M. genitalium* (ATCC 33530), *M. hominis* (ATCC 23114), *M. arthritidis* (ATCC 14152), *M. salivarium* (ATCC 14277), *M. fermentans* (ATCC 15474), *M. ovale* (ATCC 23714D), *M. penetrans* (ATCC 55252), *Acholeplasma oculi* (ATCC 27350) *Chlamydia trachomatis* (ATCC VR-902B), *Chlamydia pneumoniae* (ATCC VR-1310), *Candida albicans* (ATCC 14000), *Escherichia coli* (ATCC 25922), *Gardnerella vaginalis* (ATCC A2508), *Neisseria gonorrhoeae* (ATCC 49981), *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (ATCC 27336), *Streptococcus pneumoniae* (ATCC 27336), *Streptococcus pyogenes* (ATCC 19615), and *Haemophilus influenzae* (ATCC 9006). The following organisms were kindly provided by C. Chesa (New York State Department of Health, Albany, N.Y.): *M. pneumoniae* (ATCC 1428, ATCC 15531, ATCC 29342, and SP 300), *M. hominis* (ATCC 2331 and RRRB-NIH), and *M. arthri* (ATCC 14152). Isolates of *Streptococcus agalactiae* and the viridans streptococcus group were clinical isolates from Albany Medical Center.

**Clinical specimens.** All specimens received in the clinical laboratory for *Ureaplasma* culture from 85 patients seen at a fertility clinic between November 1998 and November 1999 were included in the present study. Specimens included 27 cervical swabs, 2 vaginal swabs, 4 female urine samples, 49 semen samples, 2 male urine samples and 1 nonspecified sample. Cervical and vaginal swabs were transported in 2 ml of 2SP medium (34). Urine samples were concentrated 10-fold by centrifugation for 30 min at 1,600 × *g* prior to testing. Specimens were cultured upon receipt, and the remaining material was frozen at −70°C for PCR

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TABLE 1. Nucleotide sequences of primers and probes used

| Analysis, organism, and primer or probe | Target or DNA sequence (5′–3′) | Length (bp) | Reference |
|---|---|---|---|
| Multiplex PCR | | | |
| *Ureaplasma* spp. | Urease gene | 429 | 4 |
| U4 primer | ACGACGTCCATAAGCAACT | | |
| U5 primer | CAATCTGCTCGTGAAGTATTAC-biotin | | |
| U9 probe | GAGATAATGATTATATGTCAGGATCA | | |
| *M. genitalium* | 140-kDa adhesion protein | 282 | 36 |
| MG1 primer | AGTTGATGAAACCTTAACCCCTTGG | | |
| MG2 primer | CCGTTGAGGGGTTTTCCATTTTTGC-biotin | | |
| MG3 probe | GACCATCAAGGTATTTCTCAACAG | | |
| *M. hominis* | 16S rRNA | 334 | 5 |
| RNAH1 primer | CAATGGCTAATGCCGGATACGC | | |
| RNAH2 primer | GGTACCGTCAGTCTGCAAT | | |
| Confirmatory test | | | |
| *U. urealyticum* | MB antigen gene | 403 or 448 | 41 |
| UMS125 | GTATTTGCAATCTTTATATGTTTTCG | | |
| UMA226 | CAGCTGATGTAAGTGCAGCATTAAATTC | | |

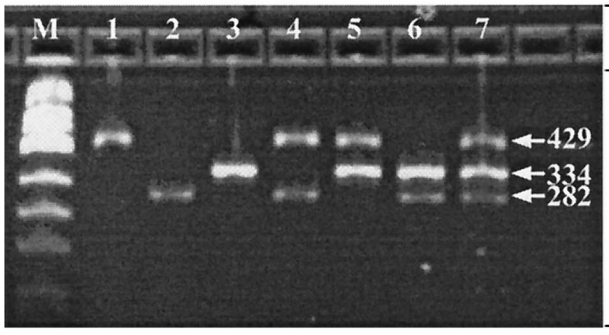
testing. Analysis of these samples was done in compliance with federal and institutional review board policies.

