ORIGINAL ARTICLE

A novel polymerase chain reaction assay to detect *Mycoplasma genitalium*

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Aims: To design and validate a polymerase chain reaction (PCR) assay targeting the 16S rRNA gene of *Mycoplasma genitalium*.

Methods: Primers were designed that were complementary to the 16S rRNA gene sequence of *M genitalium*. After optimisation of the reaction conditions, the PCR was tested against nine *M genitalium* strains, a dilution series of *M genitalium* DNA, and a panel of common microorganisms. The PCR was also challenged in parallel with a published assay against 54 urine specimens from men with urethritis.

Results: The expected 341 bp product was produced on amplification of material from all *M genitalium* strains and from none of the other microorganisms tested. The lower limit of detection was 50 genome copies. The new assay detected *M genitalium* DNA in nine of 54 men with urethritis, in comparison with eight positive specimens detected with the alternative PCR.

Conclusions: This novel PCR targeting the *M genitalium* 16S rRNA gene has been optimised and now provides a sensitive and specific alternative or addition to the available MgPa gene targeting assays.

Mycoplasma genitalium was first isolated from two men with non-gonococcal urethritis (NGU) in 1981.[1] Cultivation of the organism has proved difficult even in the complex media typically used for mycoplasma culture. Axenic primary culture can take up to three months to produce a positive result and is not sensitive diagnostically.[2] The polymerase chain reaction (PCR) has been applied to the detection of *M genitalium* and has been used to show the presence of the organism in men with NGU,[3–7] a disease for which an aetiological agent is frequently not identified. Although a proportion of healthy men carry the organism, *M genitalium* carriage is significantly associated with NGU, independent of *Chlamydia trachomatis* (reviewed in Taylor-Robinson and colleagues[8]).[2 3 6 7] The UK national guideline for the management of NGU states that *M genitalium* probably causes the disease and that treatment should reflect this.[9] *Mycoplasma genitalium* has been found in the genital tract of women, many with genitourinary disease,[10–13] and has recently been associated with both cervicitis[14] and endometritis.[15]

> "*Mycoplasma genitalium* carriage is significantly associated with non-gonococcal urethritis, independent of *Chlamydia trachomatis*"

There is currently no commercially available test for *M genitalium* and most published PCR assays target either the MgPa major adhesin gene[11 16] or the 16S rRNA gene.[17–19] The protein encoded by MgPa, the first *M genitalium* gene to be sequenced, is a virulence determinant and major antigen so that is an appropriate target for the PCR. However, surface expressed antigens often have unstable gene sequences and intraspecies variation in the MgPa gene has been reported.[20] It is likely that the 16S rRNA gene sequence is relatively stable and therefore contains few, if any, polymorphisms in the *M genitalium* population. In the absence of non-amplification tests it is in any case desirable to be able to detect more than one gene target to confirm positive results and therefore to confirm the prevalence in a specific population group.

We have designed a sensitive and specific PCR assay targeting the 16S rRNA gene of *M genitalium* and have validated the assay against previously published methods using urine specimens from men with urethritis attending a genitourinary medicine clinic.

MATERIALS AND METHODS

Polymerase chain reaction

Oligonucleotide primers corresponding to sequences within the *M genitalium* 16S rRNA gene were synthesised as follows:

- 16SFG2: 5'-CCT TAT CGT TAG TTA CAT TGT TTA A-3' (nucleotides 1096–1220; GenBank accession number X77334).
- 16SRG: 5'-TGA CAT GCG CTT CCA ATA AA-3' (nucleotides 1418–1436; GenBank accession number X77334).

