Real-time PCR detection of the *mg219* gene of unknown function of *Mycoplasma genitalium* in men with and without non-gonococcal urethritis and their female partners in England

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Real-time PCR was employed to detect a region of the *Mycoplasma genitalium mg219* gene, a gene of unknown function, in clinical samples. Amplification of DNA and signal production from 15 other species of human mycoplasmas and 14 other bacteria and viruses did not occur. Using a panel of 208 genital and rectal samples, the sensitivity when compared to the modified *mgpa* gene (encoding the major surface protein MgPa) real-time PCR assay was found to be 100 % and the specificity of the assay 99.5 % with a positive predictive value of 80 % and a negative predictive value of 100 %. The *mg219* gene was found to be in all strains of *M. genitalium* and was highly conserved. *M. genitalium* was detected in 3.9 % (11/280, 95 % CI 2.1–6.9) of all male specimens, in 7.7 % (10/130, 95 % CI 4.1–13.7) of patients with non-gonococcal urethritis (NGU) and in 0.7 % (1/150, 95 % CI <0.01–4.1) of patients without urethritis. The presence of *M. genitalium* was significantly associated with NGU ($P \leq 0.01$; 95 % CI 0.88–0.98) and non-chlamydial-non-gonococcal urethritis ($P=0.0005$; 95 % CI 0.84–0.97).

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INTRODUCTION

Mycoplasma genitalium is a sexually transmitted bacterium that is primarily found in the human urogenital tract. It has also been detected in respiratory (Baseman *et al.*, 1988), rectal (Taylor-Robinson *et al.*, 2003) and synovial (Tully *et al.*, 1995) specimens. Serological detection of *M. genitalium* is problematic due to cross-reactions with *Mycoplasma pneumoniae* antibodies (Clausen *et al.*, 2001; Lind *et al.*, 1984). Isolation via cell culture is slow and laborious and real-time PCR is currently used in the diagnosis of infected patients (Jensen, 2006). Originally isolated from men with urethritis (Tully *et al.*, 1981), *M. genitalium* has been convincingly linked to non-gonococcal urethritis (NGU) and non-chlamydial-non-gonococcal urethritis (NCNGU) (Jensen, 2006). However, the full extent of *M. genitalium* infection, epidemiology, and role in cervicitis, pelvic inflammatory disease, infertility and other infections requires investigation

(Jensen, 2006). *M. genitalium* is sexually transmitted and a high prevalence has been found (58 %) in individuals with infected sexual partners (Keane *et al.*, 2000). Sequence-based typing has identified identical isolate sequence types from sexual partners, cementing evidence for sexual transmission (Hjorth *et al.*, 2006; Ma *et al.*, 2008).

Several real-time PCR assays have been described for the molecular detection of *M. genitalium*, including those targeting *mgpa*, the 16S rRNA gene and p115 (for review see Jensen, 2006). The *mgpa* gene real-time assay has been shown to be of increased sensitivity to the 16S rRNA method (Edberg *et al.*, 2008). We sought to identify a new method for confirmation of PCR results with equal sensitivity to that of the *mgpa* gene assay. The *mg219* gene assay was developed and used to detect the presence of *M. genitalium* in parallel with the *mgpa* gene real-time PCR assay in 280 male genitourinary medicine clinic attendees and 23 female partners as part of a case-control double-blinded study in patients with and without urethritis of known chlamydia and gonococcal infection status.

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Abbreviations: C_p , crossing point threshold; IPC, internal processing control; NCNGU, non-chlamydial-non-gonococcal urethritis; NGU, non-gonococcal urethritis.

A sequence alignment of the *mg219* gene is available with the online version of this paper.

