Mycoplasma genitalium infection is associated with microscopic signs of cervical inflammation in liquid cytology specimens

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Running Title: M. genitalium elicits cervical inflammation

Conflict of Interest Statement
None of the authors have any commercial interests, financial holdings, professional affiliations, advisory board positions, board memberships, patent holdings or any other associations that pose a conflict of interest related to the subject matter presented in the manuscript.

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ABSTRACT

Cervicitis is a common clinical finding often attributed to sexually transmitted infections (STIs), but no etiologic agent is identified in the majority of cases. In this study, we comparatively assessed inflammation among the common infectious etiologies of cervicitis and assessed the potential value of liquid cytology specimens for predicting STIs. Among 473 Louisiana women at low risk for acquiring STIs, the prevalence of *M. genitalium*, *C. trachomatis*, *N. gonorrhoeae*, and *T. vaginalis* in liquid-based cytology specimens was 1.5, 2.1, 0.6, and 4.4%, respectively. *N. gonorrhoeae* and HPV18 infections were significantly more common among subjects with *M. genitalium*. Using direct microscopy, we observed significant increases in leukocyte infiltrates among subjects with mono-infections of *M. genitalium* or *C. trachomatis* compared to women with no detectable STIs. Inflammation was highest among subjects with *M. genitalium*. Using a threshold of ≥2 leukocytes per epithelial cell per high-powered field, the positive predictive value for *M. genitalium*, *C. trachomatis*, *N. gonorrhoeae* or *T. vaginalis* was 100, 70, 67 and 20%, respectively. Several novel *M. genitalium* genotypes were identified, all of which were predicted to be susceptible to macrolide antibiotics, suggesting that different strains may circulate among low-risk women and macrolide resistance is substantially lower than in high-risk populations. This study highlights the capacity of *M. genitalium* to elicit cervical inflammation and, considering the strong epidemiologic associations between *M. genitalium* and Human Immunodeficiency Virus (HIV), provides a potential mechanism for acquisition and shedding of HIV via chronic leukocyte recruitment to the cervical mucosa.

KEYWORDS

*Mycoplasma genitalium*; *Mycoplasma*; ThinPrep; PreservCyt; diagnostics; NAAT; nucleic acid amplification test; cervicitis; inflammation; sexually transmitted infection; STI; liquid-based cytology; Louisiana; Papanicolaou; Pap smear; Chlamydia trachomatis; Chlamydia; Neisseria gonorrhoeae; *Trichomonas vaginalis*; Human Papilloma Virus; HPV
INTRODUCTION

Inflammation of the uterine cervix, termed cervicitis, is characterized clinically by the presence of either mucopurulent discharge from the cervical os and/or easily-induced bleeding after endocervical sampling (cervical friability). Cervicitis appears to be a very common clinical finding [1], and has in fact been coined “the ignored counterpart in women of urethritis in men” [2]. Classically, cervicitis has been managed “syndromically” by treating the most likely etiologic agents prior to an accurate diagnosis.

This is a common practice in STD clinics to manage lower urogenital inflammation in men and women because it is imperative to initiate therapy before STI test results are available. CDC guidelines recommend such empiric therapy of cervicitis in patients suspected to have *C. trachomatis* or *N. gonorrhoeae* – particularly in populations with high STI prevalence [3]. Although no etiologic agent is identified in the majority of cases, the most common infectious causes of cervicitis are *Chlamydia trachomatis* and *Neisseria gonorrhoeae* [2, 4] followed less commonly by *Mycoplasma genitalium*, *Trichomonas vaginalis*, and Herpes Simplex Virus types I and II [5].

A diagnosis of cervicitis currently relies upon direct observation of the aforementioned clinical signs during pelvic exam. Unfortunately, algorithms for syndromic management of STIs have proven to be largely ineffective when using vaginal signs to predict cervical infection, which can be detected by less invasive methods [5]. Some specialists continue to utilize microscopic signs of cervicitis (elevated number of polymorphonuclear leukocytes per high-powered microscope field; PMNL/HPF) observed on endocervical gram stains as presumptive evidence for treatment of STIs despite conflicting data on usefulness and being removed from the CDC treatment guidelines in 1993 [6]. Over time, the threshold of inflammation to define a positive test has varied among studies from 10 to 30 PMNL/HPF, and to date no consensus or adequately justified definition of microscopically-defined cervicitis exists [6]. In addition, the positive predictive value (PPV) of inflammation on endocervical smears for identifying *C. trachomatis* or *N. gonorrhoeae* is generally less than 50% [7, 8] and declines with age [9], even in populations with high STI prevalence. Data regarding the use of microscopic signs for predicting *M.*
genitalium is comparatively sparse, but a 2013 study showed that endocervical gram stains have similarly poor utility [10]. Collectively, endocervical gram stains appear to have limited usefulness as a point of care (POC) procedure for predicting the most commonly identified etiologies of cervicitis. With the assumption that cervicitis is an important pathological condition, either as an independent syndrome or an identifiable risk factor for upper tract disease, a clear need exists to enhance the POC management of STIs in this context.

