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Development of a rotor-gene real-time PCR assay for the detection and quantification of *Mycoplasma genitalium*

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ABSTRACT

We developed and validated a real-time quantitative polymerase chain reaction (qPCR) assay to determine *Mycoplasma genitalium* bacterial load in endocervical swabs, based on amplification of the *pdhD* gene which encodes dihydrolipoamide dehydrogenase, using the Rotor-Gene platform. We first determined the qPCR assay sensitivity, limit of detection, reproducibility and specificity, and then determined the ability of the qPCR assay to quantify *M. genitalium* in stored endocervical specimens collected from Zimbabwean women participating in clinical research undertaken between 1999 and 2007. The qPCR assay had a detection limit of 300 genome copies/mL and demonstrated low intra- and inter-assay variability. The assay was specific for *M. genitalium* DNA and did not amplify the DNA from other mycoplasma and ureaplasma species. We quantified *M. genitalium* in 119 of 1600 endocervical swabs that tested positive for *M. genitalium* using the commercial Sacace *M. genitalium* real-time PCR, as well as 156 randomly selected swabs that were negative for *M. genitalium* by the same assay. The *M. genitalium* loads ranged between <300 and 3,240,000 copies/mL. Overall, the qPCR assay demonstrated good range of detection, reproducibility and specificity and can be used for both qualitative and quantitative analyses of *M. genitalium* in endocervical specimens and potentially other genital specimens.

1. Introduction

Mycoplasma genitalium, an obligate intracellular pathogen, is the smallest prokaryote capable of self-replication (Fraser et al., 1995). It has a genome consisting of 580 kb, with only 482 protein-coding genes (Fraser et al., 1995; Glass et al., 2006). *M. genitalium* was first isolated by culture in urethral specimens from two men with nongonococcal urethritis (NGU) in 1981 (Tully et al., 1981). Since then, it has proven very difficult to obtain urogenital *M. genitalium* isolates by conventional culture techniques due to the fastidious nature of this bacterium. It was not until the introduction of polymerase chain reaction (PCR)-based *M. genitalium* detection assays in the early 1990s that the role of *M. genitalium* as an aetiological agent of NGU and other reproductive tract syndromes has been rigorously explored (Jensen et al., 1991; Palmer et al., 1991). Global data suggest that *M. genitalium* is present in 21% of men with NGU and in 6% of asymptomatic men (Le Roux and Hoosen, 2010). In women, *M. genitalium* has been associated with cervicitis, endometritis, pelvic inflammatory disease and tubal factor infertility (Clausen et al., 2001; Cohen et al., 2002; Manhart et al., 2003; Simms et al., 2003). A recent review of 48 published reports among more than 27,000 women in high- and low-risk populations estimated an overall *M. genitalium* prevalence of 7.3% and 2.0%, respectively (McGowin and Anderson-Smits, 2011).

Quantitative PCR (qPCR) is a useful tool for determining *M. genitalium* bacterial loads in a range of samples, including vaginal and cervical swabs, urethral swabs and first-void urine (Blaylock et al., 2004; Deguchi et al., 2002; Dupin et al., 2003; Jensen et al., 2004, 2003; Svenstrup et al., 2005; Yoshida et al., 2002). Quantitative assays are an important tool, as they have allowed researchers and clinicians to link quantitative results with clinical and sub-clinical presentation, to monitor longitudinal changes in *M. genitalium* bacterial loads in samples from patients with chronic NGU or other syndromes, to examine the effectiveness of treatment, and to study the effect of

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storage and freeze–thaw cycles of archived patient samples and/or extracted DNA (Deguchi et al., 2002; Yoshida et al., 2002).

The MgPa (encoding the major surface protein MgPa) and 16S rRNA encoding-genes of *M. genitalium* are most commonly used as targets in *M. genitalium* qPCR. Alternative gene targets are required due to the variability which has been detected in the MgPa encoding-gene, and due to lack of specificity with the 16S rRNA primers (Hardick et al., 2006; Jensen, 2006; Jensen et al., 2004; Ueno et al., 2008; Yoshida et al., 2002). For this study, we have developed a quantitative real-time Rotor-Gene PCR (qPCR) assay targeting the *pdhD* gene of *M. genitalium*, which encodes dihydrolipoamide dehydrogenase. The authors have used the *pdhD* target gene for the qualitative real-time detection of *M. genitalium* for many years at the Centre for HIV and STIs and due to the shortcomings of the target genes described in the literature we believed this gene to be a suitable candidate for quantitative PCR. We determined the performance characteristics of our assay and then used it to quantify *M. genitalium* in endocervical swabs.

