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Endocervical swabs transported in first void urine as combined specimens in the detection of *Mycoplasma genitalium* by real-time PCR

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The aim of this study was to determine whether a patient's endocervical swab specimen can be transported in first void urine (FVU) as combined specimens for the detection of *Mycoplasma genitalium* by real-time PCR. The study also compared two different DNA extraction methods for observation of possible PCR inhibition. Three specimens, one endocervical swab specimen transported in 2-SP medium, one endocervical swab specimen transported in FVU and a FVU specimen, were collected from 329 women. All sample types underwent manual DNA extraction whereas in the DNA extraction study, 329 endocervical swab specimens transported in FVU were subjected to both manual Chelex and automated BioRobot M48 DNA extraction. A total of 100 endocervical swab specimens transported in FVU from patients PCR-negative for *M. genitalium* in the study were used in the PCR inhibition analysis. *M. genitalium* was detected in 25/329 (7.6 %) women. The endocervical swab specimens transported in 2-SP medium and transported in FVU were positive for *M. genitalium* in 17/25 (68 %) and 24/25 (96 %) women, respectively. The FVU specimens alone were positive for *M. genitalium* in 22/25 (88 %) women. In the DNA extraction study, *M. genitalium* DNA was detected in 24/329 (7.3 %) and 28/329 (8.5 %) of endocervical swab specimens transported in FVU subjected to manual Chelex extraction and automated BioRobot M48 extraction, respectively. Partial PCR inhibition was detected in 6 % of samples subjected to manual Chelex extraction whereas no inhibition was detected with the automated BioRobot M48 extraction. Thus endocervical swab specimens transported in FVU demonstrate higher sensitivity than FVU specimens only and have considerably increased sensitivity compared with endocervical swab specimens transported in 2-SP medium for detection of *M. genitalium* DNA. Moreover, automated BioRobot M48 extraction was shown to be superior to a crude manual Chelex extraction, leaving no PCR inhibition and giving a slightly higher DNA yield and/or better sensitivity.

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INTRODUCTION

The well-established pathogens *Chlamydia trachomatis* and *Neisseria gonorrhoeae* are known to cause upper genital tract infections (i.e. epididymitis in men and pelvic inflammatory disease in women) and are often associated with urethritis in men and cervicitis in women (Falk *et al.*, 2004, 2005). In many patients with symptomatic non-chlamydial, non-gonococcal urethritis (NCNGU), the aetiology remains unclear. *Mycoplasma genitalium* has, since its first isolation in 1980 (Tully *et al.*, 1981), been indicated as having a causative role in NCNGU in men and cervicitis in women (Deguchi & Maeda, 2002; Jensen, 2004; Taylor-Robinson, 2002; Uusküla & 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). Repeated attempts have been made to recover the extremely fastidious organism from clinical samples by culture techniques but isolates have been rare and difficult to obtain. With the development of PCR methods in the early 1990s, detection of *M. genitalium* infection became more feasible. Conventional sample specimens [urethra, endocervix and/or first void urine (FVU)] and transport

Abbreviations: Ct, cycle threshold; FVU, first void urine.

media (e.g. 2-SP medium containing sucrose–phosphate buffer with fetal calf serum and antibiotics) have been used in most clinical studies of *M. genitalium* published thus far. However, sampling from the urethra in women, like in men, may be uncomfortable and painful. Several studies have demonstrated the superior sensitivity of male FVU compared to urethral swabs and that an endocervical swab specimen should be supplemented with FVU in women in order to achieve higher sensitivity in *M. genitalium* detection (Jensen *et al.*, 2004a, b; Jurstrand *et al.*, 2005). Analysing two specimens separately from women (endocervical swab in transport medium and FVU) would not be economically and practically justifiable if the sensitivity of pooling the FVU with the endocervical swab proves to be equivalent to analysing the specimens separately. The aim of this study was to determine whether a patient's endocervical swab specimen can be transported in FVU as combined specimens in *M. genitalium* detection by real-time PCR. In addition, we also wanted to compare two different DNA extraction methods to observe possible PCR inhibition in the endocervical swab specimens transported in FVU.