M. genitalium is a prevalent and emerging STI linked epidemiologically to pelvic inflammatory disease, tubal-factor infertility and cervicitis (Reviewed in [11, 12]). Despite solid evidence as a cause of male non-gonococcal urethritis (NGU), additional studies are needed to unequivocally implicate M. genitalium as a cause of cervicitis and other female reproductive tract syndromes. M. genitalium has a remarkable ability to establish chronic infections of the lower genital tract [13-16] in lieu of strong antibody responses to at least two outer membrane antigens [17-27]. The data regarding M. genitalium as a cause of cervicitis have been conflicting with approximately half of published studies showing significant associations [28]. Comparative assessments of these studies indicate that M. genitalium is more commonly associated with cervicitis when microscopic criteria are considered independent of non-microscopic criteria [11, 28]. In contrast to C. trachomatis and N. gonorrhoeae, this suggests that microscopic signs may indeed be useful for predicting M. genitalium infection and further investigation is warranted.

Very little evidence has been put forth to compare the inflammatory capacity of M. genitalium to that of other STIs, and therefore its true role as a pathogen has yet to be established. Our primary goal in this study was to comparatively analyze the intensity of inflammation among the common infectious etiologies of cervicitis. Secondarily, we investigated the utility of liquid cytology specimens, routinely used for cervical cancer screening and HPV testing, for predicting M. genitalium infection. Liquid cytology specimens have been FDA-approved for the PCR-based diagnosis of HPV, C. trachomatis, N. gonorrhoeae and T. vaginalis, and are ideal to screen older and/or low-risk populations since they are an
integral component of cervical cancer management algorithms [29]. This specimen type also uniquely facilitates evaluation of cells present in the cervix, and is amendable to POC staining procedures to potentially enhance syndromic management of cervicitis.

### MATERIALS AND METHODS

#### Patient Population and Specimen Collection

A retrospective case control study was performed in accordance with an approved LSU Health Sciences Center Institutional Review Board (IRB) protocol. De-identified ThinPrep PreservCyt specimens (n=482) were received from the Interim LSU Public Hospital’s Molecular Pathology Laboratory (New Orleans, LA) from November 2012 to January 2013 for nucleic acid amplification (NAAT) of STIs. HPV infection status was available for 347 specimens as determined by cobas HPV Test (Roche Diagnostics, Inc, Indianapolis, IN). HIV infection status was unknown. The median age of the subjects was 42 years (range 20-70; mean = 42 years) and was determined using clinical laboratory testing records after de-identification of specimens. Therefore, we were unable to correlate subject age with STI testing results. Any specimen without visible turbidity, a general indicator of specimen collection and cellularity, was excluded from the study (n=9) in order to reduce the likelihood of false-negative results due to insufficient cellular material.

#### STI Screening and Quantification of *M. genitalium*

All STI testing was performed using DNA extracts from ThinPrep PreservCyt (Hologic, Inc., Bedford, MA) specimens as template. The specimens were obtained after Pap cytology and HPV testing with volumes ranging from 4-16 mL, and stored at room temperature for up to 3 months prior to DNA extraction and STI testing. After swirling, 400 uL was removed, centrifuged at 4,000 x g for 5 minutes, and the resultant pellet was used as input for DNA purification using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Inc., Valencia, CA). Purified DNA was eluted into 200 uL of the recommended elution buffer and stored at -20 °C until PCR was performed.
To detect and enumerate *M. genitalium* organisms, we employed a real-time qPCR assay targeting a 92 bp region of the MG190 (*mgpA*) gene [30] with the following reaction conditions: 12.5 µL iQ Supermix (Bio-Rad Laboratories, Inc., Hercules, CA), 1.0 µL forward primer (190F; 5 µM stock), 1.0 µL reverse primer (190R3; 5 µM stock), 1.0 µL of the MG190 TaqMan probe (MG190P; 5 µM stock), and 5 µL of DNA template. Real-time PCR was performed using the CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Inc.) with two-step cycling parameters as described previously [30]. Samples with a positive result using the MG190 TaqMan PCR assay were confirmed using a PCR assay targeting a conserved region of *mgpB* using primers 1F and 1R [16] with SYBR Green detection chemistry. Melt curve analysis was used to confirm amplicon specificity.

