A comparative study of three different PCR assays for detection of *Mycoplasma genitalium* in urogenital specimens from men and women

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The aim of this study was to compare conventional 16S rRNA gene PCR, real-time 16S rRNA gene PCR and real-time *Mycoplasma genitalium* adhesin protein (MgPa) gene PCR as detection methods for *M. genitalium* infection. The study also determined the prevalence of *M. genitalium* in male and female patients attending a sexually transmitted infections clinic in a rural area in the west of Sweden. First void urine (FVU) and/or urethral swabs were collected from 381 men, and FVU and/or cervical swabs and/or urethral swabs were collected from 298 women. A total of 213 specimens were used in the PCR comparative study: 98 consecutively sampled specimens from patients enrolled in the prevalence study, 36 consecutively sampled specimens from patients with symptoms of urethritis and 79 specimens from patients positive for *M. genitalium* by real-time MgPa gene PCR in the prevalence study. A true-positive *M. genitalium* DNA specimen was defined as either a specimen positive in any two PCR assays or a specimen whose PCR product was verified by DNA sequencing. The prevalence of *M. genitalium* infection in men and women was 27/381 (7.1 %) and 23/298 (7.7 %), respectively. In the PCR comparative study, *M. genitalium* DNA was detected in 61/76 (80.3 %) of true-positive specimens by conventional 16S rRNA gene PCR, in 52/76 (68.4 %) by real-time 16S rRNA gene PCR and in 74/76 (97.4 %) by real-time MgPa gene PCR. Real-time MgPa gene PCR thus had higher sensitivity compared with conventional 16S rRNA gene PCR and had considerably increased sensitivity compared with real-time 16S rRNA gene PCR for detection of *M. genitalium* DNA. Real-time MgPa gene PCR is well suited for the clinical diagnosis of *M. genitalium*.

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INTRODUCTION

Mycoplasma genitalium was first isolated in 1980 (Tully *et al.*, 1981). There is strong evidence of *M. genitalium* having a causative role in non-chlamydia, non-gonococcal urethritis (NCNGU) in men and cervicitis in women (Deguchi & Maeda, 2002; Jensen, 2004; Taylor-Robinson, 2002; Uuskuila & Kohl, 2002). *M. genitalium* infection has also been associated with pelvic inflammatory disease but

the exact role has not yet been determined (Cohen *et al.*, 2002). In genital tract infection, the clinical implications of *M. genitalium* infection may not differ significantly from those of the well-established pathogens *Chlamydia trachomatis* and *Neisseria gonorrhoeae* (Falk *et al.*, 2004, 2005). Repeated attempts have been made to recover this extremely fastidious organism from clinical samples by culture techniques but isolates have been rare and difficult to obtain. Serology for the diagnosis of *M. genitalium* infection has not been widely used because of cross-reactivity with other mycoplasmas (Lind *et al.*, 1984; Taylor-Robinson *et al.*, 1983). With the development of PCR methods in the early 1990s, detection of *M. genitalium*

Abbreviations: FVU, first void urine; MgPa, *M. genitalium* adhesin protein; NCNGU, non-chlamydia, non-gonococcal urethritis; NGU, non-gonococcal urethritis; STI, sexually transmitted infections.

infection became more feasible. In 1991, conventional PCR for *M. genitalium* targeting the *M. genitalium* adhesin protein (MgPa) gene was introduced (Jensen *et al.*, 1991). This method was subsequently used in *M. genitalium* studies (Gambini *et al.*, 2000; Martinelli *et al.*, 1999). Jensen *et al.* (2004b) presented a further development of the MgPa gene PCR for application in real-time PCR. Other methods are based on the 16S rRNA gene of *M. genitalium* and have also been used with both conventional and real-time PCR to detect *M. genitalium* infection (Björnelius *et al.*, 2000; Jensen *et al.*, 2003; Jurstrand *et al.*, 2005; Yoshida *et al.*, 2002). In a review by Jensen (2004), there was an urge for more studies of *M. genitalium* infection in women as fewer studies have been published on women compared with men. There is also a lack of studies addressing the prevalence in both men and women from the same catchment area. The aim of this study was to compare conventional 16S rRNA gene PCR, real-time 16S rRNA gene PCR and real-time MgPa gene PCR as detection methods for *M. genitalium* and to determine the prevalence of *M. genitalium* in patients, both male and female, attending a sexually transmitted infections (STI) clinic in a rural area in Sweden.

