Short Report: Impact of Short-Time Urine Freezing on the Sensitivity of an Established Schistosoma Real-Time PCR Assay

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Abstract. Urogenital schistosomiasis is a serious public health problem in sub-Saharan Africa. In this study, we have updated an established real-time polymerase chain reaction (PCR) routinely used in our laboratory. Schistosoma genus-specific real-time PCR was performed on DNA isolated from 85 urine samples and pellets obtained after centrifugation without and after frozen storage. The results revealed that concentration by centrifugation of the urine samples and freezing of the samples before extracting DNA improves the sensitivity of the PCR.

Urogenital schistosomiasis is a chronic disease acquired in early childhood or in adulthood. In Africa, an estimated 120 million people are infected with Schistosoma haematobium, the etiologic agent.1 Untreated infections can lead to serious complications such as liver and bladder cancer, kidney failure, and infertility. Moreover, lesions of the vagina and cervix make females more vulnerable to sexually transmitted diseases, especially to human immunodeficiency virus (HIV).2,3 Therefore, it is critical that the diagnosis of urogenital schistosomiasis be done early using specific and sensitive methods. Urine filtration, the gold standard method has a limited sensitivity in areas of low endemicity or detection of prepatent infections.4,5 Moreover, these assays cannot distinguish live parasite infections were performed in a 25 μL reaction mixture containing 1 × HotstartTaq master mix (Qiagen), 5 mM MgCl₂, 17.5 μg bovine serum albumin (BSA), 80 nM of each Schistosoma genus-specific primer, 60 nM of each PhHV-1 primers, and 80 nM of each PhHV-1 primers. Aliquots of 200 μL of (concentrated) urine were first heated at 100 °C for 10 minutes before treatment with proteinase K for 2 hours at 55 °C. The DNA extraction was performed using spin columns from the QIAamp DNA mini kit (Qiagen, Hilden, Germany). In each sample, a fixed amount of Phocin herpes virus1 (PhHV-1) was added within the isolation lysis buffer to serve as internal control.17

The presence of S. haematobium eggs in urine samples was assessed by the urine filtration method. Parasite load was expressed as the number of eggs/10 mL and classified as low (1 < eggs < 49) and high (eggs > 50). Microscopic examination was performed by two independent readers and the average recorded.

Real-time PCR was performed using primers and probes as described previously.16 Schistosoma genus-specific primers Ssp48F (5'-GGT CTA GAT GAC TTG ATY GAG ATG -3') and Ssp124R (5'-TCC CGA GCG YGT ATA ATG -3') were used to amplify S. haematobium internal transcription spacer 2 (ITS2) and the 77-bp amplicon was detected with the probe Ssp78T (FAM-5'TGG GTG CTC GAG TCG TGGC-3'-Black Hole Quencher). (Biolegio, Nijmegen, The Netherlands). Amplification reactions were performed in a 25 μL reaction mixture containing 1 × HotstartTaq master mix (Qiagen), 5 mM MgCl₂, 17.5 μg bovine serum albumin (BSA), 80 nM of each Schistosoma genus-specific primer, 60 nM of each PhHV-1 primers, and 100 nM of Schistosoma and PhHV probe, and 5 μL of DNA sample. The program consists of an initial hold step at 95 °C for 15 minutes, followed by 50 cycles at 95 °C for 15 seconds and 60 °C for 60 seconds. The amplification was performed in the Corbett thermal cycler (Corbett Research, Sydney, Australia) and the analysis with the Rotor-gene 6000 Series software 1.7.

The protocol of this study was approved by the “Comité d’Ethique Institutionnel de l’Unité de Recherches Médicales,” Lambaréné, Gabon (protocol approval no. 005/2011). An informed consent form was obtained from each parent or legal guardian.
Schistosoma haematobium eggs were detected with urine filtration microscopy in 66 of 85 samples (77.7%). The median egg output was 26.6 (range 2–861) eggs/10 mL urine indicating a light intensity of infection in this area. Of those infected, 23 (34.8%) had a heavy parasite load (>50 eggs/10 mL) of 65.2% were children, 30.4% were teenagers, and 4.4% were adults (Table 1).

In all control and study samples tested, amplification of the internal control was detected at the expected cycle threshold (Ct)-value. Hence, there was no evidence of inhibition of amplification in any of these samples. No Schistosoma-specific amplification was detected in the Dutch control samples (N=25) indicating a specificity of 100%. The PCR results of the Gabonese urine samples are summarized in Table 2. In non-frozen and frozen urine, Schistosoma-specific DNA amplification was detected in 61 of 66 samples (92.4%) in which Schistosoma eggs were detected. Concentration of the urine by centrifugation improved the sensitivity of the PCR. Specific amplification of Schistosoma DNA was detected in 64 of 66 (97.0%) and 65 of 66 (98.5%) non-frozen and frozen urine pellets from microscopy positive samples, respectively. In addition, Schistosoma-specific amplification was detected in samples in which no Schistosoma eggs were found three consecutive times by microscopy, again this shows the highest detection rates using frozen pellets of concentrated urine for DNA isolation. The Ct-values were significantly higher for DNA samples from urine with a light intensity of infection in this area. Of those infected, 2% were children, 30.4% were teenagers, and 4.4% were adults (Table 1).

Concentration of urine allows DNA extraction and subsequent DNA amplification of a larger volume of urine that has been shown to be beneficial and when samples were filtered in the field using the filter papers for DNA extraction. Freezing of urine or the pellet from concentrated urine did not show a negative effect on the amplification of Schistosoma-specific DNA as found by others. On the contrary, in this study freezing increased the number of PCR-positive samples suggesting that freezing resulted in a more successful DNA release.

The findings in this study suggest that concentration by centrifuging and freezing urine before DNA extraction improves the sensitivity of the real-time PCR assay.

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REFERENCES


### Table 1

| Intensity of S. haematobium infections categorized by number of eggs found after urine filtration (10 mL) per age group compared with the number and detected values of subjects showing Schistosoma DNA in urine (Ct values) by real-time PCR of 85 participants from Zilé, Gabon |
|----------------|----------------|----------------|----------------|----------------|
| Sample Group | Sample Group | Sample Group | Sample Group | Sample Group |
| 6–12 years | 13–19 years | 20–39 years | Total | N (% | Median Ct | Range |
| ≥50 eggs/10 mL | 15 | 7 | 1 | 23 | 23 (100%) | 18.8 | 16.3–25.7 |
| 1–49 eggs/10 mL | 22 | 19 | 2 | 43 | 42 (97.7%) | 23.3 | 18.8–34.1 |
| Negative | 14 | 4 | 1 | 19 | 11 (57.9%) | 33.7 | 30.9–36.9 |

PCR = polymerase chain reaction.

### Table 2

<table>
<thead>
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<th>PCR positives (frozen, concentrated)</th>
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<tr>
<td>Microscopy</td>
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<tr>
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PCR = polymerase chain reaction.