METHODS

Reference and validation material. The following reference strains, clinical isolates, specimens and genomic DNA were obtained from the

National Collection of type Cultures (NCTC), Colindale, London, the American Type Culture Collection (ATCC), Virginia, USA, and the authors' laboratory: *Acholeplasma laidlawii* NCTC 10116^T, *Mycoplasma amphoriforme* NCTC 11740^T, *Mycoplasma buccale* NCTC 10136^T, *Mycoplasma faucium* NCTC 10174^T, *Mycoplasma fermentans* NCTC 10117^T, *Mycoplasma genitalium* NCTC 10195^T, *Mycoplasma hominis* NCTC 10111^T, *Mycoplasma lipophilum* NCTC 10173^T, *Mycoplasma orale* NCTC 10112^T, *Mycoplasma penetrans* ATCC 55252^T, *Mycoplasma pirum* NCTC 11702^T, *Mycoplasma pneumoniae* NCTC 10119^T, *Mycoplasma salivarium* NCTC 10113^T, *Mycoplasma spermatophilum* NCTC 11720^T, *Ureaplasma parvum* ATCC 33697 and *Ureaplasma urealyticum* NCTC 10177^T (mollicute species); *Lactobacillus vaginalis* NCTC 12197^T; *Prevotella bivia* NCTC 11156^T; *Mobiluncus curtisii* NCTC 11656^T; *Mobiluncus mulieris* NCTC 11658^T; *Streptococcus pyogenes* NCTC 12067; *Streptococcus agalactiae* NCTC 12906; *Escherichia coli* NCTC 9001^T; *Staphylococcus aureus* NCTC 8532^T; clinical isolate *Neisseria gonorrhoeae* H060160180; *Chlamydia trachomatis* L2, cell culture DNA; *Treponema pallidum* H05510345; clinical samples positive for *Treponema pallidum*; *Haemophilus ducreyi*; and herpes simplex virus (HSV-1).

Extraction of DNA from bacterial cultures. Cultures of mollicute species and other bacteria (5 ml) were concentrated by centrifugation at 8000 g for 15 min, resuspended in 180 µl nuclease-free water and extracted using the Roche MagNAPure robot and DNA Isolation kit III (Roche Diagnostics) according to the manufacturer's instructions. DNA concentration was adjusted to approximately 20 pg µl⁻¹ for use in the real-time PCR assay. DNA from culture isolates of *M. genitalium* (M30 10-4, M30 early 10-2, TW10-5G 10-4, TW10-6 G10-4, R-32G 10-3, TW48-5G 10-4, M2300 10-4, M2321 10-3, M2341 10-3, G37 10-4, M2288 10-3, UTMB-10G 10-4) was kindly donated by Jørgen Jensen (Statens Serum Institut, Copenhagen, Denmark).

Real-time PCR detection of *M. genitalium*. Real-time PCR was performed using a Rotor-Gene 6000 (Corbett Life Science) and all primers and probes are listed in Table 1.

mgpa gene assay (alteration of primers). Real-time PCR detection of the *mgpa* gene based upon the method by Jensen *et al.* (2004) was adapted for use on a Rotor-Gene with a modification to the forward primer to reflect base changes between *M. genitalium* strains. The method includes amplification of a cloned internal processing control (IPC) that contains the same primer regions as the target with differing probe target and enables detection of samples inhibitory specifically to this PCR. Reaction volumes (20 µl in total) were used containing 1.25 U AmpliTaq Gold DNA polymerase

(Applied Biosystems), 0.5 µM primers (MgPa355F modified primer and MgPa432R), 0.2 µM MgPa380 probe and 0.05 µM IPCR probe, 0.1 ng IPC DNA, 5 µM MgCl₂, 0.625 mM dNTPs with dUTP (Applied Biosystems), 0.2 µl AmpErase Uracil N-glycosylase (Applied Biosystems) and 5 µl sample or positive/negative control. Uracil-DNA glycosylase was added to each reaction to eliminate carry-over contamination (Longo *et al.*, 1990). Tubes were sealed and placed into the Rotor-Gene and the following cycling conditions were applied: initial denaturation at 95 °C for 5 min (transition rate 20 °C s⁻¹; acquisition mode none); 45 quantification cycles of 95 °C for 15 s (transition rate 20 °C s⁻¹; acquisition mode none) and 60 °C for 60 s (transition rate 20 °C s⁻¹; acquisition mode single). Data were analysed using Rotor-Gene software and copy number was estimated from crossing point threshold (C_p) relative to a positive control curve consisting of 5 ng, 0.5 ng, 0.05 ng and 0.005 ng per 5 µl *M. genitalium* DNA. Samples that did not give a positive result and in which the IPC did not amplify were recorded as inhibitory and were repeated undiluted and diluted 1/10 in nuclease-free water (Sigma).