METHODS

Patients and clinical specimens. From August 2004 to June 2005, specimens were obtained from 329 women (15–65 years of age, median 24 years) attending the STI clinic at the Central Hospital Karlstad, Sweden. All new attendees who were at risk for being infected with a sexually transmitted infection, 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. Two endocervical swabs for detection of *M. genitalium* were collected using a Dacron-tipped plastic shaft Copan 159 C swab. Following the clinical examination, all women were asked to collect FVU for detection of *M. genitalium*. The FVU was distributed into two 10 ml screw-capped polypropylene tubes (Sarstedt). On a 2-week rotating schedule, the first endocervical swab was placed in 1500 µl 2-SP transport medium and the second endocervical swab was placed in one of the FVU tubes. The next week, the first endocervical swab was placed in one of the FVU tubes and the second endocervical swab was placed in 2-SP transport medium.

A specimen was considered true positive if at least one of the three specimens in a patient's set was positive for *M. genitalium* by real-time *M. genitalium* adhesin protein (MgPa) gene PCR.

All sample types underwent manual DNA extraction, whereas in the DNA extraction study, 329 endocervical swab specimens transported in FVU were subjected to both manual Chelex and automated DNA extraction and their results were compared. The study was approved by the local ethical committee.

DNA extraction

Manual extraction. A volume of 1800 µl from FVU and endocervical swab specimens transported in FVU was pelleted by centrifugation at 20 000 ***g*** for 15 min. Aliquots of swab specimen (100 µl) in 2-SP medium were mixed with 1 ml 0.85 % NaCl prior to centrifugation as 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 a 5 µl aliquot of the supernatant was analysed by real-time MgPa gene PCR.

Automated extraction. A volume of 1800 µl from endocervical swab specimens transported in FVU was pelleted by centrifugation at 20 000 ***g*** for 15 min. The pellet was resuspended in 200 µl PBS and vortexed thoroughly. The BioRobot M48 [MagAttract DNA Mini kit (Qiagen); 200 µl sample input, 100 µl output] was used according to the manufacturer's instructions. A 5 µl aliquot of template DNA was analysed in real-time MgPa gene PCR.

Real-time MgPa gene PCR. The PCR was carried out in a 25 µl SmartCycler reaction tube (Edberg *et al.*, 2008) containing 1 × reaction buffer, 3.5 mM $MgCl_2$, 200 µM dNTP mix, 0.625 U HotGoldStar *Taq* Polymerase (Eurogentec), 1.0 µM of each of the previously described (Jensen *et al.*, 2004b) forward primer MgPa-355F (5′-GAG AAA TAC CTT GAT GGT CAG CAA-3′) and reverse primer MgPa-432R (5′-GTT AAT ATC ATA TAA AGC TCT ACC GTT GTT ATC-3′) (Cybergene) and 0.1 µM MgPa-380 TaqmanMGB probe (5′-FAM-ACT TTG CAA TCA GAA GGT-MGB-3′) (Applied Biosystems) detecting a 78 bp fragment of the MgPa operon sequence (accession no. M31431). Subsequently, 5 µl template DNA was added to the mixture. Amplification was performed in a SmartCycler (Cepheid) under the following conditions: HotGoldStar *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; 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.

Inhibition analysis. In the PCR inhibition analysis, 100 endocervical swab specimens were used transported in FVU from patients negative for *M. genitalium* by real-time MgPa gene PCR in the study. The specimens were subjected to both manual Chelex and automated DNA extraction. Furthermore, by adding a 1 µl aliquot of purified *M. genitalium* DNA per patient to the real-time MgPa gene PCR mixture, a comparable cycle threshold (Ct) value was created. A reference sample was created by calculating the mean Ct value of three consecutive samples using sterile water as template. The Ct values obtained from each patient sample were then compared to the mean Ct value of the reference, $dCt = Ct_{sample} - Ct_{reference}$. A dCt value of $\leqslant 3$ was considered no inhibition, i.e. less than a 10-fold decrease in analytical sensitivity; a dCt value >3 was considered partial inhibition; and a negative sample Ct value was considered total inhibition.