METHODS

Patients and clinical specimens. From April to October 2003, specimens were obtained from 381 men (18–82 years of age, median 27 years) and 298 women (17–55 years of age, median 25 years) attending the STI clinic at the Central Hospital Karlstad, Sweden. All new attendees who were at risk of being infected with an STI due to unprotected sex with a new partner or having a sexual partner who was PCR-positive for *M. genitalium* were enrolled in the study after providing informed consent. Fewer than ten patients declined to be enrolled. Following a standard protocol, the patients were asked for historical symptoms and on-going symptoms of urethritis/cervicitis (dysuria, urethral and cervical discharge, intermenstrual or post-coital bleeding and lower abdominal pain). A specimen for a smear was obtained with either a plastic loop or a small swab from the distal urethra of men. The smears were stained with methylene blue. Patients with ≥ 5 polymorphonuclear leukocytes per high-power ($\times 1000$) microscopic field were considered to have urethritis. Following clinical examination, all men were asked to collect first void urine (FVU) for detection of *M. genitalium*. In patients with a urine bladder incubation time of ≤ 1 h, a urethral swab specimen was collected instead using a rayon-tipped wire shaft Copan 160 C and placed in 1.5 ml 2-SP medium containing 0.2 M sucrose in 0.02 M phosphate buffer (pH 7.0), 10% inactivated fetal calf serum, garamycin ($20 \mu\text{g ml}^{-1}$), vancomycin ($100 \mu\text{g ml}^{-1}$) and Fungizone ($5 \mu\text{g ml}^{-1}$). From women, an endocervical sample was obtained with a plastic loop from the posterial and lateral vaginal fornices for wet smear examination in 0.9% NaCl. Patients with more polymorphonuclear leukocytes than epithelial cells by microscopy were considered to have cervicitis. After the wet smear was taken, an endocervical swab for detection of *M. genitalium* was collected using a rayon-tipped wire shaft Copan 160 C and placed in 1.5 ml 2-SP medium. Following clinical examination, all women were asked to collect FVU for detection of *M. genitalium*. FVU was gathered in a 10 ml screw-capped polypropylene tube (Sarstedt). In patients with a urine bladder incubation time of ≤ 1 h, a urethral swab specimen was collected instead of FVU using a rayon-tipped wire shaft Copan 160 C and placed in 1.5 ml 2-SP medium.

Diagnostic tests. In the PCR comparative study, we used 213 specimens: 98 consecutively sampled specimens from patients enrolled in the prevalence study, 36 consecutively sampled specimens from patients with symptoms of urethritis and 79 selected specimens from patients positive for *M. genitalium* in the prevalence study by real-time MgPa gene PCR.

DNA extraction

For real-time PCR. A volume of 1800 μl from urine specimens was centrifuged at 20 000 *g* for 15 min. Aliquots of swab specimens (100 μl) in 2-SP medium were mixed with 1 ml 0.85% NaCl prior to centrifugation as for the urine specimens. The pellet was resuspended in 300 μl 5% (w/v) Chelex 100 slurry (Bio-Rad) in distilled water, vortexed for 60 s and incubated at 99 °C for 10 min. Finally, the specimens were centrifuged at 12 000 *g* for 5 min and the supernatant was analysed by real-time 16S rRNA gene PCR and real-time MgPa gene PCR.

