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# The impact of sample storage on molecular-based detection of *Mycoplasma genitalium*

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**Running title**: Impact of storage on *M. genitalium* detection

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Aims. *Mycoplasma genitalium* causes a common, sexually-transmitted bacterial infection. This study assessed the detection of *M. genitalium* in stored urine samples to understand the impact of sample storage on *M. genitalium* detection.

Methods. Aliquots of *M. genitalium*-positive urine (n=20 patients) were stored at either room temperature (22°C) or 4°C, without a preservative. At weekly intervals samples were tested using the commercial test ResistancePlus MG® (SpeeDx®, Australia). We report the analysis at 1 week, an acceptable collection-to-test turnaround time, with further analysis over 5 weeks to illustrate degradation trends.

Results. After storage at 4°C, the proportion of specimens that remained positive for *M. genitalium* was 100% after 1 week and 95% after 4 weeks. Storage at 22°C led to more rapid decline in detection in the first 4 weeks, with 95% detected after 1 week and 85% at two weeks onwards. At 5 weeks, samples stored at both temperatures had an 85% *M. genitalium* detection rate, with increase in crossing points (ΔCq) of 0.72 (95% confidence interval [CI] 0.01–1.43; p-trend=0.027) at 4°C, and 1.75 ([95%CI 0.79-2.71], p-trend <0.001) at 22°C.

Conclusions. Urine samples stored without preservative, and unfrozen, retained high *M. genitalium* detection levels over the short term (up to 5 weeks). To minimise degradation storage at 4°C is recommended.

Significance and Impact. There is little known about the stability of clinical samples for *M. genitalium* detection. This study found a high proportion (85-100%) of samples are still suitable for *M. genitalium* detection after storage for up to 5 weeks."

**Keywords**: Diagnosis; PCR; Molecular genetic; Degradation; Detection

## Introduction

*Mycoplasma genitalium* is a sexually-transmitted bacterium that causes urethritis in men (Horner et al., 2017). In women, infection has been associated with cervicitis, endometritis, and elevates the risk of tubular factor infertility and pregnancy complications (Lis et al., 2015). There is a high infection burden in some populations such as men-who-have-sex-with-men (MSM) where asymptomatic colonisation rates are as high as 9.5% (Read et al., 2019). There has also been a dramatic increase in resistance to antimicrobial therapies, with resistance to the first-line treatment (azithromycin) as high as 84% in MSM populations in Australia (Read et al., 2019). Together this has highlighted an increased need for diagnostic testing and research to further understand this infection. Both diagnostics and research have been advanced by the release of commercial nucleic acid amplification tests for *M. genitalium* (Tabrizi et al., 2016, Tabrizi et al., 2017).

With the increase in *M. genitalium* testing, it is important to understand the impact that delays in sample processing may have on *M. genitalium* detection, for which there is currently no information. Urine is a commonly recommended specimen for testing *M. genitalium* infection (Jensen et al., 2016). In this study, urine samples were stored *in vitro* at two temperatures (room temperature and 4°C), to assess the effect on *M. genitalium* detection by a commercial assay over a 5-week period.

## Materials and Methods

### Data extraction for sample collection-to-test times

The time between sample collection and testing for *M. genitalium* was examined for a metropolitan testing laboratory, Melbourne, Australia. The period studied was January to May 2018.

### Sample selection from existing clinical studies

First-void urine samples were collected from men attending Melbourne Sexual Health Centre between the 7th of February 2017 until the 31st May 2017 as part of ongoing clinical study (Read et al., 2019). Samples were delivered to the Royal Women's Hospital at 4°C for *M. genitalium* testing (median delivery time of 1 day), DNA was extracted from 1mL of urine as described previously (Read et al., 2018) and 5 µL of extract tested for *M. genitalium* using the ResistancePlus MG test (SpeeDx, Australia)(Tabrizi et al., 2017). For the purpose of this study, 20 sequential positive samples with a ≥10 mL residual volume were selected. The samples used in this study were collected under ethics approval from the Alfred Hospital Ethics Committee (289/17).