Each PCR reaction (in a 500 µl microcentrifuge tube) consisted of: 0.25 U superTaq polymerase (HT Biotechnology Ltd, Cambridge, UK), 5 µl 10× superTaq reaction buffer (100mM Tris/HCl, pH 9.0, 15mM $MgCl_2$, 500mM KCl, 1% Triton X-100, 0.1% wt/vol stabiliser; HT Biotechnology Ltd), 20 µM each dNTP (Pharmacia Biotech, Uppsala, Sweden), 10 nM each oligonucleotide primer, 5 µl template DNA, or negative control, and water for injections (B Braun Medical Ltd, Aylesbury, Buckinghamshire, UK) to a total volume of 50 µl. The reaction mix was protected from evaporation by a large drop (approximately 50 µl) of filter sterilised liquid paraffin (AnalaR; BDH Laboratory Supplies, Poole, Dorset, UK). Thermal cycling was carried out in an OmniGene instrument (Hybaid, Teddington, Middlesex, UK).

A modified "hot start"[21] was used, whereby the reaction mix was held at 75°C during addition of the template, and the temperature of the mix was not permitted to fall below the annealing temperature until completion of the run.

Cycling began with an initial denaturation step of three minutes at 94°C, then 45 cycles of 30 seconds at 94°C, 30 seconds at 50°C, and 60 seconds at 72°C, followed by a final

Abbreviations: HPF, high power field; IDEIA, amplified enzyme immunoassay; NGU, non-gonococcal urethritis; PCR, polymerase chain reaction; PMNL, polymorphonuclear leucocyte

Table 1 Organisms tested by 16S based PCR for *Mycoplasma genitalium*

M genitalium strains (ref)	Mollicute species	Other microorganisms
G37[1]	*Mycoplasma pneumoniae* (NCTC 10119)	*Chlamydia trachomatis* serovar L2
TW10[1]	*Mycoplasma pirum* (NCTC 11702)	*Burkholderia cepacia*
R32[1]	*Mycoplasma gallisepticum* (NCTC 10115)	*Haemophilus influenzae*
UTMB[1]	*Mycoplasma primatum* (NCTC 10163)	Coagulase negative staphylococcus sp
TW48[22]	*Mycoplasma hominis* (NCTC 10111)	*Pseudomonas aeruginosa*
M-2341[23]	*Mycoplasma orale* (NCTC 10112)	*Candida albicans*
M-2321[23]	*Mycoplasma salivarium* (NCTC 10113)	*Corynebacterium xerosus*
M-2288[23]	*Mycoplasma lipophilum* (NCTC 10173)	*Escherichia coli*
M-2300[23]	*Mycoplasma buccale* (NCTC 10136)	Group A streptococcus sp
	Ureaplasma urealyticum (NCTC 10177)	*Klebsiella pneumoniae*
	Acholeplasma laidlawii (NCTC 10116)	Group B streptococcus sp
		Enterococcus sp
		Bacillus sp
		Branhamella catarrhalis
		Bacteroides fragilis
		Micrococcus sp
		Clostridium ramosum
		Bacillus cereus
		Clostridium perfringens
		Lactobacillus casei
		Bacteroides urealyticus
		Clostridium sp

extension step of seven minutes at 72°C. Products were visualised by agarose gel electrophoresis alongside a 100 bp ladder (GibcoBRL, Life Technologies, Paisley, UK). The expected product size was 341 bp.

Assay validation

Amplification of target and non-target microorganisms

Various microorganisms were suspended in water and heated to 100°C for 10 minutes. Cell debris was removed by centrifugation and 5 µl of supernatant added to the PCR assay described above. Table 1 lists the species and strains tested.

Detection limit of the PCR

Freeze dried *M genitalium* strain TW48-5G was suspended in SP4 medium and the nucleic acid was extracted by the alkaline lysis method,[24] purified using an equal volume of phenol/chloroform/isoamyl alcohol (pH 8.0; Sigma Chemical Co, Poole Dorset, UK) (twice), and precipitated in an equal volume of ice cold isopropanol and 1/10 volume ammonium acetate (10M). Nucleic acid was pelleted at 15 000 ×*g* for 15 minutes, washed twice in 70% ethanol and once in 100% ethanol, before drying under vacuum and dissolving in 100 µl pure water (AnalaR; BDH). The quantity of DNA present was determined using a GeneQuant spectrophotometer (Pharmacia LKB Biochrom Ltd, Cambridge, UK).