For conventional PCR. DNA extraction for the conventional 16S rRNA gene PCR was performed as described previously by Jensen *et al.* (2004a). Briefly, 100 μl swab specimen in 2-SP medium was mixed with 300 μl 20% (w/v) Chelex 100 slurry in TE buffer [10 mM Tris/HCl (pH 8.0), 1 mM EDTA], vortexed for 60 s and incubated at 95 °C for 10 min. After centrifugation at 20 000 *g* for 5 min, the supernatant was analysed by conventional 16S rRNA gene PCR. Urine specimens were concentrated by centrifugation: 1800 μl was centrifuged at 20 000 *g* for 15 min. Chelex 100 slurry (300 μl) was added to the pellet and the mixture was treated as described above for the swab specimens.

Real-time MgPa gene PCR. The PCR was carried out in a 25 μl SmartCycler reaction tube containing 1 $\times$ reaction buffer, 3.5 mM MgCl_2 , 200 μM dNTP mix, 0.625 U Hot Gold Star *Taq* polymerase (Eurogentec), 1.0 μM each primer using the previously described (Jensen *et al.*, 2004b) forward primer MgPa-355F and reverse primer MgPa-432R (Cybergene) and 0.1 μM MgPa-380 Taqman MGB probe (Applied BioSystems) (Table 1). Subsequently, 5 μl template DNA was added to the mixture. Amplification was performed in a SmartCycler (Cepheid) under the following conditions: Hot Gold Star *Taq* polymerase activation at 95 °C for 10 min, followed by a touch-down protocol of 1 cycle of denaturation at 95 °C for 15 s, annealing at 64 °C for 30 s and extension at 72 °C for 30 s; 1 cycle of denaturation at 95 °C for 15 s, annealing at 62 °C for 30 s and extension at 72 °C for 30 s; and 48 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s.

Real-time 16S rRNA gene PCR. The PCR was carried out in a 20 μl LightCycler glass capillary. Primers and probes in this PCR assay were designed previously as a further development of the conventional PCR (Jensen *et al.*, 2003). The 18 μl reaction mix contained FastStart DNA Master Hybridization Probe mix, containing FastStart *Taq* polymerase, reaction buffer, dNTP mix, 10 mM MgCl_2 (Roche Diagnostics), 0.6 μM forward primer MG16-45F and 0.4 μM reverse primer MG16-447R (Scandinavian Gene Synthesis). Two hybridization probes, Mg16S-137 and Mg16S-169 (TIB-Molbiol), were used at a final concentration of 0.2 μM each (Table 1). Additional MgCl_2 was added to a final concentration of 5 mM. An aliquot (2 μl) of template DNA was added to the mixture. Amplification was performed on a LightCycler PCR system (Roche Molecular Biochemicals) under the following conditions: FastStart *Taq* polymerase activation at 95 °C for 10 min, followed by 50 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 10 s and extension at 72 °C for 16 s (Jurstrand *et al.*, 2005).

Conventional 16S rRNA gene PCR. The conventional 16S rRNA gene PCR was performed as described previously by Jensen *et al.* (2003). In brief, the PCR was carried out with a final reaction volume

Table 1. Primers and probes used for *M. genitalium* PCR assays

Primer/probe	Primer/probe name	Sequence 5'→3'	Nucleotide position
Primers for SmartCycler assay	MgPa-355F	GAGAAATACCTTGATGGTCAGCAA	1420–1443*
	MgPa-432R	GTTAATATCATATAAAGCTCTACCGTTGTTATC	1497–1465*
Primers for conventional and LightCycler assay	MG16-45F	TACATGCAAGTCGATCGGAAGTAGC	45–69†
	MG16-447R	AAACTCCAGCCATTGCCTGCTAG	469–447†
Probe for SmartCycler assay	MgPa-380	FAM-ACTTTGCAATCAGAAGGT-MGB	1445–1462*
Probes for LightCycler assay	Mg16S-137	LC-red 640-AATTCATGCGAACTAAAGTTCTTATGCGGTATTAGCT-phosphate	181–145†
	Mg16S-169	AATAACGAACCCCTGCAGGTCTTTCAACTT-fluorescein	213–183†

*MgPa operon sequence (GenBank accession no. M31431).