*C. trachomatis* testing was performed using a real-time qPCR assay targeting the cryptic plasmid as described previously [31]. *N. gonorrhoeae* testing was performed using a SYBR Green qPCR assay targeting a 102 bp region of the *porA* pseudogene [32]. The PCR setup was as follows: 12.5 µL iQ SYBR Green Supermix (Bio-Rad Laboratories, Inc.), 1.0 µL forward primer (5 µM stock), 1.0 µL reverse primer (5 µM stock), and 5 µL of DNA template. The cycling parameters consisted of an initial denaturation at 95 deg for 5 min, followed by 50 cycles of 95 deg for 1 sec, 60 deg for 20 sec. Melt curve analysis was used to confirm amplicon specificity. *T. vaginalis* testing was done using a laboratory-developed quantitative real-time PCR assay targeting the single-copy glycine hydroxymethyltransferase gene (TVAG_109540). The PCR setup volumes were identical as outlined for the *N. gonorrhoeae* using forward primer 5'-CCATCAAGAGCATGCTAGCTGC-3', reverse primer 5'-GTTCATCAACGTATTTGGTGCCTCCA-3' (5 µM stock), and TaqMan probe 5'–AGTATGCGGAAGGATATCCAGGTGCTCGC-3’. Cycling parameters consisted of an initial denaturation at 95 deg for 5 min, followed by 35 cycles of 95 deg for 10 sec, 53 deg for 20 sec, and 72 deg for 30 sec.
Cervical Leukocyte Quantification

Approximately 100 uL of each ThinPrep PreservCyt specimen was placed onto a glass microscope slide, air-dried for 15 min at room temperature, and then stained with Diff-Quik reagents per the manufacturer’s instructions (Siemens Healthcare Diagnostics, Ltd., Deerfield, IL). Leukocytes and epithelial cells were quantified in parallel from 5 representative high-powered fields by an ASCP-certified cytologist; cell counts were entered into a customized requisition form. First, all subjects mono-positive for M. genitalium (n=5) and a randomly selected subset of subjects negative for all tested STIs (n=40) were stained for evaluation of M. genitalium-specific modulation of cervical leukocyte counts. Next, all specimens testing positive for mono-infections of C. trachomatis (n=9), N. gonorrhoeae (n=2), and T. vaginalis (n=18) were processed for comparison to M. genitalium-positive subjects and those testing negative for all STIs. The prevalence of each STI, stratified by variable thresholds of leukocyte/epithelial cell ratios (≥2 or ≥2.5), was used to calculate PPVs (Table 2). In order to eliminate experimental bias, the cytologist was masked to the NAAT results throughout the study.

M. genitalium Genotyping

DNA specimens from subjects with positive results from both the MG190 TaqMan and MG191 SYBR PCR assays were used as templates for the MG191 (mgpB) genotyping assay [15]. Conventional PCR using the MgPa-1 and MgPa-3 primers [33] followed by agarose gel electrophoresis, PCR clean up (QIAquick PCR Purification Kit; Qiagen, Inc.) and bi-directional Sanger sequencing was performed and analyzed using standard methods. Sequences were compared to those from published M. genitalium strains using NCBI’s BLASTn program.

Macrolide Resistance Screening of M. genitalium

Subjects with positive results from the MG190 TaqMan and MG191 SYBR PCR assays were screened with a third PCR targeting the 23S rRNA gene (rrlA). Mutations in rrlA known to confer macrolide resistance were identified using a PCR assay previously developed by Jensen and colleagues [34]. Amplification was detected by agarose gel electrophoresis followed by PCR clean up and Sanger
sequencing as described above. Sequence data was analyzed using MacVector version 12.0.3 (MacVector, Inc.) to identify \textit{M. genitalium} nucleotides corresponding to \textit{M. genitalium} G37 (GenBank Ref. NC_000908) positions 173,798 and 173,799 that indicate macrolide resistance (\textit{E. coli} positions A2058 and A2059 of the rrlA gene).

\textbf{Statistical Analysis}

The ANOVA was used to determine whether STIs were associated significantly with increased ratios of cervical leukocytes to epithelial cells whereby significant differences were noted when p<0.05. Compiled contingency tables and calculated odd ratios (OR) were used for univariate analysis of the associations between \textit{M. genitalium} infection and other STIs. The Fisher’s Exact test was used to determine whether significant associations exist, and P values are reported in Table 1. Similarly, contingency tables were assembled to determine the PPV of variable leukocyte/epithelial cell ratios among STIs, followed by the Fisher’s exact test (Table 2). PPV was calculated using Prism (Version 6; GraphPad Software, Inc., La Jolla, CA).