Ten and twofold dilution series were made of the DNA, and used to determine the detection limit of the PCR.

Usefulness of the test with clinical specimens

First catch urine specimens were taken from consecutive male patients with urethritis attending the Bristol Royal Infirmary genitourinary medicine clinic. Patients were diagnosed with NGU if there were more than five polymorphonuclear leucocytes (PMNLs) in each high power (×1000) microscope field (HPF) in five or more fields of a Gram stained urethral smear, or > 10 PMNLs/HPF in five or more fields of a Gram stained thread from 15–20 ml of a first passed urine specimen and *Neisseria gonorrhoeae* was not cultured from a urethral swab. Seven of 54 men had a history of urethritis in the previous three months, suggesting that they were suffering from chronic or recurrent urethritis.

Before storage at –20°C, the urine specimens were tested for *Chlamydia trachomatis* by standard laboratory methods: positive results with IDEIA, an amplified enzyme immunoassay (Novo Nordisk Diagnostics, Cambridge, UK), were confirmed using direct fluorescent antibody (MicroTrak; Syva, Palo Alto, California, USA).

The research ethics committee of the United Bristol Healthcare NHS Trust granted permission to test urine specimens from men with urethritis for *M genitalium*.

Urine specimens were brought to room temperature and mixed thoroughly. One millilitre of urine was centrifuged at 15 000 ×*g* for five minutes. The pellet was washed twice in phosphate buffered saline, resuspended in 20 µl 10% Chelex 100® resin (BioRad Laboratories, Hemel Hempstead, Hertfordshire, UK) in deionised water and held at 100°C for 10 minutes. The slurry was pulse centrifuged to pellet the resin, and 5 µl of the supernatant used in the PCR.[25]

Both the 16S rRNA gene PCR and an adhesin gene based PCR[11] were performed on each specimen. The adhesin gene based assay was performed using reagent concentrations and cycling times optimised for assay components and cyclers used locally (those used for the 16S PCR). The concentration of $MgCl_2$ was 4.5mM (as published) and the concentration of each primer was reduced from 200nM to 20nM. The concentration of the dNTPs was reduced from 125µM to 20µM. The annealing temperature for the primers was 60°C and 40 cycles of PCR were performed (as recommended by JS Jensen, personal communication, 1996). Primer sequences were as follows:

- MgPa1: 5′-AGT TGA TGA AAC CTT AAC CCC TTG G-3′.[11]
- MgPa3: 5′-CCG TTG AGG GGT TTT CCA TTT TTG C-3′.[11]

The modified hot start was not used. Sensitivity of the PCR was confirmed to be comparable to that obtained in the author's laboratory by use of positive control DNA kindly donated by JS Jensen (Statens Seruminstitut, Copenhagen, Denmark).

Specimens positive by either or both the adhesin gene and 16S based tests were retested by a heminested adhesin gene PCR.[5 16] Again, the assay was adapted to local practice: primer concentrations were reduced from 1 µg/reaction to 20nM and dNTP concentrations from 200µM to 20µM in a reaction volume of 50 µl. Thirty five cycles were performed at each stage of the PCR, with hold times as in the 16S PCR. Primer sequences were as follows:

- MgP1: 5′-GGT TAA CTT ACC TAG TGG CTT TGA TC-3′.[5]
- Mg2: 5′-CTG CTT TGG TCA AGA CAT CA-3′.[16]

Table 2 Comparison of *Mycoplasma genitalium* detection in men with urethritis by two different PCR assays

	16S based PCR	
	Positive	Negative
MgPa based PCR (non-nested)		
Positive	8	0
Negative	1	55

Table 3 *Mycoplasma genitalium* and *Chlamydia trachomatis* detection in men with urethritis

	M genitalium (16S based PCR)	
	Positive	Negative
C trachomatis (EIA)		
Positive	2	6
Negative	7	49

EIA, enzyme immunoassay; PCR, polymerase chain reaction.

- Mg3: 5′-GTA ATT AGT TAC TCA GTA GA-3′.[16]

The assay retained a very low minimum level of detection.

