Influence of storage time on DNA of *Chlamydia trachomatis*, *Ureaplasma urealyticum*, and *Neisseria gonorrhoeae* for accurate detection by quantitative real-time polymerase chain reaction

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Abstract

The shipment and storage conditions of clinical samples pose a major challenge to the detection accuracy of *Chlamydia trachomatis* (CT), *Neisseria gonorrhoeae* (NG), and *Ureaplasma urealyticum* (UU) when using quantitative real-time polymerase chain reaction (qRT-PCR). The aim of the present study was to explore the influence of storage time at 4°C on the DNA of these pathogens and its effect on their detection by qRT-PCR. CT, NG, and UU positive genital swabs from 70 patients were collected, and DNA of all samples were extracted and divided into eight aliquots. One aliquot was immediately analyzed with qRT-PCR to assess the initial pathogen load, whereas the remaining samples were stored at 4°C and analyzed after 1, 2, 3, 7, 14, 21, and 28 days. No significant differences in CT, NG, and UU DNA loads were observed between baseline (day 0) and the subsequent time points (days 1, 2, 3, 7, 14, 21, and 28) in any of the 70 samples. Although a slight increase in DNA levels was observed at day 28 compared to day 0, paired sample t-test results revealed no significant differences between the mean DNA levels at different time points following storage at 4°C (all P > 0.05). Overall, the CT, UU, and NG DNA loads from all genital swab samples were stable at 4°C over a 28-day period.

Key words: Storage time; *Chlamydia trachomatis*; *Ureaplasma urealyticum*; *Neisseria gonorrhoeae*; Stability; qRT-PCR

Introduction

*Chlamydia trachomatis* (CT) and *Neisseria gonorrhoeae* (NG) are the two most common bacteria responsible for sexually transmitted diseases (STD) worldwide, accounting for 131 million and 78 million of the total estimated 357 million new infections per year, respectively, according to the World Health Organization (http://www.who.int/mediacentre/factsheets/fs110/en/). *Ureaplasma* species, consisting of 14 serovars, which can be divided into two biovars – *U. parvum* and *U. urealyticum* (UU), are also frequently found in STD (1). However, *U. urealyticum* has been reported to be more pathogenic than *U. parvum* in several studies, and is the etiological agent in nongonococcal urethritis (NGU) (2). Other agents, such as the herpes simplex virus and Epstein Barr Virus, are found in a very small ratio, and their association with NGU is only suspected (3). Therefore, UU, CT and NG have been considered the major causes of urethritis and adverse pregnancy outcome, and are listed as routine test items in STD diagnosis (4–7).

Traditionally, detection of CT, NG, and UU in clinical specimens relies on selective culture methods that are labor intensive, time consuming, and have a low sensitivity. The application of quantitative real-time polymerase chain reaction (qRT-PCR) has revolutionized pathogen diagnostics, having much higher sensitivities than the respective culture methods (95.7–98.7% vs 60–85%, 100 vs 86.8%, and 89.5 vs 47.4% in CT, NG, and UU detection, respectively) (8–10). Furthermore, qRT-PCR can be performed on a variety of samples obtained through non-invasive methods (including urethral, urine, vaginal, and cervical specimens) (11), is rapid, and offers a high degree of automation. There are several commercially available RT-PCR systems allowing the analysis of 96–384 samples in 35–55 min (12). It has been previously reported that qRT-PCR enables a higher rate of UU detection compared to the culture method (13–15), mainly due to a substantial improvement in the detection of very low levels of pathogens. In addition, it has been used to detect and distinguish the two biovars of UU.
Data are reported as means ± SD. L-group: low concentration group; I-group: intermediate concentration group; H-group: high concentration group.
Genital swab samples

Genital swabs (made of medical absorbent cotton, urethral swabs from males and vaginal swabs from females) were collected from all participants and placed into separate transport tubes (Yangzhou JiKang Medical Instrument Ltd., China) and were immediately transported to the Department of Clinical Laboratory in a dry tube environment, where they were hydrated and tested for CT, UU, and NG by qRT-PCR. Nucleic acid extraction and qRT-PCR were performed using CT, UU, and NG DNA Fluorescence Diagnostic Kits (Sansure Biotech, China; Product No. 3400143, 3400146, and 3400142, respectively, with Registration License (2011) by China Food, Drug and Medical Device Administration). DNA and nucleic acid extraction of the samples were performed following the manufacturer’s recommended protocol. Briefly, the genital swab was rehydrated with 1 mL 0.9% normal saline and swirled vigorously for 1 min; then, 500 μL of the rehydrated sample was transferred to a 1.5-mL reaction tube (DNA LoBind Tubes, Eppendorf, Germany) and centrifuged at 15,800 g for 5 min at 4°C. 50 μL DNA extraction buffer was added to each tube after the supernatant was removed. Samples were mixed thoroughly, spun down briefly, and heated at 95°C for 10 min. Finally, each sample was centrifuged at 15,800 g for 5 min at 4°C and divided into 5-μL aliquots (DNA LoBind Tubes, Eppendorf). One aliquot of each sample was used immediately for qRT-PCR analysis, and the remaining aliquots were stored at 4°C and processed on days 1, 2, 3, 7, 14, 21, or 28.