\textbf{RESULTS}

\textbf{Performance of the \textit{M. genitalium} diagnostic PCR system}

The MG190 TaqMan PCR assay was described previously [30]. In the current study, we evaluated performance of the MG191 assay with liquid cytology specimens. Reproducibility of \textit{M. genitalium} detection was determined by measuring inter-assay precision among 10 independent PCR runs. Liquid cytology specimens spiked with serial dilutions of \textit{M. genitalium} G37 organisms showed a linear range of detection from \(1 \times 10^4\) to \(3 \times 10^7\) organisms per mL with \% coefficient of variations ranging from 1.3 to 2.7 (Fig. 1A). The reproducible limit of detection (LOD), was \(3 \times 10^4\) organisms per mL, which was detected in 100\% of reactions. Concentrations of \(1 \times 10^6\) organisms per mL were detected in 60\% of specimens (Fig. 1A). Spiking \textit{M. genitalium} organisms into liquid cytology specimens from 5
different subjects showed similar reproducibility of the combined extraction and PCR system with coefficient of variation values between 3.1 and 5.3% (Fig 1B).

**STI Prevalence and co-infections in low-risk Louisiana women**

Among 473 subjects, 35 were positive by the MG190 TaqMan PCR assay of which seven were confirmed positive using the MG191 SYBR assay (1.5%; 7/473). The titers from confirmed *M. genitalium*-positive specimens ranged from $2 \times 10^2$ to $6 \times 10^4$ genomes per mL of ThinPrep PreservCyt fluid (mean ± SEM, $1.8 \times 10^4 \pm 1.0 \times 10^4$). The prevalence of *C. trachomatis, N. gonorrhoeae*, and *T. vaginalis* was 2.1 (10/473), 0.6 (3/473) and 4.4% (21/473), respectively. The titers of *T. vaginalis* ranged from $4 \times 10^5$ to $1 \times 10^7$ genomes per mL (mean ± SEM, $3.1 \times 10^6 \pm 8.4 \times 10^5$). *N. gonorrhoeae* infection was significantly more common among subjects with *M. genitalium*, but this was not the case for *T. vaginalis* or *C. trachomatis* (Table 1). No co-infections with *C. trachomatis* were identified.

Human papillomavirus (HPV) results were available for a subset of subjects in this study (n=347). The prevalence of high-risk HPV infection was determined to be 32% (111/347) with HPV16 and HPV18 present at 5.2% (18/347) and 3.5% (12/347), respectively. The combined prevalence of 12 other high-risk HPV types (31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68) was 28.8% (100/347). No associations were observed between *M. genitalium* and high-risk HPV infection (OR 3.2, 95% CI 0.5-19.7), *M. genitalium* and HPV16 (OR 1.6, 95% CI 0.1-30.0) or *M. genitalium* with a combination result of 12 high-risk HPV types other than types 16 and 18 (OR 1.7, 95% CI 0.3-10.0). However, HPV18 detection was associated significantly (p = 0.01, Fisher’s exact test) with *M. genitalium* infection (OR 22.1, 95% CI 3.3-147.5). With exception to HPV infections, all subjects with co-infections were excluded from these calculations for purposes of enumerating pathogen-specific modulation of cervical leukocytes.
M. genitalium and C. trachomatis infections were associated with microscopic signs of cervical inflammation

We evaluated whether M. genitalium and other STIs are associated with cervical inflammation using a ratio of leukocytes to epithelial cells quantified from liquid cytology specimens. A representative stained preparation is shown in Figure 2A. Subjects with mono-infections of M. genitalium (3.4 ± 0.7 leukocytes/epithelial cell/HPF; n=5) and C. trachomatis (3.0 ± 0.7; n=9) had significantly higher (p<0.05) mean leukocyte/epithelial cell ratios compared to a representative subset of women without STIs (0.8 ± 0.15; n=40) (Fig. 2B). The largest ratio of leukocytes to epithelial cells was observed in subjects with M. genitalium infection. Subjects with N. gonorrhoeae (2.2 ± 0.3; n=2) or T. vaginalis (1.4 ± 0.5; n=18) infections also showed increased leukocyte/epithelial cell ratios, but these increases were not significantly different than subjects with no detectable STIs (p>0.05, ANOVA).

