Mycoplasma genitalium PCR: Does Freezing of Specimens Affect Sensitivity?*

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Mycoplasma genitalium is an established cause of sexually transmitted infections. Studies of disease associations are often performed on archived specimens, but little is known about the effect of storage of specimens on the detection of M. genitalium. Genital swab and first-void urine specimens submitted for detection of M. genitalium were tested on the day of receipt. Remnants of positive original specimens as well as DNA preparations were stored at −20°C for up to 18 months. A total of 361 M. genitalium-positive specimens were available. PCR after repeat DNA preparation was performed for 262 specimens. The sensitivity after repeat DNA preparation was 90%, and the median decrease in DNA load was 155 genome equivalents (geq) (P < 0.0001). For 327 specimens, PCR could be repeated on the primary DNA preparation. The sensitivity of PCR after storage was 95%, and the median decrease in DNA load was 13.5 geq (P < 0.0001). The specimens yielding negative results at repeat testing had a significantly lower median DNA load in the primary analysis than those with a repeat positive test (P < 0.0001). For 228 specimens, PCR could be performed both on the primary DNA preparation and after repeat DNA preparation. The median DNA load was lower after repeat DNA extraction than after repeat testing of the stored DNA extract (P < 0.0001). In conclusion, the M. genitalium DNA load as well as the detection rate decreased after storage. This was more pronounced in clinical specimens stored frozen than in stored DNA extracts, particularly in those with an initial low DNA load.

MATERIALS AND METHODS

Specimens with requests for diagnostic M. genitalium PCR testing were received from general practitioners, STD clinics, and private specialists. First-void urine (FVU) specimens were submitted in sterile polypropylene or polystyrene tubes; cervical, vaginal, and urethral swab specimens were collected and transported in a variety of transport media used for C. trachomatis testing, primarily ProbeTec medium (BD, Sparks, MD), Amplicor UTM (Roche Molecular Diagnostics, Pleasanton, CA), Copan Universal Transport Medium (UTM) (Copan, Brescia, Italy), and 2SP chlamydia transport medium (Statens Serum Institut [SSI], Hilleroed, Denmark). A few specimens were collected in Stuart’s transport medium (SSI). M. genitalium was detected by an inhibitor-controlled quantitative MGPa-gene TaqMan real-time PCR (9) on the day of receipt, and all positive results were subsequently confirmed using a conventional gel-based 16S rRNA gene PCR (10). The standard curve for the quantitative PCR was generated from 10-fold dilution series of M. genitalium genomic DNA ranging from 1 genome equivalent (geq) per μl to 100,000 geq/μl. All quantitative results were expressed as the number of geq/5 μl template used in the PCR and were determined as the mean of two wells. The same stock of M. genitalium DNA was used to prepare the standards throughout the study. Sample preparation was performed essentially as described previously (8). In brief, 1.9 ml of the FVU specimens was centrifuged at 20,000 × g for 15 min, the pellet was resuspended in 300 μl of a 20% Chelex 100 slurry (Bio-Rad, Hercules, CA) in TE (Tris-EDTA) buffer, and the suspension was incubated at 95°C for 10 min. From swab specimens collected in BD ProbeTec transport medium, 950 μl was treated as described above for FVU specimens. For swab specimens collected in Roche STM, 2SP chlamydia transport, and Copan UTM medium, 100 μl of the transport medium was aspirated and added directly to 300 μl of Chelex slurry. For specimens collected in Stuart’s transport medium, the charcoal-impregnated cotton swab was placed in 1 ml of 2SP chlamydia transport medium and vortexed thoroughly, and 100 μl of the medium was added directly to 300 μl of Chelex slurry. Remnants of M. genitalium-positive original specimens as well as DNA preparations were stored at −20°C for 1 to 18 months (median, 296 days). The freezers were standard commercial models without automatic defrosting, thus avoiding repeated, unneeded freeze-thaw cycles. Furthermore, they were centrally temperature monitored in order to document uninterrupted function. Clinical specimens were subjected to repeat DNA preparation and quantitative PCR (repeat DNA), and the corresponding primary DNA preparations were thawed, vortexed, and briefly centrifuged before repeat quantitative PCR (repeat PCR).

