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).^{2–4} 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.^{5,6} Other diagnostic tools such as immunodiagnostic assays are sensitive but not very specific, and do cross-react with other helminth infections.^{7,8} Moreover, these assays cannot distinguish live parasite from dead parasite as antibodies raised against the antigen crude extracts used in these assays (adult worm antigen and soluble egg antigen) persist for several months even after successful treatment.^{9,10} Molecular approaches like real-time polymerase chain reaction (PCR) and loop-mediated isothermal amplification are increasingly used for the detection of *Schistosoma* infections.^{5,11–14}

In this study, we are assessing the effect of concentration by centrifugation and frozen storage of urine samples on the sensitivity of a *Schistosoma* real-time PCR assay routinely used in our laboratory.

Eighty-five participants (age range 6–39, median age 12 years, 59% male) residing in Zilé, a village located 15 Km from Lambaréné (Gabon) were recruited in this study. The village is rural and highly endemic for urogenital schistosomiasis.¹⁵ In addition, 25 healthy controls living in the Netherlands were included to assess the specificity of the PCR assay.

Urine containers (50 mL) were distributed to consenting parents or legal guardians at night and collected the following morning. Ten milliliters (10 mL) of fresh urine sample were analyzed through a 12- μ m pore size filter (EMD Millipore, Darmstadt, Germany) under the microscope for the presence of *S. haematobium* eggs. Another 10 mL of urine were thoroughly mixed. Next, two aliquots (200 μ L) of the mixed urine were collected; one was immediately processed for PCR and

the other frozen at -20°C . After mixing, urine samples were centrifuged for 5 minutes at $710 \times g$ in a Rotanta 460 R centrifuge (Hettich, Tuttlingen, Germany) and 9 mL of supernatant discarded. Two hundred microliters of the concentrated urine was immediately processed for PCR and the remaining stored frozen at -20°C for 7 months.

The DNA was isolated from urine samples as described previously.¹⁶ Aliquots of 200 μ L 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 ($1 < \text{eggs} < 49$) and high ($\text{eggs} > 50$). 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 CT-3') and Ssp124R (5'-TCC CGA GCG YGT ATA ATG TCA TTA-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 GTT GTG CTC GAG TCG TGGC-3'-Black Hole Quencher). (Biolegio, Nijmegen, The Netherlands). Amplification reactions were performed in a 25 μ L reaction mixture containing $1 \times$ HotstartTaq master mix (Qiagen), 5 mM MgCl_2 , 17.5 μ g 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 μ L 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 to 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.

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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

	Microscopy				PCR positives (frozen, concentrated)			
	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.

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 in which no *S. haematobium* eggs were found by microscopy compared with DNA samples from urine in which eggs were found (Mann-Whitney test; $P < 0.0001$). The difference between the Ct-values of DNA from urine with a low number of eggs and urine with a high number of eggs was significant as well (Mann-Whitney test; $P < 0.0001$).

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.¹⁴

TABLE 2

Number (%) of positive *Schistosoma*-specific real-time PCR results performed on DNA isolated from urine samples and pellets obtained after centrifugation without and after frozen storage in *Schistosoma haematobium* egg-positive and egg-negative urine samples from Zilé, Gabon ($N = 85$)

Samples from Linc, Sussex (n = 85)					
		PCR positives			
		Not frozen		Frozen storage	
		Mixed	Concentrated	Mixed	Concentrated
Microscopy					
Positive	N = 66	61 (92.4%)	64 (97%)	61 (92.4%)	65 (98.5%)
Negative	N = 19	4 (21%)	7 (36.8%)	6 (31.6%)	11 (57.9%)
Total	N = 85	65	71	67	76

PCR = polymerase chain reaction.

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.¹⁸ 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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