The PPV of measuring leukocyte/epithelial cell ratios from stained smears of liquid cytology specimens ranged from 20 to 100% among STIs using a threshold of positivity of >2.0 leukocytes/epithelial cell/HPF (Table 2). As expected, the PPV decreased if the threshold was raised to the more stringent cutoff of >2.5 leukocytes/epithelial cell/HPF. Using either threshold of positivity, the prevalence of M. genitalium or C. trachomatis infection was significantly higher in subjects with inflammatory signs compared to those without signs. Similar trends were not observed for subjects with N. gonorrhoeae or T. vaginalis infections (Table 2).

Macrolide resistance-mediating mutations and novel M. genitalium genotypes

Several recent studies have highlighted that AZM resistant M. genitalium strains are emerging worldwide [35-42], two of which provide molecular evidence that resistance can be induced with 1 gram STAT dosing [38, 42]. Using conventional PCR and Sanger sequencing, analysis of the M. genitalium-positive subjects in this study showed that none of their infecting strains possessed mutations that confer macrolide resistance (data not shown). Using a previously validated genotyping system that targets a relatively conserved region of the MG191 gene (mgpB) [15], we observed only a single infecting strain
to be 100% identical to a previously defined strain (M6283; Miyazaki, Japan). The remaining six genotypes had unique MG191 sequences differing from all previously published sequences in the NCBI database, as well as among each other (Fig. 3). These nucleotide sequences have been submitted to NCBI with accession numbers KF995739-KF995744.

**DISCUSSION**

In this study, approximately 1 in 65 women presenting to women’s clinics in Louisiana for cervical cancer screening tested positive for *M. genitalium* infection (1.5%). As expected, this prevalence was in between that of *N. gonorrhoeae* (0.6%) and *C. trachomatis* (2.1%), and substantially lower than previous observations of high-risk subjects that include STD clinic attendees, subjects attending family planning clinics for termination of pregnancy, or commercial sex workers [11]. On average, urogenital *M. genitalium* infection occur in approximately 2% of low-risk women recruited from fertility clinics, otherwise healthy subjects enrolled in population-based studies, or subjects enrolled in studies of adverse pregnancy outcomes [11]. In a study by Manhart and colleagues, 1.3% of otherwise healthy young adults aged 18-27 in the southern region of the USA tested positive for *M. genitalium* infection [43]. Our observed prevalence of 1.5% from Louisiana women’s clinics was on par with these studies, however, women enrolled in our study were older (median age = 42) and samples were obtained during routine cervical cancer screening visits to either rural or urban women’s clinics. The finding that *M. genitalium* is more common than *N. gonorrhoeae* and similar to *C. trachomatis* in this population highlights the potential need to screen older populations such as the case for *T. vaginalis*.

The convention of categorically defining cervicitis based on elevated levels of PMNL/HPF on endocervical gram stains has prevented an accurate assessment of inflammation by distilling the findings into a qualitative endpoint of cervicitis/no cervicitis. Perhaps even more troubling with this method is the lack of an adequate control for sampling efficiency. Using liquid cytology specimens in the current study, direct leukocyte quantification as a ratio to epithelial cells accounts for sampling efficiency and
highlights the observation that women with M. genitalium have approximately three times more leukocytes in the cervix compared to women without STIs. The significance of this finding is somewhat unclear since no pathologic threshold of cervical leukocytes has been established. Previous studies have utilized varied microscopic definitions of cervical inflammation [12] and those that employed high thresholds of inflammation (>20 or >30 PMNL/HPF) showed fewer associations between M. genitalium and cervicitis [11]. Combined with results of the 2010 Swedish study where no (0/6) M. genitalium-positive subjects had >30 PMNL/HPF [44], cumulative evidence suggests that M. genitalium infections may be characterized by relatively low intensity inflammation. Therefore, the concept of utilizing a defined microscopic threshold of leukocyte infiltrates without a measure of sampling efficiency is inadequate for detecting inflammation and/or predicting infection. Importantly, increases in cervical leukocytes among M. genitalium-positive women in our study were highest among the STIs and substantiate the role of M. genitalium as an inflammatory pathogen. Using a cutoff ratio of >2, the PPV of the leukocyte/epithelial cell ratio for predicting M. genitalium was very high (100%), but this and the PPVs for other STIs should be interpreted with caution since the STI prevalence was very low. Collectively, despite screening almost 500 low-risk women, expansion of the study to include high-risk women and differing age groups is warranted to better understand the utility of this method for predicting STIs.