**Culture for genital mycoplasmas.** Specimens were inoculated onto A7 agar (Becton Dickinson, Cockeysville, Md. 21030) and incubated at 37°C in 5% $CO_2$ for 5 days. Cultures were examined microscopically daily for 5 days for the appearance of typical mycoplasma colonies. A7 agar incorporates a direct test for urease that allows the differentiation of ureaplasma from the other *Mycoplasmatales* (34).

**Multiplex PCR assay for genital mycoplasma infection.** Bacterial DNA from 100 μl of specimen or transport media was isolated by lysis in 400 μl of NucliSens lysis buffer (Organon Teknika, Durham, N.C.), extracted with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1), and extracted again with chloroform-isoamyl alcohol. DNA was then precipitated in 100% isopropanol, washed in 70% ethanol, and suspended in 15 μl of RNase-DNase free sterile deionized water (Sigma, St. Louis, Mo.). Multiplex PCR was performed with primers specific for highly conserved regions in the urease gene of *Ureaplasma* spp., the 140-kDa adhesion protein gene of *M. genitalium*, and the 16S rRNA gene of *M. hominis* (4, 5, 36). Hot-start PCR was performed on the equivalent of 25 μl of sample in 50-μl reactions containing a 0.2 mM concentration of deoxynucleoside triphosphate mixture, 10 mM Tris, 3 mM $MgCl_2$, 25 pmol of each unlabeled forward primers, and 25 pmol of biotin-labeled reverse primer (Table 1) (Synthetic Genetics, San Diego, Calif.) and 1.25 U of Gold *Taq* (Applied Biosystems, Foster City, Calif.). All reactions were performed in a GeneAmp PCR System 9600 Thermocycler (Perkin-Elmer, Norwalk, Conn.) under the following conditions: 1 cycle of 10 min at 95°C, followed by 35, two-step cycles of 95°C for 15 s and 60°C for 60 s, followed by 5 min at 72°C.

**PCR product detection.** *Ureaplasma* and *M. genitalium* PCR products were detected by enzyme-linked oligosorbent assay (ELOSA) as previously described (38). Briefly, 5 μl of heat-denatured biotinylated-PCR product was added to 100 μl of hybridization solution containing 1 pmol of horseradish peroxidase-labeled probe specific for the target region (Table 1) (Synthetic Genetics), 7.5× Denhardt solution, 3.5× SSPE, and 0.2 mg of herring sperm DNA (Invitrogen Corp., Carlsbad, Calif.)/ml in streptavidin-coated microtiter plates (Roche Molecular, Indianapolis, Ind.). Each probe was used in separate ELOSAs. Plates were incubated at 42°C for 60 min and washed six times with Coulter wash buffer (Coulter Corp., Miami, Fla.) in a Dynatech microtiter plate washer (Guernsey, United Kingdom). A color substrate, *ortho*-phenylenediamine (Abbott Diagnostics, Abbott Park, Ill.), was added to each well, followed by incubation at room temperature for 30 min before substrate development was stopped with 1 M $H_2SO_4$. The absorbance was measured at 490 nm, and data were analyzed by using a UVMax Microplate reader (Molecular Devices, Menlo Park, Calif.). Specimens were considered positive if the optical density at 490 ($OD_{490}$) was greater than the mean $OD_{490}$ of the negative amplification control plus a cutoff factor. The cutoff factor was derived from the mean $OD_{490}$ of 50 negative specimens plus two standard deviations. In addition, amplified products were visualized for specific fragment size bands (Fig. 1) under UV light after electrophoresis for 1 h at 100 V through a 7% acrylamide gel stained with ethidium bromide. PCR products with 334-bp bands, which are consistent with amplification of *M. hominis*, were further evaluated by digestion with NarI, which results in the digestion of *M. hominis* PCR product to fragments of 62 and 272 bp.