A total of 361 M. genitalium-positive specimens were received from July 2007 through January 2009; 166 were collected from 127 women and 195 were collected from 178 men. Figure 1 shows the number of specimens tested after storage under different conditions. From women, 25 FVU specimens, 33 urethral swab specimens, 92 cervical swab specimens, and 12 vaginal swab specimens were received. For four swab specimens, the anatomical site was not available. From men, 106 urethral swab specimens and 89 FVU specimens were examined.
RESULTS

Considering the DNA load on primary testing, the 166 specimens from women had a median DNA load of 25 geq. There was no difference in the median DNA loads in the 92 cervical swab specimens (median, 23 geq), the 23 urethral swab specimens (median, 25 geq), or the 25 FVU specimens (median, 38 geq). However, the 12 vaginal swab specimens contained a median of 107 geq, which was significantly higher than the DNA load in the cervical swab specimens ($P = 0.046$) but not significantly different from the loads in the other specimen types. Among the 195 specimens from men, the median DNA load for all specimens was 342 geq; FVU and urethral swab specimens had similar DNA loads (416 and 257 geq, respectively; $P = 0.7$). No differences in the DNA load could be found when the results for different transport media with similar specimen types were compared (data not shown).

PCR could be performed again after repeat DNA preparation for 262 (73%) of the 361 specimens, comprising 166 specimens from 127 women (median age, 24 years; age range, 16 to 57 years) and 195 specimens from 178 men (median age, 27 years; age range, 15 to 63 years). After repeat DNA preparation and subsequent PCR, 25 were negative and the median decrease in the DNA load was 155 geq ($P < 0.0001$). Thus, the sensitivity of PCR after repeat DNA preparation was 95%, which was significantly lower than that of the PCR performed after primary analysis ($P < 0.0001$).

In 228 (63%) of the 361 specimens with an initial median DNA load of 78 geq, PCR could be repeated both on the primary DNA preparation and after repeat DNA preparation. For this group of specimens where test results were available for both storage conditions, repeat PCR on the primary DNA extract had a sensitivity of 94% and showed a median DNA load of 37 geq. PCR on the repeat DNA extract had a sensitivity of 89% and showed a median DNA load of 21 geq. The median DNA load was lower after repeat DNA extraction than after repeat testing of the stored DNA extract (median difference, 46 geq; $P < 0.0001$), and the sensitivity of testing after repeat DNA extraction was significantly lower than that after testing of the stored DNA preparation ($P = 0.035$).

The results of primary PCR, repeat PCR, and repeat DNA, stratified by sex, specimen type, and the different transport media used, are presented in Table 1.

DISCUSSION

The present study aimed to quantify the effect of freezing at $-20^\circ$C on the detection of \textit{M. genitalium} by PCR. We previously used a conventional gel-based PCR (10) to evaluate the effect of freezing on 102 \textit{M. genitalium}-positive FVU specimens from males and 22 FVU specimens from females extracted by the Chelex method (repeat PCR) and found that 94% of the male FVU specimens and all of the female FVU specimens remained positive after storage (8). Similarly, repeat DNA preparation was performed for 68 of the corresponding original male FVU specimens and for 15 female FVU specimens which had been stored for 1 to 18 months at $-20^\circ$C. A sensitivity of 93% for the male FVU specimens was found after repeat DNA extraction, while only 73% of the female FVU specimens remained positive after the specimens were frozen. However, only FVU specimens were studied and the gel-based assay did not allow quantitation. In the present study, the sensitivities found for repeat PCR on male and female FVU specimens as well as the sensitivities for testing of repeat DNA were not statistically different from the results found previously, and since similar sample preparation methods were applied, pooling of the two data sets would provide a better estimate of the sensitivity. If this approach is followed, the sensitivity for repeat PCR on DNA extracted before freezing of male FVU specimens would be 96% (95% confidence interval [CI], 92 to 98%; 174/182 specimens), and for female FVU specimens the sensitivity would be 98% (95% CI, 88 to 99.9%; 42/43 specimens). For repeat DNA extraction, the sensitivity for male FVU specimens would be 92% (95% CI, 86 to 96%; 121/132 specimens) and that for female FVU specimens would be 83% (95% CI, 67 to compared to the lowest quartile) and a sensitivity of 86% (57 of 66 specimens) ($P = 0.079$). Although the difference in sensitivity was not statistically significant, these findings suggest that the decrease in sensitivity caused by freezing may increase with the length of storage.