Previous studies have shown that vaginal swabs may have a greater relative sensitivity for M. genitalium detection than cervical swabs [45, 46], and a combination of these sites yields a sensitivity of more than 95% [45]. Liquid cytology specimens predominately contain squamous cells of the ectocervix but because the anatomical target of sampling is the transition zone between the ecto- and endocervix, columnar cells of the endocervix are also present. We routinely observed vaginal microflora suggesting that this specimen type may be ideal for M. genitalium detection as it offers a composite sampling of the lower urogenital tract. Regarding analytical sensitivity, the defined lower threshold of reproducible detection was 3x10^4 organisms/mL of ThinPrep PreservCyt fluid and represents a potential limitation to
the current study. We hypothesize that this is due to the relatively small 5 μL template used in the PCR and that additional optimization of the system would likely enhance sensitivity. With this potential limitation, it is possible that subjects with low *M. genitalium* titers were not detected, and therefore the observed microscopic signs of inflammation are representative of subjects with high titers. Additionally, several specimens identified as positive with the MG190 screening test were not confirmed with a second test suggesting that specificity of the MG190 PCR is sub-par and should be enhanced to reduce false-positive results. Additional investigation of this specimen type and detection system is warranted considering the widespread use of liquid cytology specimens for cervical cancer screening and HPV testing in women aged 21-65 [29].

We identified six previously un-discovered genotypes all of which were susceptible to macrolides suggesting that *M. genitalium* strains circulating in low-risk women may differ from those in high-risk populations. This study, to our knowledge, is the first to investigate macrolide susceptibility among low-risk women and suggests that *M. genitalium* strains circulating in this population are more susceptible to these antibiotics compared to higher-risk populations whereby AZM is less effective [12, 47]. AZM treatment failures using the one gram dose have ranged from 15-60% [47-49]. Other studies have highlighted the hazard of stat one gram AZM therapy by describing the molecular mechanisms of induced resistance and subsequent treatment failure [35, 38, 42]. Since AZM is commonly used to treat male NGU and is also included in the current CDC treatment guidelines for gonorrhoeae, it remains imperative to monitor resistance patterns for *M. genitalium* and other bacterial STIs since virtually all men with urethritis receive AZM. In patients with AZM-resistant *M. genitalium* infections, moxifloxacin is the recommended and most commonly used antibiotic [12, 47], but reports of resistance are emerging [50].

This study highlights the importance of *M. genitalium* as an etiology of cervicitis and the potential utility of liquid cytology specimens for diagnosis and measuring inflammation. The major limitation to interpreting the significance of the findings is the low prevalence of *M. genitalium* and
other STIs, and the inability to stratify our results by age. A larger cohort will be required to corroborate
the results in both high and low-risk populations, particularly with regard to the PPV of this method as a
POC procedure. Since the prevalence of STIs would be substantially higher in high-risk populations, our
findings may not be generalizable to these women since co-infections, frequent re-infection, BV, and
other factors may complicate the validity of measuring leukocyte/epithelial cell ratios. Importantly,
cervicitis has been associated with increased HIV shedding, and treatment of the condition reduces both
viral shedding and the presence of virus-infected cells in cervical secretions [51-53]. Having been shown
to enhance susceptibility to HIV infection [54, 55], and with cross-sectional associations with HIV
infection in more than 20 studies [56], continued investigation of *M. genitalium* remains important as
antibiotic resistant strains continue to emerge worldwide.

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**REFERENCES**

as a contributor to the multiple etiologies of cervicitis in women attending sexually transmitted

2. Brunham RC, Paavonen J, Stevens CE, Kiviat N, Kuo CC, Critchlow CW, and Holmes KK.


FIGURE LEGENDS

Figure 1. Reproducibility of the MG190 TaqMan PCR assay. (A) M. genitalium G37 organisms were spiked into ThinPrep PreservCyt specimens at concentrations ranging from 1x10^4-3x10^7 organisms per mL. DNA was purified from each spiked specimen and used as template in ten replicate PCR reactions run on separate plates. (B) In a separate study, after spiking M. genitalium G37 organisms at the indicated concentrations, five independent DNA purifications and PCRs were performed once weekly over a five week period to evaluate inter-assay precision of the assay system. Data are presented as cycle of quantification (Cq) with % coefficient of variation for each group.

Figure 2. Quantification of cervical leukocytes from liquid cytology specimens. ThinPrep PreservCyt specimens from low-risk Louisiana women were screened for M. genitalium using the TaqMan PCR system described herein, and for C. trachomatis, N. gonorrhoeae, and T. vaginalis. All subjects with co-infections among M. genitalium, C. trachomatis, N. gonorrhoeae, and T. vaginalis were excluded from the calculation of leukocyte modulation. Those specimens that were mono-positive for each STI and a randomly selected subset of specimens negative for all tested STIs (n=40) were Diff-
Quik stained followed by quantification of cervical epithelial cells and leukocytes. (A) Representative microscope field from a stained ThinPrep PreservCyt specimen illustrating squamous epithelial cells and cervical leukocytes. Differing ratios of leukocytes to epithelial cells are readily observed using the preparation and staining paradigm. (B) Comparison of cervical leukocyte counts among women with and without STIs presented as the ratio of leukocytes to epithelial cells per high powered field (HPF) compiled from five representative microscope fields. Significant differences between leukocyte/epithelial cell ratios among subjects are denoted with an asterisk (p<0.05, ANOVA).