### Sample processing for sample stability analysis

The processing of samples is outlined in Figure 1. Urine samples were vortex-mixed, turbidity noted by eye (non-standardised method, either "turbid" or "not turbid"), then ten 1 mL aliquots transferred to sterile 1.5 mL polypropylene screw-capped tubes (Sarstedt, Germany). Aliquots were stored in the dark, at either room temperature (approx. 22°C, in a cupboard in an air-conditioned room with limited temperature variation) or 4°C. At one-week intervals for 5 weeks, an aliquot of each sample was taken and extracted as above, then stored at -30°C (for up to 6 months) before batch testing for *M. genitalium*, as described above (week 1 to week 5). Cycle threshold (Cq) values were determined for samples based on detection of the MGPA gene (channel A, FAM), with an

increase in Cq value indicative of sample degradation. The distribution of initial Cq values for samples was: Cq<20, 2 samples; Cq=20-24, 9 samples; Cq=25-29, 6 samples; Cq=30-35, 3 samples. A subset of samples (the first 14 samples collected) was also analysed for β-globin detection by quantitative PCR, as described previously (Cornall et al., 2013). Culture analysis of urine samples was not performed.

### Data analysis

Tests that had no detectable analyte were assigned an arbitrary Cq value at the limit of detection (Cq=35 for *M. genitalium* detection, Cq=40 for β-globin detection). The change in Cq values was analysed for difference by Student's t-test and for trend by fixed effects regression analysis using STATA (v14.2, StataCorp LLC).

## Results

### Sample collection-to-test times can exceed 2 weeks

Collection-to-test times were analysed for a diagnostic laboratory over a period of 5 months. For 3623 *M. genitalium* tests, 96.6% of samples were tested within one week of collection, 3.1% (n=114) one to two weeks after collection, and 0.25% (n=9) more than two weeks after collection. The oldest sample for this period was tested 44 days after collection. Of note, these collection-test times reflect delays in the sample reaching the laboratory, not delays in the testing laboratory (only one sample in this period had a laboratory turn-around-time of more than 7 days). Anecdotally, clinics blamed delays on sample misplacement and misdirection. This illustrates that prolonged collection-test times are common as a result of delays in sample delivery to the pathology laboratory, and consideration should be given to the impact this has on false negative results. In the following sections we analysed the impact of sample storage for 1 week (an acceptable collection-to-test turnaround time), then for an additional 4 weeks to establish stability trends.

### Detection of *M. genitalium* in urine specimens remains high after several weeks of storage

Samples had an average starting Cq value of 24.4 (range: 17.7 to 33.0). After storage at 4°C, the level of detection of *M. genitalium* was: week 1, 100% positive; weeks 2-4, 95% positive; week 5, 85% positive (Figure 2). Storage at 22°C led to an earlier decline in detection: week 1, 95% positive; weeks 2-5, 85% positive. The mean increase in Cq value over 5 weeks at 4°C was 0.72 (95% confidence interval [CI] 0.01–1.43; p-trend = 0.027) and at 22°C it was 1.75 (95%CI 0.79-2.71; p-trend <0.001). The change in Cq value at 22°C was not significantly different to that at 4°C (p=0.20, Student's t-test).

Samples that crossed beyond the threshold of *M. genitalium* detection had higher starting Cq values (mean 29.1, compared with 22.8; p=0.0032). Only one individual had samples testing negative after storage in both conditions at 5 weeks.