†16S rRNA gene sequence (GenBank accession no. X77334).

of 100 µl containing 1× PCR buffer (Super *Taq* buffer; HT Biotechnology) with 2.5 mM MgCl₂; 0.4 mM each primer (Table 1); 62.5 µM each dATP, dGTP and dCTP; 125 µM dUTP; 10 µl of the appropriate dilution of internal process control; and 2 U *Taq* polymerase (Platinum *Taq*; Life Technologies). A Perkin Elmer 9600 thermal cycler was used with 0.2 ml MicroAmp tubes. After denaturation at 94 °C for 2 min, 40 cycles were performed. The first ten cycles were used in a touch-down procedure and consisted of denaturation at 94 °C for 15 s, annealing at 72–62 °C for 30 s with a 1 °C decrement per cycle and extension at 72 °C for 15 s. The following 30 cycles consisted of denaturation at 92 °C for 15 s, annealing at 62 °C for 30 s and extension at 72 °C for 30 s. Amplicons were visualized after electrophoresis on 2% agarose gels containing 1 µg ethidium bromide ml⁻¹ and examined by UV transillumination.

DNA sequencing. The PCR products were purified by a standard ethanol precipitation method. In brief, 50 µl PCR product was mixed with 5 µl 3 M sodium acetate (pH 4.6) and 100 µl 95% ethanol in a 1.5 ml microcentrifuge tube. The tubes were left at –20 °C for 30–40 min to precipitate the PCR products. Following the freeze precipitation, the tubes were centrifuged at 20 000 *g* for 20 min. The supernatant was carefully aspirated and the pellets were rinsed with 300 µl 70% ethanol. The tubes were then centrifuged at 20 000 *g* for 5 min. Again, the supernatant was carefully aspirated and discarded. The pellets were left to dry at room temperature and then resuspended in 50 µl sterile water. The purified PCR products were then DNA sequenced using an ABI BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) using the same primers used for the MgPa gene real-time PCR assay. Sequencing reactions were purified using a DyeEx 2.0 Spin kit (Qiagen) before separation using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The nucleotide sequences were analysed using ChromaPro version 1.33 software and compared with sequence databases at the National Center for Biotechnology Information, using the basic local alignment search tool (BLAST) at <http://www.ncbi.nlm.nih.gov/BLAST/>.

RESULTS

Prevalence

Patients. *M. genitalium* was detected in 27/381 men (7.1%) and in 23/298 women (7.7%) by real-time MgPa gene PCR.

Specimens from men. *M. genitalium* was found in 27/361 (7.5%) single urine specimens tested. Five men provided a urethral swab specimen together with a urine specimen, whereas 15 men submitted only a urethral swab specimen. None of these specimens was positive for *M. genitalium*. Clinical information from the 27 *M. genitalium* PCR-positive men showed a 59% concordance between a positive PCR result and symptoms of urethritis and/or a positive urethral smear indicating urethritis.

Specimens from women. A cervical swab specimen together with a urine specimen was provided by 255 women and 19 (7.5%) were positive for *M. genitalium* in either specimen (ten urine- and endocervical-positive; eight urine-positive and endocervical-negative; one urine-negative and endocervical-positive). A FVU specimen only was submitted from four women, none of which was positive for *M. genitalium*. A cervical swab specimen only was obtained from 30 women from whom *M. genitalium* was found in two (6.7%). Nine women provided a urethral swab specimen together with a cervical swab specimen. Of these, two were positive in either specimen (one cervical-positive and urethral-negative; one cervical-negative and urethral-positive) for *M. genitalium* (Table 2). Clinical information from the 23 *M. genitalium* PCR-positive women showed a 30% concordance between a positive PCR result and symptoms of cervicitis and/or a positive wet smear indicating cervicitis.

PCR comparative study

A total of 213 specimens were used in the PCR comparative study. The real-time MgPa gene PCR assay established a 92 and 89.7% agreement in comparison with analysis results obtained with the conventional 16S rRNA gene PCR and real-time 16S rRNA gene PCR assays, respectively. A 93% agreement was demonstrated in analysis results in comparison between real-time 16S rRNA gene PCR and conventional 16S rRNA gene PCR assays. Seventy-six

Table 2. Distribution according to specimen type of 23 *M. genitalium* PCR-positive specimen sets from 298 women included in the prevalence study

ND, Not determined.