Figure 3. Nucleotide alignment of *M. genitalium* genotypes. Using a previously described genotyping system [15], we PCR-amplified and sequenced the specific genotyping region of MG191 from all specimens confirmed to be positive for *M. genitalium* infection (n=7). Sequencing reads were trimmed using Sequencher version 4.10.1 and aligned using the Clustal W in MacVector version 12.0.3. Sequences were compared to previously described *M. genitalium* strains using NCBI’s BLASTn program. *M. genitalium* strain G37 was processed in parallel to confirm accuracy of the system, and presented as a reference. Nucleotide reference numbers are relative to G37 (GenBank Ref. NC_000908).
Table 1. Prevalence and univariate associations between *M. genitalium* infection and other STIs

<table>
<thead>
<tr>
<th>STI</th>
<th>NAAT Prevalence (%)</th>
<th>M. genitalium-positive</th>
<th>M. genitalium-negative</th>
<th>Odds Ratio (95% CI)</th>
<th>p value&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. genitalium</em></td>
<td>1.5 (7/473)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td><em>C. trachomatis</em></td>
<td>2.1 (10/473)</td>
<td>0 (0.0)</td>
<td>10 (2.1)</td>
<td>2.9 (0.2-54.2)</td>
<td>1.00</td>
</tr>
<tr>
<td><em>N. gonorrhoeae</em></td>
<td>0.6 (3/473)</td>
<td>1 (14.3)</td>
<td>2 (0.4)</td>
<td>38.7 (3.1-486.6)</td>
<td><strong>0.04</strong></td>
</tr>
<tr>
<td><em>T. vaginalis</em></td>
<td>4.4 (21/473)</td>
<td>1 (14.3)</td>
<td>20 (4.3)</td>
<td>3.7 (0.04-32.4)</td>
<td>0.27</td>
</tr>
<tr>
<td>High-Risk HPV&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>32 (111/347)</td>
<td>3 (60.0)</td>
<td>108 (31.6)</td>
<td>3.2 (0.5-19.7)</td>
<td>0.33</td>
</tr>
<tr>
<td>HPV16&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5.2 (18/347)</td>
<td>0 (0.0)</td>
<td>18 (5.3)</td>
<td>1.6 (0.1-30.0)</td>
<td>1.00</td>
</tr>
<tr>
<td>HPV18&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.5 (12/347)</td>
<td>2 (40.0)</td>
<td>10 (2.9)</td>
<td>22.1 (3.3-147.5)</td>
<td><strong>0.01</strong></td>
</tr>
<tr>
<td>OHR HPV types&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>28.8 (100/347)</td>
<td>2 (40.0)</td>
<td>98 (28.7)</td>
<td>1.6 (0.3-10.0)</td>
<td>0.63</td>
</tr>
</tbody>
</table>

<sup>a</sup>Fisher’s Exact Test

<sup>b</sup>Combined HPV results for 14 high-risk HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68)

<sup>c</sup>HPV results were available for 347 subjects

<sup>d</sup>OHR, Combination result for 12 other high-risk HPV types (31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68)
Table 2. Value of leukocyte to epithelial cell ratios detected in liquid cytology specimens for predicting *M. genitalium* and other STIs

