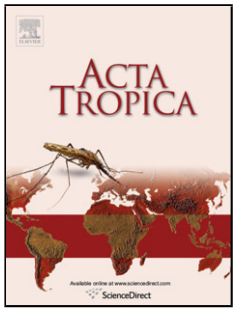


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# A diagnostic study comparing conventional and real-time PCR for *Strongyloides stercoralis* on urine and on faecal samples

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**ABSTRACT**

*Strongyloides stercoralis* is a soil-transmitted helminth with a wide distribution in tropical and subtropical areas. The diagnosis of *S. stercoralis* infection can be challenging, due to the low sensitivity of microscopic examination of stool samples and coproculture. In the last decade, different in-house molecular biology techniques for *S. stercoralis* have been implemented. They demonstrated good accuracy, although sensitivity does not seem sufficiently high yet. Recently, a novel PCR technique has been evaluated for the detection of *S. stercoralis* DNA in urine. Aim of this work was to compare the sensitivity of the real-time PCR (qPCR) on feces routinely used at the Centre for Tropical Disease (CTD) of Negrar, Verona, Italy, with that of the novel based PCR on urine. As secondary objective, we evaluated a Urine Conditioning Buffer ® (Zymoresearch) with the aim of improving nucleic acid stability in urine during sample storage/transport at ambient temperatures. Patients attending the CTD and resulting positive at routine screening with serology for *S. stercoralis* were invited, previous written consent, to supply stool and urine samples for molecular biology. A convenience sample of 30 patients was included. The sensitivity of qPCR on feces resulted 63%, and that of based PCR on urine was 17%. In all the samples treated with the Urine Conditioning Buffer ® there was no detectable DNA. In conclusion, the sensitivity of the novel technique resulted low, and needs further implementation before being considered as a valid alternative to the validated method.



**Keywords**: *strongyloides stercoralis*, DNA, diagnostic test, diagnosis, urine specimen, fecal specimen

## 1. INTRODUCTION

*Strongyloides stercoralis* is a soil – transmitted helminth (STH) that affects between 100 and 370 million people worldwide (Bisoffi et al., 2013; Schar et al., 2013). The parasite leads to a chronic infection that mainly causes no or mild symptoms. However the immunosuppressed hosts is at risk of a syndrome that is invariably fatal if not promptly treated and sometimes despite the treatment (Buonfrate et al., 2013; Greaves et al., 2013; Lam et al., 2006). Diagnosis is challenging: stool microscopy, commonly used for the other STH, has low sensitivity for the detection of *S. stercoralis*, while serology is highly sensitive but can have false positive results due to cross-reactions and long-term persistence of antibodies after treatment (Bisoffi et al., 2014). Over the last few years, DNA - based methods have been developed aimed to detect intestinal parasites in stool samples and in urine (Formenti et al., 2017; Friesen et al., 2018; Ibironke et al., 2012; Menu et al., 2018; Qvarnstrom et al., 2018; Schuurs et al., 2018), increasing the sensitivity and the specificity of the diagnosis (Lodh et al., 2013; Verweij et al., 2009). At the moment, molecular protocols for helminthic infections are mostly in-house methods, available at referral centers, although their use is expanding. Recently, a PCR based on the amplification of a highly repeated sequence of *S. stercoralis* in urine has been evaluated for the detection of *S. stercoralis* in specimens collected in an endemic area in Northern Argentina, and showed promising results (Lodh et al., 2016b).

The primary objective of this work was to compare the sensitivity of qPCR on feces for the diagnosis of strongyloidiasis with based PCR amplification on filtered urine. As secondary objective, we evaluated a Urine Conditioning Buffer ® Zymoresearch which aims to preserve DNA on urine samples up to 30 days before analysis.

## 2. METHODS

### 2.1.Study design and participants

This preliminary prospective study has been conducted on a convenience sample of 30 patients. All consecutive patients aged > 18 years attended at the Centre for Tropical Diseases, CTD – IRCCS Sacro Cuore Don Calabria Hospital, Negrar, Verona, Italy and presenting a positive serology for *S. stercoralis* were offered to participate to the study. Patients who gave written informed consent were asked to supply a stool and a urine sample. The qPCR on stool was done at the CTD, as for normal practice. Based PCR on urine was performed at the John Hopkins Bloomberg School of Public Health, Baltimore, United States.

**Ethical issues**

The study protocol received ethical clearance by the local competent Ethics Committee, Comitato Etico per la Sperimentazione Clinica delle Province di Verona e Rovigo, protocol number 7406 and cleared for anonymous use by Johns Hopkins University IRB number 6199.