Cervical swab	Urethral swab	First void urine	<i>M. genitalium</i> -positive
+	+	ND	0
+	—	ND	1
—	+	ND	1
—	ND	+	8
+	ND	+	10
+	ND	—	1
+	ND	ND	2

specimens were considered to be true-positive specimens defined as either a specimen positive in any two PCR assays or a specimen whose PCR product was verified by DNA sequencing. Forty-nine specimens were positive in all three assays (Fig. 1). Ten specimens were positive only by conventional 16S rRNA gene PCR and real-time MgPa gene PCR. Three specimens were positive only by real-time 16S rRNA gene PCR and real-time MgPa gene PCR. Twelve specimens were positive for *M. genitalium* only by real-time MgPa gene PCR. Four of these specimens came from women with another true-positive sample. All 12 specimens were retested with the real-time MgPa gene PCR and the PCR products were verified by DNA sequencing (data not shown). Two specimens were positive for *M. genitalium* only by conventional 16S rRNA gene PCR. These two specimens also came from women with another true-positive sample. Both specimens were able to be retested by real-time MgPa gene PCR and the PCR products were verified by DNA sequencing.

DISCUSSION

The main purpose of this study was to compare different PCR assays for the detection of *M. genitalium* in urogenital specimens from men and women. We also wanted to determine the prevalence of *M. genitalium* in patients, both male and female, attending an STI clinic in the study catchment area. Many clinical studies published to date have used primers targeting the *M. genitalium* adhesin gene according to Jensen (2006). Moreover, the majority of studies to date on *M. genitalium* have used conventional PCR as the detection method. Yoshida *et al.* (2002) published the first real-time PCR assay for detection of *M. genitalium*. In the current study, 213 specimens were used to compare two different real-time PCR assays targeting the MgPa gene and the 16S rRNA gene of *M. genitalium*. We also compared results with conventional 16S rRNA gene PCR. The real-time MgPa gene PCR assay, using the MgPa-355F/432R primers published by Jensen *et al.* (2004b), detected 12 specimens in the PCR comparative study that were not able to be detected by the two other assays tested. These specimens were retested and the PCR products were verified by DNA sequencing as containing *M. genitalium* DNA. Four of the retested specimens came from women with another true-positive sample, which verified the result. However, the possibility of contamination rendering the remaining eight specimens positive could not be excluded. Two specimens were found to be positive for *M. genitalium* only by conventional 16S rRNA gene PCR. These two specimens also came from women with another true-positive sample. Both specimens were able to be retested with real-time MgPa gene PCR and the PCR products were verified by DNA sequencing. No retested specimens could be subjected to a new DNA extraction due to insufficient specimen material left after three previous DNA extractions. Previous studies have also demonstrated lower sensitivity for real-time 16S rRNA gene PCR compared with conventional 16S rRNA gene PCR. Jurstrand *et al.* (2005) showed 72.2 % sensitivity and

Fig. 1. Distribution of 213 specimens (76 positive and 137 negative) in a comparison of real-time 16S rRNA gene PCR, conventional 16S rRNA gene PCR and real-time MgPa gene PCR assays for detection of *M. genitalium*. Positive specimens are indicated within the circles.