qRT-PCR analysis

A 5-μL template DNA from CT, UU, or NG as prepared by the previously described DNA extraction method was subjected to the commercially available PCR kits mentioned above. Amplification was performed using a SLAN-96P Real-Time PCR System (Sansure Biotech) with the following protocol: an initial 50°C, 2-min step for uracil N-glycosylase enzyme reaction followed by a 94°C, 5-min Taq enzyme activation step, 45 cycles at 94°C for 15 s and 57°C for 30 s for denaturation, an annealing (with fluorescence monitoring) step, and an elongation phase. Considering the possibility of false-positive results and the presence of qRT-PCR inhibition, both a negative and a positive control were included in each run. Finally, CT, UU, and NG concentrations were determined based on a four-point standard curve generated by amplifying 1 x 10^3, 1 x 10^4, 1 x 10^5, and 1 x 10^6 copies each of CT, UU, or NG during each amplification. The detection limit of these PCR kits was 4 x 10^3 copies/mL, and their inter- and intra-assay coefficients of variation were all within 10%. Each qRT-PCR analysis for CT, UU, and NG samples was carried out in the same run by one technician using the same batch kit.

Statistical analyses

Before analyses, the data were log10 transformed. A paired-sample t-test was performed using SPSS version 16.0 (SPSS Inc., USA) to assess any significant differences in concentrations of CT, UU, and NG at different storage times. All data are reported as means ± SD and the significance level was set at P < 0.05.

Results

CT, UU, and NG samples from the 70 patients were successfully analyzed, without any inhibition or false-positive results. The DNA concentrations at days 0, 1, 2, 3, 7, 14, 21, and 28 are shown in Table 2. For CT DNA detection, the mean level in the low-concentration group ranged from 3.34 to 3.92 log10 copies/mL. Interestingly, an increased DNA level was observed at day 28 compared to

| Groups                  | Day 0    | Day 1    | Day 2    | Day 3    | Day 7    | Day 14   | Day 21   | Day 28   |
|-------------------------|----------|----------|----------|----------|----------|----------|----------|----------|
| **Chlamydia trachomatis** |          |          |          |          |          |          |          |          |
| L-group                 | 3.34 ± 0.36 | 3.78 ± 0.67 | 3.84 ± 0.54 | 3.82 ± 0.69 | 3.68 ± 0.55 | 3.75 ± 0.76 | 3.61 ± 0.73 | 3.92 ± 0.92 |
| P                       | 0.162    | 0.114    | 0.130    | 0.281    | 0.193    | 0.378    | 0.069    |          |
| P-group                 | 4.54 ± 0.28 | 4.71 ± 0.47 | 4.85 ± 0.41 | 4.83 ± 0.56 | 4.65 ± 0.47 | 4.51 ± 0.48 | 4.76 ± 0.43 | 4.62 ± 0.37 |
| P                       | 0.363    | 0.107    | 0.118    | 0.544    | 0.883    | 0.261    | 0.677    |          |
| H-group                 | 6.53 ± 0.83 | 6.55 ± 0.89 | 6.62 ± 1.02 | 6.69 ± 0.77 | 6.67 ± 0.86 | 6.53 ± 0.83 | 6.59 ± 0.85 | 6.49 ± 0.87 |
| P                       | 0.956    | 0.810    | 0.684    | 0.721    | 0.998    | 0.858    | 0.922    |          |
| **Ureaplasma urealyticum** |          |          |          |          |          |          |          |          |
| L-group                 | 3.47 ± 0.29 | 3.93 ± 0.56 | 3.57 ± 0.45 | 3.99 ± 0.42 | 3.69 ± 0.61 | 3.64 ± 0.81 | 3.71 ± 0.64 | 3.74 ± 0.80 |
| P                       | 0.095    | 0.725    | 0.075    | 0.422    | 0.527    | 0.386    | 0.320    |          |
| P-group                 | 4.56 ± 0.29 | 4.72 ± 0.38 | 4.79 ± 0.51 | 4.84 ± 0.45 | 4.72 ± 0.32 | 4.56 ± 0.53 | 4.62 ± 0.42 | 4.76 ± 0.39 |
| P                       | 0.424    | 0.236    | 0.395    | 0.952    | 0.787    | 0.311    |          |          |
| H-group                 | 5.53 ± 0.44 | 5.53 ± 0.39 | 5.70 ± 0.59 | 5.85 ± 0.38 | 5.71 ± 0.46 | 5.68 ± 0.48 | 5.66 ± 0.36 | 5.75 ± 0.44 |
| P                       | 1.000    | 0.399    | 0.139    | 0.388    | 0.469    | 0.524    | 0.275    |          |
| **Neisseria gonorrhoeae** |          |          |          |          |          |          |          |          |
| One group               | 6.29 ± 1.39 | 6.39 ± 1.45 | 6.17 ± 1.50 | 6.32 ± 1.38 | 6.33 ± 1.39 | 6.30 ± 1.31 | 6.25 ± 1.34 | 6.34 ± 1.29 |
| P                       | 0.883    | 0.840    | 0.965    | 0.951    | 0.994    | 0.945    | 0.933    |          |