RESULTS

Specimens

All 329 women provided three specimens: one endocervical swab specimen transported in 2-SP, one endocervical swab specimen transported in FVU and also a FVU specimen only. *M. genitalium* was detected in 25/329 (7.6 %) women by real-time MgPa gene PCR. The endocervical swab specimens transported in 2-SP medium and transported in FVU were positive for *M. genitalium* in 17/25 (68 %) and 24/25 (96 %) women, respectively (Fig. 1). Two specimens were positive for *M. genitalium* only in the endocervical swab specimens transported in FVU. Both specimens were able to be retested and were found to be repetitively positive. The FVU specimens alone were positive for *M. genitalium* in 22/25 (88 %) women.

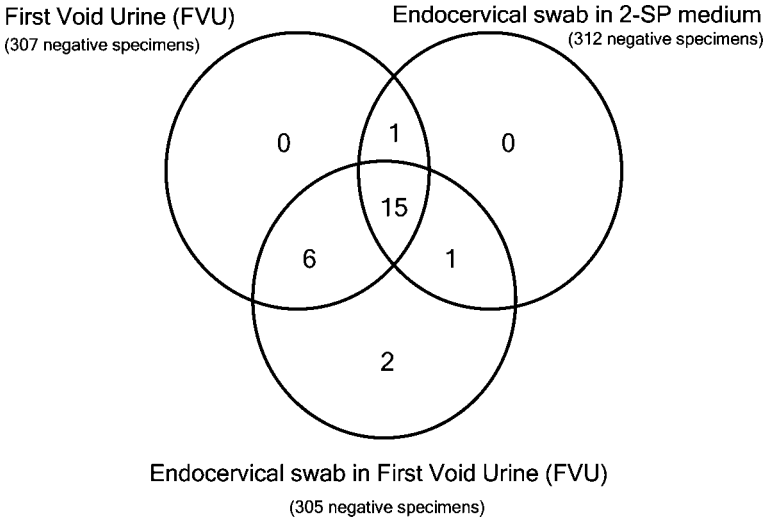

Fig. 1. Distribution of 329 specimen sets from women as determined by real-time MgPa gene PCR. *M. genitalium*-positive specimens are indicated within the circles.

DNA extraction and inhibition analysis

A total of 329 endocervical swab specimens transported in FVU were used in the DNA extraction comparative study. *M. genitalium* DNA was detected in 24/329 (7.3 %) and 28/329 (8.5 %) endocervical swab specimens transported in FVU subjected to manual Chelex extraction and automated BioRobot M48 extraction, respectively. Four specimens were found to be positive for *M. genitalium* only by automated BioRobot M48 extraction. All four specimens were able to be retested and found to be positive. One of these specimens came from a woman with other *M. genitalium*-positive samples.

One hundred PCR-negative endocervical swab specimens transported in FVU were used in the PCR inhibition analysis. Partial PCR inhibition was detected in 6 % of samples subjected to manual Chelex extraction whereas no inhibition was detected with the automated BioRobot M48 extraction (Table 1).

DISCUSSION

To the best of our knowledge, this is the first comparison between endocervical swab specimens transported in 2-SP medium, endocervical swab specimens transported in FVU and FVU alone for detection of *M. genitalium* infection in women. It is also the first comparison of manual Chelex extraction and automated BioRobot M48 extraction using the MagAttract DNA kit for detection of *M. genitalium*. The majority of clinical studies on *M. genitalium* published to date have used traditional sample specimens and transport media, e.g. 2-SP medium. In women, swab specimens from the urethra and/or the endocervix and/or FVU have been used and, in men, swab specimens from the urethra and/or FVU are most commonly used. In a recent study by Jensen *et al.* (2004a), significantly more (88 %) *M. genitalium* infections were detected in FVU specimens than in urethral (57 %) and endocervical (71 %) swab specimens of infected women. However, if the FVU was supplemented with an endocervical swab specimen the sensitivity of *M. genitalium* detection could be improved to 96 %. In 2005, Jurstrand *et al.* (2005) illustrated the need to analyse both endocervical swabs and FVU since *M. genitalium* DNA was detected in only one of the two specimens in 50 and 31 % of *M. genitalium*-infected women by real-time LightCycler PCR and conventional PCR, respectively. However, analysing two separate specimens from women is not cost-effective and efficient if the sensitivity of combining the FVU with the endocervical swab is equivalent to that when the specimens are analysed separately. The main purpose of the present study was to determine whether womens' endocervical swab specimens can be transported in FVU for detection of *M. genitalium* by real-time MgPa gene PCR. This method was shown to be superior to transporting the endocervical swab specimens in 2-SP medium. FVU specimens only were somewhat less sensitive