mg219 gene assay. The *mg219* gene was identified as being specific to *M. genitalium* and absent from all other completed mycoplasma genomes (using ORFAnage; Siew *et al.*, 2004) and from other bacterial sequences within GenBank. A real-time PCR was developed targeting a 251 bp fragment of the *mg219* gene of *M. genitalium* strain G37 NCTC 10195^T (2 655 586–265 837 in GenBank accession no. NC000908). The assay was run with identical reagents and conditions to those for the *mgpa* gene assay except for the primers and probes (Table 1), enabling running both assays together in the same tube or both assays on the same machine simultaneously and reducing testing time. To ensure that the *mg219* gene assay could be used as a confirmatory test, all samples in this study were performed in separate tubes to the *mgpa* gene assay.

mg219 gene assay validation. The sensitivity of the *mg219* gene assay was calculated in parallel by testing serial dilutions (in triplicate, 100 µg ml⁻¹–100 pg ml⁻¹) of a DNA extract of *M. genitalium* strain G37 NCTC 10195^T. This DNA panel was also tested using the modified *mgpa* gene assay. In addition, a panel of DNA extracts from cultured isolates of *M. genitalium* (listed above) was tested using the *mg219* gene assay. A panel of 208 anonymized clinical samples submitted to the laboratory were tested using the *mg219* gene assay and *mgpa* gene assay to ascertain the efficacy of the *mg219* gene assay with differing sample types and to determine inhibition using the *mgpa* gene assay. A total of 30 liquid-based cytology samples, 18 genital ulcer swabs (16 penile, 2 vulval), 1 penile swab, 13 urethral swabs, 22 rectal swabs, 38 vaginal swabs, 40 endocervical swabs and 46 urines were analysed in both assays.

Table 1. Primers and probes

Name	Sequence (5'→3')
MgPa355F modified (Jensen <i>et al.</i> , 2004)	GAG AA(A/G*) TAC CTT GAT GGT CAG CAA
MgPa432R (Jensen <i>et al.</i> , 2004)	GTT AAT ATC ATA TAA AGC TCT ACC GTT GTT ATC
MgPa380 probe (Jensen <i>et al.</i> , 2004)	FAM-ACT TTG CAA TCA GAA GGT-BHQ1
IPCR probe†	ROX-TCC TTC GTG ATA TCG GAC GTT GGC TG-BHQ2
Mg219F (forward primer)	CAT AGT TCA TTA TGC GCA CCA GTT ACT TG
Mg219R (reverse primer)	CTC TTT AAC AAC AGG GGT TGG GAT TAG
Mg219 probe	Cy5-GGT GTG GAT CGA GCG GC-BHQ3

*A/G wobble – due to 15.6 % of isolates having G at this position (underlined bases are A, A, A and G in 4.4, 4.4, 2.2 and 2.2 % of isolates, respectively, modified in this study from Jensen *et al.*, 2004).

†IPCR probe, internal processing control probe. The method includes amplification of a cloned internal processing control that contains the same primer regions as the target with differing probe target, enabling detection of samples inhibitory specifically to this PCR (Jensen *et al.*, 2004).