**Analytical sensitivity.** The analytical sensitivity was determined by amplification of twofold serial dilutions of bacterial DNA, either individually or as a




| Lane | Sample | U9 probe OD* | U9 probe (Cut-off) | MG3 probe OD | MG3 probe (Cut-off) |
|---|---|---|---|---|---|
| | Water | 0.054 | (0.108) | 0.048 | (0.102) |
| 1 | A. *U. urealyticum* | 4.200 | (0.108) | 0.002 | (0.102) |
| 2 | B. *M. genitalium* | 0.040 | (0.108) | 2.525 | (0.102) |
| 3 | C. *M. hominis* | 0.020 | (0.108) | 0.030 | (0.102) |
| 4 | A and B | 4.146 | (0.108) | 2.346 | (0.102) |
| 5 | A and C | 4.200 | (0.108) | 0.059 | (0.102) |
| 6 | B and C | 0.069 | (0.108) | 2.172 | (0.102) |
| 7 | A, B, and C | 4.200 | (0.108) | 1.809 | (0.102) |

FIG. 1. Performance of multiplex PCR in mixed specimens. Amplification of DNA from 100 CFU of *U. urealyticum*, *M. hominis*, and *M. genitalium*, either individually or in combination, was carried out. The results are shown on a ethidium bromide-stained agarose gel, and the $OD_{490}$ was determined by ELOSA.

mixture of all three organisms. Dilutions ranged from 3.13 to 100 CFU. The lower limit of detection (LOD) was the CFU equivalent of DNA in the last sample positive in the dilution series.

**Expanded "gold standard" and resolution of discordant results.** All samples were amplified in a second PCR targeting the multiple-banded antigen (MBA) gene of ureaplasma (41) under the conditions described above. Detection involved visualization on gel electrophoresis, which offered the additional benefit of speciation by amplicon size. *U. parvum* (formerly biovar 1 or parvo biovar) (31, 43) produces an amplicon of 403 bp, and *U. urealyticum* (biovar 2 or T960 biovar) produces an amplicon of 448 bp. Determination of a true-positive case was based on an expanded gold standard for infected patients, which was defined by a positive result by culture or by both amplification assays. Samples that were positive by culture and negative by PCR were investigated for the presence of inhibitors to amplification by PCR. These samples were spiked with ca. 50 CFU of *U. urealyticum* DNA and analyzed for DNA recovery by PCR.

**Quality control.** Published guidelines were followed to guard against nuclease and nucleic acid contamination (22, 26), including maintaining a strict physical separation between PCR setup and analysis, the use of sterile filter tips, and the regular decontamination of all surfaces with hypochlorite (27). Assay controls included a negative extraction control, derived from pooled negative specimens, and a negative amplification control, comprised of RNase-DNase-free sterile deionized water. Positive extraction and amplification controls were derived from the DNA equivalent of 125 CFU of ATCC strains of *U. urealyticum*, *M. genitalium*, and *M. hominis*.

**Statistical analysis.** Clinical data were evaluated to determine how many samples were positive by each test, as well as those positive by two or more tests and those positive by only one test. True positives for genital mycoplasma infection were patients with *Mycoplasmatales* detected by two or more tests. Sensitivity, specificity, positive and negative predictive values were calculated by Bayesian analyses. Confidence intervals were calculated by using the exact binomial method.

## RESULTS

**Analytical sensitivity and specificity of the multiplex PCR.** This assay amplified and differentiated between *Ureaplasma* spp., *M. hominis*, and *M. genitalium*, whether present as individual organisms or as a mixture (Fig. 1). The analytical sensitivity was established by the amplification of serial dilutions of bacterial DNA in three separate experiments. The average LODs for each organism amplified individually was 10.8 CFU for *U. urealyticum* and *M. genitalium* and 8.8 CFU for *M. hominis*. When these organisms were mixed together, there was a minimal loss in sensitivity, with LODs of 12.5 CFU for *U. urealyticum*, 11.3 CFU for *M. genitalium*, and 10 CFU for *M. hominis*.