Table 1. Comparison of PCR inhibition in 100 endocervical swab specimens transported in FVU subjected to manual Chelex and automated MagAttract DNA extraction, including dCt distribution as determined by real-time MgPa gene PCR

dCt, Delta cycle threshold ($Ct_{sample} - Ct_{reference}$).

	Chelex extraction	MagAttract DNA extraction
No inhibition	94 %	100 %
Partial inhibition	6 %	<1 %
Total inhibition	<1 %	<1 %
dCt mean	0.99	−0.12
dCt max	9.27	0.41
dCt min	−0.23	−0.46

than endocervical swab specimens transported in FVU. Although there were few positive patients, the results indicate that pooling a cervical swab with FVU has several advantages in the diagnosis of *M. genitalium* infection, such as sensitivity of the diagnostic test, economy and comfort for the woman. Transportation of the endocervical swab specimen in the patient's FVU has previously been shown favourable for detection of *C. trachomatis*, with a sensitivity of 97.9 % in pooled specimens of FVU and endocervical swabs compared to 93.3 % in FVU alone (Airell *et al.*, 2000). Data were in agreement with the present study for *M. genitalium* detection.

Four endocervical swab specimens transported in FVU were found positive for *M. genitalium* by real-time MgPa gene PCR when subjected to automated BioRobot M48 extraction, but not when manual Chelex extraction was used, indicating a higher sensitivity for the automated DNA extraction method. In the present study, 200 µl of endocervical swab specimen transported in 2-SP medium was used for manual Chelex DNA extraction in comparison to 1800 µl of endocervical swab specimen transported in FVU and FVU specimens only. This could partly explain the lower sensitivity for the endocervical swab specimen transported in 2-SP medium.

In the present PCR inhibition analysis, two different DNA extraction methods were compared to observe possible PCR inhibition in the endocervical swab specimens transported in FVU. We demonstrated a slightly higher DNA yield and/or better sensitivity in terms of PCR mean dCt values (mean dCt −2.38, data not shown) using automated BioRobot M48 extraction compared to manual Chelex extraction, where partial inhibition was observed in 6 % of samples. No inhibition was detected with automated BioRobot M48 extraction, using the MagAttract DNA kit. Other studies have found inhibitory activities when analysing *M. genitalium* by PCR, most of which have used the crude Chelex extraction method (Jensen *et al.*, 2004a, b; Jurstrand *et al.*, 2005). Jensen *et al.* (2004a) demonstrated that 28 % of urethral swab specimens and 14 % of FVU specimens contained less than 10 genome equivalents of *M. genitalium* DNA. Moreover, 20 and 13 % of the two specimen types had less than 5 genome equivalents. Inhibitors and the probability of low DNA load in specimens emphasize a need for improved protocols for specimen preparation to increase the sensitivity in assays for clinical purposes.

In conclusion, endocervical swab specimens transported in FVU demonstrate higher sensitivity than FVU specimens only and considerably increased sensitivity compared to endocervical swab specimens transported in 2-SP medium for detection of *M. genitalium* DNA by real-time MgPa gene PCR. Moreover, automated BioRobot M48 extraction using the MagAttract DNA kit was shown to be superior to a crude manual Chelex extraction, leaving no PCR inhibition and giving a slightly higher DNA yield and/or better sensitivity. Endocervical swab specimens transported in patients' FVU will save the cost of the 2-SP medium, reduce the analytical cost as two specimens become one and relieve logistic difficulties in distributing the 2-SP medium out to clinics.

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