The specificity of the *mg219* gene assay was determined using a panel of DNAs from all human mollicute species, additional bacterial species, common genital bacteria and viruses and clinical samples of known viral and bacterial status as listed above. Clinical samples were heat-inactivated at 50 °C for 10 min and swabs were then agitated in 1 ml PBS for 5 min prior to centrifugation. A total of 1.8 ml urine was concentrated by centrifugation at 8000 g for 15 min, resuspended in 200 µl of nuclease-free water and extracted using the Roche MagNAPure robot using DNA isolation kit III (Roche Diagnostics) according to the manufacturer's instructions.

Clinical screening. Between 26 March 2007 and 8 June 2007, a total of 286 males and 25 females were recruited to the study from a local Sexual Health Department. Six male patients were diagnosed with *N. gonorrhoeae* and excluded from the study. Two female recruits were subsequently excluded as their male partner's sample was unavailable. No urine specimen was provided for one of the females tested thus only an endocervical swab was tested for this patient. This resulted in a total of 280 consenting male attendees, 130 case male recruits with NGU and 150 control male recruits without urethritis, and 23 female partners. Ethical approval was given by the local National Health Service organization. Patients were excluded from the study if they: (i) were under the age of 16 years, (ii) had taken antibiotics during the last month or (iii) had passed urine within 1 h of their appointment. Patients were screened for *N. gonorrhoeae* by culture, for *C. trachomatis* by PCR (BD ProbeTec ET Amplified DNA Assay; Becton Dickinson) and for urethritis (defined as the presence of $\geq$ five polymorphonuclear lymphocytes per high-power field). A first void urine specimen was provided by all test subjects and in addition an endocervical swab from women. The DNA was extracted from these specimens using a MagNA Pure LC instrument and MagNA Pure LC Total Nucleic Acid isolation kit (Roche) and tested for *M. genitalium* using both the *mg219* gene and *mgpa* gene assays.

***mg219* gene sequencing and alignments.** The entire *mg219* gene (2 655 597–266 043 in GenBank accession no. NC000908) and flanking regions were amplified from cultures and clinical isolates by gradient PCR using primers upstream and downstream of *mg219* [primer MG219-upstream mapped to position –76 to –54 (upstream of the *mg219* start codon) and primer MG219-downstream mapped to position 503–525 (downstream of the *mg219* stop codon)], with an annealing temperature of 57–68 °C and Roche High Fidelity Taq. The resulting amplified fragments were purified and both strands of the PCR products were sequenced using the oligonucleotides MG219-upstream and MG219-downstream. Sequencing was performed using the Dye Terminator Cycle Sequencing (DTCS) Quick Start kit (Beckman Coulter) following the manufacturer's instructions and using the CEQ 8000 Genetic Analysis System, and the consensus sequence for each strain was determined using the BioNumerics version 4.5 software package (Applied Maths) and CLUSTAL W software (Chenna *et al.*, 2003).

Statistical analysis. All statistical analysis (Fisher's exact test and confidence intervals) was performed with GraphPad Quickcalcs (<http://www.graphpad.com/quickcalcs/index.cfm>) (GraphPad Software) or in Microsoft Excel. The probability of a type I error (α) of 0.05 was taken for all analyses and 95 % confidence intervals are included where relevant.

RESULTS AND DISCUSSION

mg219 gene assay sensitivity

M. genitalium was consistently detected in all DNA replicates at a concentration of 100 µg ml⁻¹–100 pg

ml⁻¹, corresponding to a lower detection limit of 0.5 pg per reaction in the *mg219* gene assay. Replicates less than 10 pg ml⁻¹ could not be consistently detected. The *mg219* gene assay detected 825 copies or more of the target sequence in the 5 µl samples used in the assay corresponding to 0.5 pg per reaction (of extracted concentrated DNA from *M. genitalium* strain G37 NCTC 10195^T). This was equal to the modified *mgpa* gene assay tested in parallel on the same DNA samples. All positive clinical male urine samples included in the study contained on average 9.91×10^5 (from 7.0×10^0 to 6.43×10^6) copies of the target sequence per 5 µl concentrated extracted DNA. This corresponds to 7.79×10^1 – 7.15×10^7 copies per ml patient urine. The *mg219* gene assay was found to have 100 % sensitivity in comparison to the *mgpa* gene assay. Positive samples amplified on average 2.0 cycles earlier in the *mg219* gene assay (C_p 32.6 ± 5.2) than in the *mgpa* gene assay (C_p 34.6 ± 4.2).