<table>
<thead>
<tr>
<th>Infection Status</th>
<th>Threshold of Leukocyte / EC Ratio</th>
<th>&gt;2.0</th>
<th>&gt;2.5</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. genitalium</em> Positive (n=7)</td>
<td>No.</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>%</td>
<td>100.0</td>
<td>71.4</td>
</tr>
<tr>
<td><em>M. genitalium</em> Negative (n=70)</td>
<td>No.</td>
<td>16</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>%</td>
<td>22.9</td>
<td>14.3</td>
</tr>
<tr>
<td></td>
<td>P value&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.0001</td>
<td>0.0025</td>
</tr>
<tr>
<td></td>
<td>PPV (%)</td>
<td>100.0</td>
<td>71.4</td>
</tr>
<tr>
<td><em>C. trachomatis</em> Positive (n=10)</td>
<td>No.</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>%</td>
<td>70.0</td>
<td>50.0</td>
</tr>
<tr>
<td><em>C. trachomatis</em> Negative (n=67)</td>
<td>No.</td>
<td>16</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>%</td>
<td>23.9</td>
<td>14.9</td>
</tr>
<tr>
<td></td>
<td>P value&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.0062</td>
<td>0.0205</td>
</tr>
<tr>
<td></td>
<td>PPV (%)</td>
<td>70.0</td>
<td>50.0</td>
</tr>
<tr>
<td><em>N. gonorrhoeae</em> Positive (n=3)</td>
<td>No.</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>%</td>
<td>66.7</td>
<td>66.7</td>
</tr>
<tr>
<td><em>N. gonorrhoeae</em> Negative (n=74)</td>
<td>No.</td>
<td>21</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>%</td>
<td>28.4</td>
<td>17.6</td>
</tr>
<tr>
<td></td>
<td>P value&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.211</td>
<td>0.0952</td>
</tr>
<tr>
<td></td>
<td>PPV (%)</td>
<td>66.7</td>
<td>66.7</td>
</tr>
<tr>
<td><em>T. vaginalis</em> Positive (n=20)</td>
<td>No.</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>%</td>
<td>20.0</td>
<td>15.0</td>
</tr>
<tr>
<td><em>T. vaginalis</em> Negative (n=57)</td>
<td>No.</td>
<td>19</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>%</td>
<td>33.3</td>
<td>21.1</td>
</tr>
<tr>
<td></td>
<td>P value&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.3952</td>
<td>0.7466</td>
</tr>
<tr>
<td></td>
<td>PPV (%)</td>
<td>20.0</td>
<td>15.0</td>
</tr>
</tbody>
</table>

<sup>a</sup>Fisher’s Exact Test
Figure 1. Reproducibility of the MG190 TaqMan PCR assay. (A) *M. genitalium* G37 organisms were spiked into ThinPrep PreservCyt specimens at concentrations ranging from $1 \times 10^4$-$3 \times 10^7$ organisms / mL. DNA was purified from each spiked specimen and used as template in ten replicate PCR reactions run on separate plates. (B) In a separate study, after spiking *M. genitalium* G37 organisms at the indicated concentrations, five independent DNA purifications and PCRs were performed once weekly over a five week period to evaluate inter-assay precision of the assay system. Data are presented as cycle of quantification (Cq) with % coefficient of variation for each group.
Figure 2. Quantification of cervical leukocytes from liquid cytology specimens. ThinPrep PreservCyt specimens from low-risk Louisiana women were screened for M. genitalium using the TaqMan PCR system described herein, and for C. trachomatis, N. gonorrhoeae, and T. vaginalis. All subjects with co-infections among M. genitalium, C. trachomatis, N. gonorrhoeae, and T. vaginalis were excluded from the calculation of leukocyte modulation. Those specimens that were mono-positive for each STI and a randomly selected subset of specimens negative for all tested STIs (n=40) were Diff-Quik stained followed by quantification of cervical epithelial cells and leukocytes. (A) Representative microscope field from a stained ThinPrep PreservCyt specimen illustrating squamous epithelial cells and cervical leukocytes. Differing ratios of leukocytes to epithelial cells are readily observed using the preparation and staining paradigm. (B) Comparison of cervical leukocyte counts among women with and without STIs presented as the ratio of leukocytes to epithelial cells per high powered field (HPF) compiled from five representative microscope fields. Significant differences between leukocyte/epithelial cell ratios among subjects are denoted with an asterisk (p<0.05, ANOVA).
Using a previously described genotyping system [15], we PCR-amplified and sequenced the specific genotyping region of MG191 from all specimens confirmed to be positive for *M. genitalium* infection (n=7). Sequencing reads were trimmed using Sequencher version 4.10.1 and aligned using the Clustal W in MacVector version 12.0.3. Sequences were compared to previously described *M. genitalium* strains using NCBI's BLASTn program. *M. genitalium* strain G37 was processed in parallel to confirm accuracy of the system, and presented as a reference. Nucleotide reference numbers are relative to G37 (GenBank Ref. NC_000908).

Figure 3. Nucleotide alignment of *M. genitalium* genotypes. Using a previously described genotyping system [15], we PCR-amplified and sequenced the specific genotyping region of MG191 from all specimens confirmed to be positive for *M. genitalium* infection (n=7). Sequencing reads were trimmed using Sequencher version 4.10.1 and aligned using the Clustal W in MacVector version 12.0.3. Sequences were compared to previously described *M. genitalium* strains using NCBI's BLASTn program. *M. genitalium* strain G37 was processed in parallel to confirm accuracy of the system, and presented as a reference. Nucleotide reference numbers are relative to G37 (GenBank Ref. NC_000908).