Multiplex amplifications were performed with ca. 500,000 CFU of *M. arthritidis*, *M. salivarium*, *M. fermentans*, *M. ovale*, *M. penetrans*, and *Acholeplasma oculi*, as well as 11 other urogenital and respiratory microorganisms. In all cases, no bands were visible on gel electrophoresis, and all yielded negative results by ELOSA, demonstrating that the multiplex PCR assay did not cross-react with other *Mycoplasmatales* or other common genitourinary and respiratory microorganisms tested.

**Clinical specimens.** Genital and urine specimens were obtained for culture of genital mycoplasma from 85 patients seen at a fertility clinic. The results of one cervical swab were excluded from analysis due to bacterial overgrowth in the mycoplasma culture. Of the 84 patient results analyzed, 21 were culture positive (25%). Seventeen culture-positive specimens were also multiplex PCR positive. An additional 11 specimens were PCR positive, but culture negative (PCR detection rate = 33%). Of the 21 culture-positive specimens, *Ureaplasma* spp. were isolated from 17 and *Mycoplasma* spp. were isolated from 4. Of the 28 PCR-positive specimens, 23 were positive for *Ureaplasma* spp., 3 were positive for *M. hominis*, and 2 were positive for both.

TABLE 2. Number of specimens positive for genital *mycoplasma* spp.

| Specimen type | No. of specimens | | |
|---|---|---|---|
| | Tested | Culture positive (%) | PCR positive (%) |
| Cervical swab | 26 | 6 (23) | 13 (50) |
| Vaginal swab | 2 | 1 (50) | 2 (100) |
| Semen | 49 | 12 (24) | 10 (20) |
| Urine (female) | 4 | 2 (50) | 2 (50) |
| Urine (male) | 2 | 0 (0) | 1 (50) |
| Not specified | 1 | 0 (0) | 0 (0) |
| Total | 84 | 21 (25) | 28 (33) |

An analysis of test results according to specimen type demonstrated interesting differences in the performance of the two test systems (Table 2). In cervical or vaginal specimens, PCR detected mycoplasma in 15 of 28 specimens, whereas culture detected mycoplasma in only 8 specimens. In semen specimens, the culture was positive in 12 of 49 specimens, whereas PCR was positive in only 10 (only 8 of which were culture positive). In fact, the only specimens that were culture positive, PCR negative were these 4 semen specimens.

**Resolution of discordant results.** All discordant samples were at a minimum discrepant for ureaplasma. To determine true positive specimens, all samples were amplified with a set of primers targeting the MBA gene in the ureaplasma genome. Ureaplasma was detected in 23 specimens with the PCR for MBA. Ten appeared to be *U. parvum*, twelve were *U. urealyticum*, and one was mixed with both species. Patients were considered to have genital mycoplasmas if specimens were culture positive or positive for both amplification assays. Based on this definition, 30 patients were determined to be infected with genital mycoplasma, resulting in a prevalence rate of 36%. The multiplex PCR detected mycoplasmas in 26 true-positive specimens, as well as 2 specimens that were not confirmed positive (Table 3).

Thirteen semen specimens, including the four culture-positive, multiplex PCR-negative specimens, were analyzed for the presence of inhibitors of PCR. Samples were spiked with *U. urealyticum* DNA and amplified in the multiplex PCR. In all cases, the spiked DNA was recovered by amplification and detected by ELOSA. In fact, the $OD_{490}$ obtained from the PCR products of the spiked samples, compared to the values of the PCR product of the stock of DNA used for spiking, demonstrated complete recovery of DNA (data not shown). These results indicate that these semen specimens did not contain inhibitors of PCR.

**Clinical sensitivity, specificity, and predictive values.** After discrepant results were resolved, the sensitivity and specificity were determined to be 87 and 96%, respectively, for PCR. Hence, PCR improves test sensitivity by 24% compared to culture, which has a sensitivity of 70% (Table 4). Based on a prevalence of 36%, positive and negative predictive values of multiplex PCR were determined to be 94 and 93%, respectively, whereas the negative predictive value for culture was only 86%. These results demonstrated that the multiplex PCR enhances ureaplasma detection.