mg219 gene assay specificity

The *mg219* assay was found to be highly specific in that DNA extracted from reference cultures (all human mollicute species and other common genital bacteria and viruses) did not give a positive signal in the assay. No significant homology of the target sequence was found with any other known genes with BLAST (Altschul *et al.*, 1990). Of the clinical samples used to validate the assay, 4/208 (1.9 %, 95 % CI 0.6–5.0) were PCR-positive for *M. genitalium* in both the *mg219* gene and *mgpa* gene assays (0/46 urine, 2/22 rectal swabs, 0/13 urethral swabs, 0/18 genital ulcer swabs, 0/30 liquid-based cytology samples, 0/38 vaginal swabs, 2/40 endocervical swabs, 0/1 penile swab). One rectal swab sample was found to be positive only in the *mg219* gene assay and insufficient sample prevented further investigation. These results could represent either a false-positive reaction, pipetting error or a genuine positive reaction that was only identified by the *mg219* gene assay. The study included a low number of positive samples; nonetheless, the *mg219* gene assay was found to have 99.5 % specificity, a positive predictive value of 80 % and a negative predictive value of 100 % in comparison to the *mgpa* gene assay.

mg219 gene sequence data

The *mg219* gene was found to be both present in all DNA of cultured isolates examined and highly conserved. Of the DNA of cultured strains, M30 10-2 and 10-4, TW10-5G, TW10-6G, R-32G, TW48-5G, M2300, M2321, UTMB 10G and G37 (resequenced in this study) were all 100 % identical to the NCTC 10195^T G37 control strain (the alignment is shown in Supplementary Fig. S1 in JMM Online). Interestingly, all the base differences were found within two urethral strains only: M2341 10-3 was 93.1 % identical with 40/569 bases differing and M2288 10-3 was 93.2 % identical with 39/569 bases differing. Of these, 30 bases consisted of an insertion in M2341 and M2288 with

the sequence AAGCAACTAAACAAACCAAAACAAAGC-CAA located between positions 504 and 505 in the control strain. Of the remaining 10 bases, all were identical in both strains (position 72 G to A, 284 T to C, 346 T to C, 354 C to T, 362 C to A, 426 G to A, 458 T to C, 463 G to A, 485 C to A) except for an A insertion at position 524–525 in M2341 only.

Clinical data

Overall, *M. genitalium* was detected by the *mg219* gene assay and the *mgpa* gene assay in 3.9 % (11/280, 95 % CI 2.1–6.9) of all male specimens, in 7.7 % (10/130, 95 % CI 4.1–13.7) of patients with NGU and in 0.7 % (1/150, 95 % CI <0.01–4.1) of patients without urethritis. The presence of *M. genitalium* was significantly associated with NGU ($P \leq 0.01$, 95 % CI 0.88–0.98). *M. genitalium* was not detected in any of the 23 females or corresponding 23 male sexual partners. Patients with *M. genitalium* had a median age of 26.0 years. Inhibition was detected in 1/280 (0.36 %, 95 % CI <0.01–2.2) male urine specimens, 4/23 female urine specimens and 4/23 endocervical swabs (17.4 %, 95 % CI 6.4–37.7), of which one female had inhibition in both samples. Repeat testing of dilutions of these samples resulted in removal of inhibition. Concordant results were found for all 11 samples positive in both the *mgpa* gene and *mg219* gene assays. A further five males were positive in the *mgpa* gene assay only and on initial analysis and all had crossing points of 40 cycles or more. Repeat testing of these equivocal specimens was negative in the *mgpa* gene assay and the initial positive *mgpa* gene result was unrepeatable on further testing.