2. Materials and methods

2.1. Development and validation of the quantitative PCR assay

Mycoplasma genitalium G37 genomic DNA (ATCC 33530D) was used as a quantification standard in optimizing and validating our qPCR. The amount of genomic DNA in this preparation was confirmed by measuring the optical density at 260 nm using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, USA). The concentration in genome copies/reaction was then calculated. Briefly, we identified the genome size of *M. genitalium* as determined by Celera Genomics (580 076 bp) and we used this number to identify the mass of DNA per *M. genitalium* genome. We then calculated the mass of genomic DNA (gDNA) containing the copy numbers of *M. genitalium*, ranging between 10 and 1,000,000 copies and then the concentrations of gDNA needed to achieve the required copies of *M. genitalium*. Finally, we prepared serial dilutions of the *M. genitalium* gDNA using a stock concentration of *M. genitalium* gDNA with known concentration (ATCC 33530D; 1 ng/μL) to a final volume of 200 μL. A standard 10-fold dilution series corresponding to 10^6, 10^5, 10^4, 10^3, 10^2, 10 and 1 genome copies/reaction (5 replicates of each dilution/run) of *M. genitalium* G37 genomic DNA were prepared and used as standards in each Rotor-Gene PCR run. The primers and probe targeting the *pdhD* gene were developed by researchers at the Centers for Disease Control and Prevention, Atlanta, USA and the technology was subsequently transferred to the Centre for HIV and STIs in Johannesburg. The following primers and probe were used to amplify an 82 bp fragment of the *pdhD* gene of *M. genitalium* (nucleotides [nt] corresponding to the sequence with Genbank number L43967.2): forward primer, MG-041 (5′-CGG ATC AAG ACC AAG ATA CTT AAC TTT-3′; nt 329417-329391), reverse primer MG-042 (5′-AGC TTG GGT TGA GTC AAT GAT AAA C-3′; nt 329336-329360) and a JOE-labelled probe, MG-048 (5′-[AminoC6 + Joe] CCA GGG TTT GAA AAA GCA CAA CAA GCT G [BHQ1a]-3′; nt 329389-329362). The 4,5-dichloro-dimethoxy-fluorescein (JOE) is a dichlorinated, dimethoxylated version of the fluorescent dye fluorescein. The primers were synthesized by the University of Cape Town, South Africa and the probe by Eurofins MWG Operon, Germany.

The qPCR assay was performed on the Rotor-Gene 6000 real-time PCR platform (Corbett Research, Mortlake, Australia). Each 50 μL reaction volume contained 25 μL of DNA template, 5 units of Amplitaq Gold Taq polymerase (Applied Biosystems, Foster City, USA), 1x PCR buffer without $MgCl_2$ (Applied Biosystems, Foster City, USA), 4 mM of $MgCl_2$ (Applied Biosystems, Foster City, USA), 400 μM of dNTP (Bioline, London, United Kingdom) and 300 nM of each primer and probe. The cycling conditions were as follows: an initial incubation step at 50 °C for 2 min and Taq polymerase activation at 95 °C for 10 min followed by 40 cycles of denaturation at 95 °C for 20 s and annealing/extension at 60 °C for 60 s. Fluorescence was acquired on the JOE channel after each annealing/extension step.

A standard curve was constructed by plotting the log of the starting quantity of template against the cycle threshold (Ct)-value obtained during amplification of each dilution. The equation of the linear regression line, along with Pearson's correlation coefficient (R-value) and the coefficient of determination (R^2-value), was used to evaluate optimisation of the qPCR assay. The R- or R^2-value of the standard curve signified how well the experimental data fit the regression line, while linearity gave an indication of the variability across assay replicates and whether the amplification efficiency was the same for different starting template copy numbers. In order to construct this standard curve, we amplified each standard dilution 5 times in one run. The Ct was determined automatically by the instrument software in order to calculate the Ct-value for each of the dilutions. The Ct-value indicated the cycle at which the amplification reached a critical fluorescence level and could be related directly to the starting copy number of the sample by means of this standard curve. The specimens, a negative control as well as a standard dilution of known quantity, were run simultaneously. The Rotor-Gene instrument software allows for the importation of the optimised standard curve in consecutive runs which eliminates the need to re-run a new standard curve for each new experiment/run.

2.2. Determination of qPCR assay reproducibility

We determined the repeatability and reproducibility of the assay by measuring the intra- and inter-assay variabilities, respectively. For intra-assay variability we tested 4 standard series (5 replicates of each 10-fold serial dilution) in one run. We evaluated 3 standard curves on 3 different days to determine inter-assay variability. The coefficient of variation (CV) was determined for crossing points and concentration using the formula $CV = \sigma/\mu \times 100$, where CV is defined as the ratio of the standard deviation (σ) to the mean (μ) and expressed as a percentage. In order to evaluate the effect of differences in clinical sample matrices and determine reproducibility within clinical specimens, 8 randomly chosen endocervical swabs that tested negative for *M. genitalium* with both the Sacace screening assay and the qPCR assay were spiked with 10^3 copies/μL of *M. genitalium* standard DNA and tested using the qPCR assay.

2.3. Determination of qPCR assay specificity

The primer and probe specificities were determined by performing a BLAST search (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi), using the *M. genitalium* qPCR primer and probe sequences. The BLAST search tool identified all target sequences that were similar to those of the *pdhD* gene in *M. genitalium*. As primers could amplify pseudo genes or prime unintended regions, and to confirm the assay specificity for *M. genitalium* DNA, we tested genomic DNA preparations of 14 species of mycoplasmas and ureaplasmas with the qPCR assay. Genomic DNA preparations were obtained from the Statens Serum Institut, Copenhagen, Denmark, and included *M. genitalium*, *M. salivarium*, *M. buccale*, *M. faucium*, *M. penetrans*, *M. pneumoniae*, *M. fermentans*, *M. hominis*, *M. lipophilum*, *M. pirum*, *M. orale*, *M. primatum*, *Ureaplasma urealyticum* serotype 6 and *U. urealyticum* serotype 8. Genomic DNA from *M. genitalium* G37 (ATCC 33530D), at a defined concentration of 1 ng/μL, was obtained from the American Type Culture Collection (ATCC), Manassas, USA.

2.4. Testing endocervical swab-derived DNA for M. genitalium bacterial load

A total of 1600 freezer-stored endocervical swabs, collected from women enrolled in a clinical trial in Zimbabwe between 1999 and