RESULTS

Assay validation

DNA preparations from all nine strains of *M genitalium* and none of the other organisms were positive by PCR, producing the expected 341 bp product. Preparations from all other organisms tested gave no observable PCR products.

The nucleic acid obtained from *M genitalium* strain TW48-5G was estimated by the GeneQuant instrument as 66% pure. The A_{260}/A_{280} ratio was 1.190, and the concentration of DNA was calculated as 72 µg/ml. Each genome copy is 580 kbp or 0.476 fg. Using these figures the sensitivity of the PCR is 24 fg *M genitalium* DNA, equivalent to about 50 genome copies. This was similar to the sensitivity of 7 fg achieved using both the comparator and confirmatory adhesin gene based tests when challenged with a dilution series of the same DNA extract.

Usefulness of the test with clinical specimens

Tables 2 and 3 show the results of these investigations. Nine of 54 specimens (16.7%) gave a positive result with the 16S rDNA based PCR for *M genitalium* when products were examined by gel electrophoresis. Eight of these men were also positive by the MgPa gene PCR method of Jensen *et al*,[11] and all were confirmed using the MgPa gene heminested PCR.[16] Eight men were positive for *C trachomatis*. Two of the *M genitalium* positive men were also infected with *C trachomatis*. One patient had Gram negative diplococci (presumptively *N gonorrhoeae*) on a urethral smear but was not positive for *M genitalium* or *C trachomatis*.

DISCUSSION

The *M genitalium* 16S based PCR was tested against a range of target and non-target microorganisms. A product of the correct size was obtained from all *M genitalium* isolates tested and from no other mollicute. The modified hot start procedure was necessary to prevent amplification of DNA from a bacillus sp isolate. No amplification was seen with the other organisms tested, either with or without the hot start.

> "This assay is useful as a confirmatory assay or as an alternative to MgPa based assays, and may be less susceptible to intraspecies genetic polymorphism"

Take home messages

- We have developed and optimised a novel PCR targeting the *Mycoplasma genitalium* 16S rRNA gene
- This assay provides a sensitive and specific alternative or addition to the available MgPa gene targeting assays

In the study of urine specimens, 16S rDNA based PCR performed slightly better than did the non-nested adhesin gene based PCR. This could result from heterogeneity in the adhesin gene, although no such heterogeneity has been reported in this region. The proportion of men identified as *M genitalium* positive is low compared with that found in many of the previous PCR based studies of comparable subjects; the range for men with urethritis being 10–50%.[8] The specimens had been stored at −20°C, and both the storage conditions and the freeze thaw cycle may have caused degradation of *M genitalium* DNA. This is unlikely to have affected the comparative sensitivities of the assays and the prevalence is compatible with a later, unpublished, survey of unfrozen samples from men in the Bristol area. The low prevalence may be accounted for by the small size of the study, but is also compatible with variations of *M genitalium* prevalence in men with NGU between populations or over time caused by geographical variation, ethnic composition, local sexual practices, the prevalence of virulent strains, or other, unknown, factors. Such factors may also account for the low prevalence of *C trachomatis* compared with other studies of comparable subjects in Bristol. It is possible that this is because we used IDEIA, which has a reported sensitivity of 80% in a similar population.[26] However, this loss of sensitivity is unlikely to account for the large discrepancy between the observed and expected *C trachomatis* prevalence in the group studied. It has been proposed that the prevalence of chlamydial infection in men may be lower in those without symptoms or signs[9 27]; we did not collect this information and it is possible that the inclusion of men with NGU but no signs or symptoms is the cause of the unexpectedly low rates of both *M genitalium* and *C trachomatis*.

In conclusion, on a small sample of patients, the novel PCR assay appears to be at least as sensitive as a previously published non-nested assay, detects DNA from a range of *M genitalium* isolates, and shows no crossreactivity with the common microorganisms tested. It is useful as a confirmatory assay or as an alternative to MgPa based assays, and may be less susceptible to intraspecies genetic polymorphism.

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