C. trachomatis infection was detected in 54/280 (19.3 %, 95 % CI 15.1–24.3) male recruits, considerably higher than for *M. genitalium*. This equates to a prevalence of 33.8 % (44/130, 95 % CI 26.3–42.4) in males with NGU compared to a prevalence of 6.7 % (10/150, 95 % CI 3.5–11.9) in the control group ($P \leq 0.001$, 95 % CI 0.6–0.8). The median age of patients positive for *C. trachomatis* was 22.5 years. Dual infection with both *M. genitalium* and *C. trachomatis* was detected in 2/280 (0.7 %, 95 % CI 0.2–2.7) male patients. A total of 86 males had NCNGU. The prevalence level of *M. genitalium* in patients within this group was 10.5 % (9/86, 95 % CI 5.4–18.9; $P=0.0005$).

C. trachomatis was detected in 26.1 % (6/23, 95 % CI 12.3–46.8) of women and in their corresponding male partner (26.1 %, 6/23, 95 % CI 12.3–46.8). All *C. trachomatis*-negative females had *C. trachomatis*-negative male partners and *C. trachomatis* was detected in the partners of all *C. trachomatis*-infected males with partners in the study (100 %, 6/6, 95 % CI 55.7–100).

The *mg219* gene assay showed excellent specificity and sensitivity and had an equal lower detection level to that of the modified *mgpa* gene assay. The *mg219* gene is expressed as part of a polycistronic message from the *mg218* operon. This operon encodes phosphorylated proteins involved in

cytadherence, gliding and terminal organelle curving and motile response (Burgos *et al.*, 2007; Dhandayuthapani *et al.*, 2002; Musatovova *et al.*, 2003; Pich *et al.*, 2008; Su *et al.*, 2007). Using both NetPhos (Blom *et al.*, 1999) and Phosida (Gnad *et al.*, 2007), several potential phosphorylation sites are predicted in the Mg219 conserved hypothetical protein and it is likely that Mg219 is also phosphorylated. It could be involved in cytadherence and gliding, similar to the other proteins encoded by the *mg218* operon, and may function in response to environmental signals. The *mg219* gene is unique to and highly conserved in *M. genitalium*. *M. genitalium* was isolated most frequently from rectal swabs (9.1 %) and endocervical swabs (5 %) in this study and previous studies have isolated *M. genitalium* from the rectum of men (Taylor-Robinson *et al.*, 1985, 2003). A higher prevalence in men suffering from urethritis has been repeatedly demonstrated (Björnelius *et al.*, 2000; Deguchi *et al.*, 2002; Horner *et al.*, 1993; Jensen, 2006; Maeda *et al.*, 2001). The significant association of *M. genitalium* with NGU (7.7 %) and NCNGU (10.5 %) seen in this study is in line with previous reports (Jensen, 2006; Moi *et al.*, 2009). Few studies on the prevalence of *M. genitalium* in England have been undertaken, and *M. genitalium* was detected in 0.7 % male patients without urethritis in this study. A larger study of female patients in England is needed to establish prevalence in this group. Additional data are required to determine whether widespread screening of asymptomatic patients is beneficial for epidemiological analysis; however, symptomatic NCNGU patients can now be and should be screened for *M. genitalium* when clinically relevant. No data are available regarding the prevalence of *M. genitalium* in the population of England in relation to differing areas and differing patient demographics, typing, antibiotic resistance and optimal treatment.

Conclusion

The *mg219* gene assay for detecting *M. genitalium* in clinical samples is reproducible, highly specific and sensitive and is applicable to a wide range of specimen types. The *mg219* gene is highly conserved and present in all strains tested. Dual infections with *C. trachomatis* were found in 0.7 % samples tested and overall 3.9 % samples were PCR-positive for *M. genitalium*. *M. genitalium* was found in 0.7 % asymptomatic males in this study and was associated with both NGU (7.7 %) and NCNGU (10.9 %).

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