**Human DNA degradation at both temperatures**

Human DNA stability was assessed by quantitative PCR targeting a 110 bp region of the β-globin gene. The amplicon size for this assay is similar to that in the ResistancePlus MG test (approximately 120 bp). While detection rates for β-globin were generally higher, there was a stronger trend and magnitude for increasing Cq values, indicative of sample degradation; over 5 weeks at 4°C there was a ΔCq of 1.82 (95%CI 1.11-2.54), p-trend <0.001, and over 5 weeks at 22°C there was a ΔCq of 3.00 (95%CI 2.13-3.86), p-trend <0.001 (Figure 2). However, when compared to the change in Cq values for *M. genitalium* detection, the change in Cq values for β-globin detection over 5 weeks was not significantly different (p≥0.16).

**Sample turbidity did not impact *M. genitalium* detection**

Urine turbidity was used as an indicator for bacterial contamination of samples. Twenty-five percent (5/20) samples had observable turbidity at the start of the experiment, and all turbid samples tested positive for *M. genitalium* throughout the experiment.

## Discussion

Delays in testing samples at pathology clinics or research laboratories may occur as a result of multiple factors, including transport, sample processing, and human error. For research studies such

as surveillance, sampling may take place in remote locations necessitating sample transport to the laboratory. The effect of delays on sample integrity for the detection of *M. genitalium* by nucleic acid amplification methods is not well characterised. We found that the rate of *M. genitalium* detection in positive urine samples remained high, despite incubation at room temperature or 4°C for 5 weeks. While storage at 4°C appeared to result in superior preservation of bacterial DNA compared to storage at room temperature, this did not reach statistical significance. Samples that became "undetectable" generally had lower *M. genitalium* DNA content at the outset, reflected in higher starting Cq values. Additionally, samples with lower concentration of analyte showed greater variation in Cq values; this may be a consequence of errors introduced during the preparation of the samples (e.g. stochastic sampling error, loss of sample during removal of supernatant from pellets, incomplete resuspension of samples in PBS). The growth of other bacteria in the sample (e.g. from a urinary tract infection) may accelerate degradation of the sample; however, in this study visible turbidity of the urine sample did not impact the detection of *M. genitalium*. Of note, however, turbidity may also result from the presence of immune cells.

There has been very little work analysing the stability of primary clinical samples for *M. genitalium* detection. A study investigating the effect of transport of urine samples found that neat urine and urine adsorbed to swabs (n=5 patients) retained detectable *M. genitalium* by an in-house qPCR assay 10 days after incubation at 37°C, without use of a transport/stabilisation buffer (Costa et al., 2017). Another study on sample integrity found that freezing primary urine samples at -20°C reduces the detection rate for *M. genitalium*, yielding a 9% false negative rate (Carlsen et al., 2010) (of note this study used a smaller 78 bp amplicon, compared to the 120 bp amplicon of the ResistancePlus MG assay). While our study used a smaller number of samples, results suggest that storage of neat urine at 4°C over the short term (0-4 weeks) would preserve samples better than freezing.

The analysis of only one sample type (urine) collected from male participants is a limitation of this study, meaning results may not be generalizable to women or other sample types. Including two temperatures and the longer incubation period are the strengths of the study.

In conclusion, this study shows that samples remain "detectable" for *M. genitalium* after significant periods of storage. While clinical specimens should be tested in the shortest time possible in accordance with good laboratory practice, for short term storage (1-4 weeks) 4°C is preferable to

room temperature. More work is needed to determine optimum storage conditions for longer periods.

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**Conflict of Interest**

The authors have jointly received Government funding with SpeeDx to conduct this work, and SpeeDx provided detection kits for the assays.

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**Fig. 1.** Sample processing procedure.

**Fig. 2.** Detection of analytes after sample storage at two different temperatures. (a) *M. genitalium* detection, storage at 4°C; (b) *M. genitalium* detection, storage at 22°C; (c) β-globin detection, storage at 4°C; (d) β-globin detection, storage at 22°C. (○) Percent detection in samples (left axis); (●) mean change in Cq values (right axis). Error bars indicate 95% confidence intervals, p-trend for ΔCq was 0.027 (panel A) and <0.001 (panels B-D).