TABLE 3. Confirmatory PCR discordant result analysis

| Discordant result set | *Ureaplasma* spp. | | | *M. hominis* | | *n* (*U. parvum*/*U. urealyticum*) | Result[a] |
|---|---|---|---|---|---|---|---|
| | Multiplex PCR | Culture | Confirmatory PCR | Multiplex PCR | Culture | | |
| 1 | + | + | + | − | − | 12 (10/2) | TP |
| 2 | + | + | − | − | − | 1 | TP |
| 3 | − | + | − | − | − | 4 | TP |
| 4 | + | − | + | − | − | 8 (0/8) | TP |
| 5 | + | − | + | + | + | 1 (0/1) | TP |
| 6 | + | − | + | + | − | 1 (Mixed)[b] | TP |
| 7 | − | − | + | + | + | 1 (0/1) | TP |
| 8 | − | − | − | + | + | 2 | TP |
| 9 | + | − | − | − | − | 2 | TN |
| 10 | − | − | − | − | − | 52 | TN |

[a] TP, true positive; TN, true negative.
[b] Mixed, mixed infection of *U. parvum* and *U. urealyticum.*

## DISCUSSION

We developed a multiplex PCR assay for the simultaneous detection of *Ureaplasma* spp., *M. genitalium*, and *M. hominis* in clinical specimens. This assay appeared to be very sensitive, with an analytical LOD of <12.5 CFU for all three organisms. Furthermore, this assay demonstrated high analytical specificity. DNA from other closely related *Mycoplasma* species, as well as from a variety of other common urogenital organisms, did not amplify in this multiplex.

In addition, the multiplex PCR was more sensitive than culture. In clinical specimens, PCR enhanced the detection rate of genital mycoplasma by 24%. This increased sensitivity was seen primarily in female specimens, where 78% more true-positive samples were detected by PCR than by culture (16 versus 9 true-positive specimens, respectively). The importance of genital mycoplasma detection in women is augmented by the high rate of vertical transmission of these organisms and their association with neonatal morbidity and mortality (3, 7, 8, 30, 42).

Enhanced sensitivity for genital mycoplasma detection with PCR is consistent with the literature (2, 23, 40). This finding is not surprising given the fact that mycoplasmas are labile organisms lacking a cell wall. PCR has an advantage in that it can still detect nonviable organisms. It is also important to point out that mycoplasma culture can be enhanced by the use of a broth medium in addition to solid medium, with an 8% increase in the ability to isolate *Ureaplasma* spp. and a 76% increase in the ability to isolate *Mycoplasma* spp. (11). Because our laboratory does not use a broth medium, one would expect a significant effect on our ability to isolate *Mycoplasma* spp.; however, use of the multiplex PCR assay did not increase our ability to detect this organism.

The analysis of the four apparently false-negative specimens by PCR is interesting. These four specimens were defined as true positive based on the fact they were culture positive; however, that was the only system for which they were positive. There are many factors known to cause false-negative PCR results. Inhibitors of *Taq* polymerase can be found in clinical specimens, which could result in false-negative results for both the multiplex PCR and the confirmatory test. The four PCR-negative, culture-positive specimens were all semen, suggesting an inhibitory component in this specimen type. However, we demonstrated that inhibitors were not present in these four specimens, as well as numerous other semen specimens. Sequence variability is an alternative source for potential false-negative PCR results. However, the genes targeted for the multiplex PCR and the confirmatory assay are different. Hence, the chance of divergence occurring in both genes is small. Furthermore, sequence divergence has not been reported for either of these target regions.

Historically, culture identification is considered definitive; however, any system has the potential for false-positive results. Mycoplasmal colony identification is difficult and subjective, since it is based on visual observation under a dissecting microscope. Hence, it is possible to obtain false-positive results by culture due to the misidentification of artifacts as colonies. Indeed, attempts to subculture organisms obtained from one of the four semen samples in question failed, as did PCR analysis of this isolate. Unfortunately, isolates from the other three specimens were not analyzed similarly. If the definition of a true positive were changed to at least two positive results from any three tests, PCR would demonstrate an enhancement of testing specificity in addition to sensitivity. The sensitivity and specificity would be 100 and 97%, respectively, for PCR and 65 and 93%, respectively, for culture. Likewise, the positive and negative predictive values of the multiplex PCR would be 93 and 100%, respectively, whereas the same indices for culture would be only 81 and 86%, respectively.