Data are reported as means ± SD (log10 copies/mL). L-group: low concentration group; I-group: intermediate concentration group; H-group: high concentration group. A paired-sample t-test was used for statistical analyses.
day 0, but this difference was not statistically significant (P=0.069). Data from all 30 samples tested in days 0 and 28 are also shown in Figure 1, which shows a near overlap for sample 2 between days 0 and 28. With regards to the intermediate concentration group, the mean concentration ranged from 4.51 to 4.85 log₁₀ copies/mL, showing a similar non-significant increase between days 0 and 28 (P=0.677). Three overlaps (samples 15, 16, and 19) were found among the samples tested at days 0 and 28 in this group (Figure 1). The high-concentration group had a high CT DNA level in the range 6.53–6.69 log₁₀ copies/mL, which decreased from 6.53 to 6.49 log₁₀ copies/mL over the 28-day period. However, the differences were not statistically significant and half of the sample was overlapped between days 0 and 28.

For the UU DNA analyses, the concentration of the low UU DNA group ranged from 3.47 to 3.99 log₁₀ copies/mL, increasing from 3.47 to 3.74 log₁₀ copies/mL over the 28-day period, although the difference was not significant; two overlaps (samples 2 and 6) were observed (Figure 2). The intermediate and high UU DNA concentration samples showed similar results, with DNA levels ranging from 4.56 to 4.84 and 5.53 to 5.85 log₁₀ copies/mL, respectively, and with no differences being statistically significant (Table 2).

The mean NG DNA concentration ranged from 6.17 to 6.39 log₁₀ copies/mL. During the 28-day period, the mean NG DNA increased from 6.29 to 6.34 log₁₀ copies/mL, but similar to the previous results, the difference was not statistically significant (P=0.933). Three overlaps between samples tested at days 0 and 28 were observed (samples 4, 5, and 6; Figure 3).

Discussion

Given the rapid, specific, sensitive, and quantitative detection of CT, NG, and UU DNA, qRT-PCR is becoming the method of choice in research and screening programs with a centralized batch processing of clinical specimens (19). However, hospitals in remote areas and with limited laboratory capacities still have to transport samples to distant clinical laboratories that offer DNA testing. Thus, optimized sample collection, transportation, and storage of the specimens are of great importance. The detection ability of untreated specimens of CT, NG, and UU following various storage conditions using qRT-PCR has been explored in several studies (17,18,21), albeit with controversial results. The storage of extracted DNA should be practical both for sample transportation and further sample reanalysis. Nevertheless, the effect of different storage conditions on the detection of the extracted DNA load of these organisms using qRT-PCR has not been thoroughly assessed in clinical specimens. The present study therefore assessed the optimum DNA storage conditions to ensure the accuracy and reproducibility of test results with qRT-PCR.
The results presented herein show that no significant differences in CT, NG, and UU DNA loads were observed between immediate detection and detection following storage, in any of the 70 samples. In addition, for CT and UU detection, the collected samples were further divided into three categories based on their range of DNA concentration, including samples with low, intermediate, and high CT or UU DNA loads. Importantly, similar nonsignificant results were observed after stratification. These findings indicate that the DNA of CT, NG, and UU stored at 4°C is stable for at least 28 days, regardless of the initial DNA concentration. Thus, if samples cannot be immediately assayed, their DNA could be extracted and stored at 4°C for up to 28 days, and subsequently transported if necessary.

Freise et al. (22) explored the influence of DNA storage on the sensitivity of detection limits of CT elementary bodies (EB) at −20°C for up to 4 months; however, dramatically decreased detection limits (10- to 1000-fold) for CT EBs were observed. These results are inconsistent with those presented herein, and may be attributed to the following observations: first, the different DNA extraction methods used could lead to varying detection sensitivities after DNA storage. As reported by Freise et al. (22), an average 1000-fold decrease in detection rate was observed when DNA was extracted using Qiaex II Gel Extraction Kit and CTAB, while a 10-fold decrease was found when using alkaline lysis. Secondly, only one time-point (4 months) was used in their study. Finally, samples with serial dilutions of cultured CT EB were used in their study, which may not reflect real clinical samples. Therefore, our results are not comparable to those of that study, given the different DNA extraction methods, storage temperature, and time-points used. Given that few studies have assessed the impact of storage conditions on the accuracy of qRT-PCR DNA detection, further studies with larger numbers of clinical specimens should be conducted to confirm these results as well as to determine whether sample stability would be observed for CT, NG, and UU DNA loads after much longer term storage.

Overall, the CT, UU, and NG DNA loads from all genital swab samples were stable at 4°C over a 28-day period. These findings can therefore be applied in the shipment of clinical specimens, when analysis is postponed due to a laboratory backlog, or when samples require reevaluation to confirm prior results.

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