For 327 (91%) of the 361 specimens, PCR could be repeated on the primary DNA preparation; of these, 15 were negative and those that were repeat negative had a lower median DNA load in the primary analysis (3.5 geq versus 107 geq for those that were repeat positive; $P < 0.0001$). The sensitivity of PCR performed after storage of the primary DNA preparation was 95%, which was significantly lower than that of the PCR performed after primary analysis ($P < 0.0001$).

Statistical analysis. Fisher’s exact test was used to test for differences in proportions; McNemar’s test was used to compare paired proportions, but the exact $P$ value of McNemar’s test statistic, based on the binomial distribution, was used (Liddell’s test); the Mann-Whitney test was used to test for differences in continuous variables between groups; the Wilcoxon signed-ranks test was used to test for differences between matched pairs (i.e., the DNA loads before and after freezing). These tests were performed with the StatsDirect (version 2.6.6) program (StatsDirect Ltd., Cheshire, United Kingdom).
The present study was based on specimens submitted for detection of *M. genitalium*. Consequently, a wide range of different transport media were used; however, no significant difference in the sensitivity after freezing and repeat DNA extraction could be found. This indicates that the decay of target DNA is largely independent of the transport medium and is an effect of the freezing alone. It is not surprising that *M. genitalium* cells may lyse after only one freeze-thaw cycle, and is an effect of the freezing alone. It is not surprising that *M. genitalium*,
Lock medium (Sierra Molecular Corporation) or Aptima transport medium (Gen-Probe Inc.), are more efficient in protecting the DNA remains to be determined. These transport media supposedly contain nucleic acid stabilizers and should provide less degradation. It is important, however, that the extraction procedure also extract liberated nucleic acid, and consequently, the centrifugation step included in the Chelex DNA extraction method may be less efficient than extraction procedures accommodating a large volume of urine or transport medium. Furthermore, since the length of storage without freeze-thaw cycles appears to have a significant effect on the decrease in DNA load, future studies should consider including storage at −80°C for comparison.

The findings of the present study are important for the interpretation of the findings of studies based on specimens that have been stored for a longer period of time at −20°C. Manhart et al. recently estimated the prevalence of *M. genitalium* infection in a nationally representative sample of young adults in the United States using stored frozen urine specimens from 1,714 women and 1,218 men. The prevalence in women was found to be as low as 0.8% (13), but using the knowledge obtained from the present study, the true prevalence may actually have been closer to 1%. Similarly, the prevalence of *M. genitalium* in pregnant women was found to be 0.7% when it was determined with frozen stored urine specimens (15). If fresh material had been used, the prevalence would probably have been higher, in particular, when considering that the DNA load in asymptomatic subjects tends to be lower than that in symptomatic patients and that those specimens failing amplification after DNA extraction of frozen material were those with the lowest DNA loads.

The decrease in sensitivity and DNA load should have implications for planning of future studies; DNA extraction should obviously be performed on fresh material, and if at all possible, testing should be performed before the DNA preparation is frozen.

The present study has some limitations. No clinical information could be obtained, so a possible difference in sensitivity between symptomatic and asymptomatic patients could not be determined. Apparently, specimens form women contained a lower median number of genome copies than specimens from men, leading to a larger decrease in sensitivity for female specimens than for male specimens after storage. Whether this gender bias can be generalized or is an effect of a larger proportion of symptomatic men being tested would need further studies. Furthermore, the decrease in sensitivity may to some extent reflect a Poisson distribution leading to sampling error for specimens with a low concentration of template DNA. We did not perform repeat testing of frozen *M. genitalium*-negative specimens, but in a previous study (8), 2 out of 100 negative swab specimens were positive when they were tested with a concentration step consisting of centrifugation. When FVU specimens from these two patients were retested, they were actually *M. genitalium* positive, suggesting that some false-negative results should be expected with this rather crude DNA extraction procedure.

In conclusion, freezing of clinical specimens as well as Chelex-extracted DNA leads to a significantly lower *M. geni-