Historically, evaluation of amplification assays has been difficult in terms of determining true-positive detection because the experimental test often has higher detection rates than the gold standard. Using additional tests to resolve discordant results (discrepant analysis) has led to considerable discussion (13–15, 20, 32, 33). Much of the criticisms focused on the process of analysis of only discordant samples, which is poten-

TABLE 4. Sensitivity, specificity, and predictive values of genital mycoplasma culture and multiplex PCR

| Method | No. TP[a] | % (95% confidence interval) | | | |
|---|---|---|---|---|---|
| | | Sensitivity | Specificity | PPV | NPV |
| Culture | 21 | 70 (59–70) | 100[b] (94–100) | 100[b] (84–100) | 86 (80–86) |
| Multiplex PCR | 26 | 87 (74–92) | 96 (89–99) | 94 (80–99) | 93 (86–96) |

[a] TP, true positive.
[b] By definition, the value is 100%.

tially biased due to selective confirmation of initial test results, leading to overestimating the absolute sensitivity of the experimental assay. In the present study, we addressed this criticism by applying the confirmation test to all of the specimens. We chose to amplify a second target of ureaplasma for the analysis of discordant results, since all discrepancies occurred with ureaplasma detection. Confirmatory testing did not result in additional discordant specimens and enabled the determination of true-positive specimens.

The MBA PCR also enabled ureaplasma speciation. *U. parvum* is most commonly isolated by culture (1, 25). However, *U. urealyticum* is isolated significantly more often from women with infertility, miscarriages, and pelvic inflammatory disease (1, 28, 29). In the present study, *U. parvum* was also isolated most often by culture (83%, Table 3). Interestingly, the more-invasive species, *U. urealyticum*, was detected in all true positives that were culture negative, suggesting that culture often misses potentially more serious infections.

In the urogenital specimens, ureaplasma DNA was detected in 25 of 84 specimens (11 men and 14 women), whereas *M. hominis* was detected in 5 specimens, 2 of which were dually infected with ureaplasma and all of which were from women. No specimens were shown to contain *M. genitalium*. Similar ratios for the prevalence of these three organisms have been demonstrated by others (2, 23). Although *M. genitalium* was detected in vitro, further testing of a larger population or a population with a higher incidence would be required to achieve the power necessary to adequately assess this assay for this organism. A recent paper by Skov-Jensen demonstrated enhanced sensitivity for the detection of *M. genitalium* by targeting the rRNA gene for amplification (35).

Interestingly, the multiplex assay is just as sensitive for *M. hominis* as it is for ureaplasma and *M. genitalium*, although gel electrophoresis is used for detection of this target. Probe sequences for this target have been published (4); however, this probe never worked well in our analyses. In an effort to determine appropriate sequences for a probe that may perform better, we recently analyzed the 16S RNA gene sequences published in GenBank for the *Mycoplasmatales*. From this analysis, we determined that, indeed, the region chosen by Blanchard et al. is the superior region for species-specific hybridization. However, it was apparent that the sequence published by this group was missing a nucleoside, resulting in only 12 bp of homology for the probe. The appropriate sequence should read: CGCTGTAAGGCGNCACTAAA. This information will be necessary as we develop a real-time assay for this multiplex PCR.

Another advantage of the multiplex PCR is that the presence of other microorganisms does not interfere with testing. Indeed, the specimen excluded from study due to bacterial overgrowth was actually positive for ureaplasma by PCR. Finally, the multiplex PCR is a relatively rapid assay that can be performed in less than 8 h. Isolation by culture may take 2 to 5 days to obtain a result for *Ureaplasma* spp. and *M. hominis* and as long as 8 weeks for *M. genitalium*. The rapid detection of genital mycoplasmas is very important, particularly in the management of low-birth-weight infants, in whom these organisms are a significant cause of meningitis, respiratory disease, and death.

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