99.7% specificity for real-time PCR compared with conventional PCR for detection of *M. genitalium* in urogenital specimens from men. Recently, Svenstrup *et al.* (2005) evaluated real-time MgPa gene PCR, real-time *gap* gene PCR and conventional 16S rRNA gene PCR as detection methods for *M. genitalium* in 246 urethral swab specimens from men. Real-time MgPa gene PCR proved to be the most sensitive method, detecting *M. genitalium* DNA in three specimens that were negative by real-time *gap* gene PCR (range 0.03–2.65 copies μl^{-1}). A low load of *M. genitalium* DNA in clinical specimens underlines the need for highly sensitive assays. Jensen *et al.* (2004a) demonstrated that 28% of urethral swab specimens and 14% of FVU specimens contained less than ten genome equivalents of *M. genitalium* DNA. In our PCR comparative study, the real-time 16S rRNA gene PCR was performed on a LightCycler using only 2 μl template DNA, whilst the real-time MgPa gene PCR was performed on a SmartCycler using 5 μl template DNA. This could explain the lower sensitivity for the real-time 16S rRNA gene assay. However, the conventional 16S rRNA gene PCR had the advantage of being performed with a total volume of 100 μl , allowing 10 μl template DNA to be analysed. The conventional assay increased the sensitivity of the 16S rRNA gene assay somewhat, but clearly using the adhesin gene as the target provided a more sensitive PCR method for detection of *M. genitalium*. All clinical specimens in this study were subjected to a crude Chelex extraction of DNA as the sample preparation method. In a recent study at the Department of Clinical Microbiology, Central Hospital, Karlstad, Sweden, we demonstrated a 6% partial inhibition compared with <1% partial inhibition in specimens prepared by Chelex extraction compared with an automated extraction using a MagAttract DNA Mini M48 kit (Qiagen) on a GenoM48 Biorobot (F. Aronsson, personal communication). In our PCR comparative study, only the conventional 16S rRNA gene PCR assay used an internal control for inhibition in the set-up. Inhibitors and the probability of low DNA load in specimens emphasize the need for improved protocols for specimen preparation to increase the sensitivity in assays for clinical purpose. There is an urge for more studies of *M. genitalium* infection in women as claimed by Jensen (2004), as more studies have been published on men. There are only a few studies addressing the prevalence in both men and women from the same catchment area. A study of *M. genitalium* in non-gonococcal urethritis (NGU) in Swedish male STI patients showed a high occurrence of 26 and 36% in patients with NGU and with NCNGU, respectively, compared with 10% in control patients without urethritis (Björnelius *et al.*, 2000). A prevalence study on *M. genitalium* in relation to the number of life-time sexual partners in patients visiting STI clinics in western Sweden showed considerably lower numbers of infected patients. Seven per cent of the examined men, 14% of men with urethritis, 3.5% of the examined women and 1% of the control patients without urethritis were infected with *M. genitalium* (Johannisson *et al.*, 2000). Other Swedish studies have shown a prevalence

in concordance with our results (Anagrius *et al.*, 2005; Falk *et al.*, 2004, 2005). Mellenius *et al.* (2005) presented slightly lower numbers of prevalence compared with our study: 4.1% in men and 3.8% in women. The clinical information from men and women in our study showed a 59% concordance for men and a 30% concordance for women between a positive PCR result for *M. genitalium* and symptoms of urethritis/cervicitis and/or a positive urethral/wet smear indicating urethritis/cervicitis. This is a lower proportion than previous reports for men but is congruent with other reports for women (Falk *et al.*, 2004, 2005). The lower percentage for men could be attributed to the fact that a plastic loop or a swab was used instead of a blunt curette in sampling. Several recent studies on *M. genitalium* have utilized real-time PCR as the detection method. The real-time MgPa gene PCR can be used in a quantitative setting, determining the *M. genitalium* load in clinical specimens, which may prove to be useful in clinical studies, giving information about the number of organisms. To our knowledge, this is the first comparison between two real-time PCR methods for detection of *M. genitalium* using specimens from both men and women. In conclusion, real-time MgPa gene PCR demonstrated higher sensitivity compared with conventional 16S rRNA gene PCR and considerably increased sensitivity compared with real-time 16S rRNA gene PCR for detection of *M. genitalium* DNA. It has a number of advantages over conventional PCR as it is a closed format, decreasing the contamination risk, it is less labour-intensive and the use of probes increases specificity. With the implementation of an internal processing control, this method will be well suited for clinical diagnostics of *M. genitalium* in urogenital specimens from men and women.

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