### 2.2.Test methods: Serology

The serological test used is an immunofluorescence IFAT test routinely used at the CTD. It is an in-house method that detects IgG antibodies against *S. stercoralis*. For antigen preparation, intact *S. stercoralis* filariform larvae are obtained from a positive charcoal fecal culture, as it has been described previously (Boscolo et al., 2007). Negative and positive controls are placed in the wells of a slide. The same slides contains patient's sera at different dilutions. The reaction is read using a fluorescence microscope. More details can be found at (http://www.tropicalmed.eu). Samples with antibody titers ≥1:20 are considered positives.

### 2.3.DNA extraction from feces

Stool specimens were collected as described previously (Formenti et al., 2015) according the protocol procedure of our laboratory. The DNA was extracted using the MagnaPure LC.2 instrument Roche Diagnostic, Monza, Italy, following the protocol "DNA I Blood_Cells High performance II", using the kit "DNA isolation kit I" Roche. The DNA was eluted in a final volume of 100 µl.

### 2.4.Filtered urine preparation

Two aliquots of approximately 40 ml of urine were collected between 8 and 11 am, as described by Lodh et al., 2016. Briefly; one aliquot was filtered through a 12.5 cm Whatman No. 3 disc, at the latest after 2-3 h from the collection of urine specimen. The second aliquot was treated with a urine preservative ,Urine Conditioning Buffer, Zymo Research and filtrated after 24 h. The filter disc was dried under a fly proof cover and stored in a sealed plastic bag with desiccant at 4˚C for the shipment to Baltimore.

### 2.5.DNA extraction from urine for conventional PCR

Filter papers received in Baltimore were treated similarly to the previous extraction (Lodh et al., 2016b): papers obtained by the use of Zymo Urine Conditioning Buffer were processed separately. Each filter disc was removed from its plastic sheath, and 15 1.0 mm diameter discs were removed from filter, transferred to a 2.0 ml Eppendorf tube and 800µL nuclease free water was added. After incubation at 95 ºC for 10 min, the samples were subjected to gentle agitation over night at room temperature. Following the overnight stand, tubes were centrifuged at 4000 rpm to pack the paper, and the supernatant removed. Each sample was processed using QIAmp DNA Blood Mini Kit Qiagen,MD according to manufacturers protocol. The amount of recovered DNA was measured by NanaDrop ND-1000 spectorphotometer Thermo Scientific Milwaukee, WI and stored at -20 ºC.

### 2.6.qPCR

The protocol for qPCR followed the one described by (Verweij et al., 2009). Positive and negative controls were included in all the experiments; positive controls were two pools of positive DNA for the targets included in the qPCR. One had a low Ct ( 30<Ct<36) and the other a high Ct (37<Ct<39.9). For all the qPCR analysis, the threshold was set at 200. As a control for qPCR inhibitors and amplification, the exogenous PhHV-1 DNA was amplified with the the following primers and probe mix. (PhHV-267s F 5'-GGGCGAATCACAGATTGAATC -3', PhHV-337as R 5'-GCGGTTCCAAACGTACCAA -3', PhHV-305tq Cy5 -5'-TTTTTATGTGTCCGCCACCATCTGGATC-3'-BHQ2).

**Conventional PCR**

Primers were designed specifically to amplify a 125bp fragment from **a** *S. stercoralis* dispersed repetitive sequence ,GenBank: AY08262 (Lodh et al., 2016a). The forward primer was SSC-F 5' CTC AGC TCC AGT AAA GCA ACA G 3' and reverse primer was SSC-R 5' AGC TGA ATC TGG AGA GTG AAG A 3'. PCR amplification was in a 15µL volume with Taq 2X Master-mix, New England Biolabs, Ipswich, Mass., 0.75 µL, of 10µL of each primer, 1-2 µL 20-100 ng/ µL of DNA, $McCl_2$ and PCR grade water. The amplification profile was initial denaturation at 95 C for 10 min, and 35 cycles at 95C for 1 min 63 C for 1 min 30 sec, 72C for 1 min and a final extension at 72 C for 10 min. To confirm amplification and amplicon size the PCR products were resolved on a 2% agarose gel stained with Ethidium Bromide, Sigma-Aldrich, St. Louis, Mo.

### 2.8 Statistical analysis

The results of qPCR on feces and conventional PCR on filtered urine are presented in a contingency tables. Sensitivities (Se) and false negative probabilities (FNP) for each test were calculated comparing their results with the results of IFAT. The results of the two techniques were also stratified based on an

antibody titer threshold of 160, that defines results close to 100% specificity, as previously described (Bisoffi et al., 2014). The concordance between the two tests were estimated by the overall, positive and negative agreements. Ratio of sensitivities, the McNemar's test and Kappa coefficient are presented to further assess the agreement between the two tests. All estimated parameters are presented with their respective 95% confidence intervals (CI) and statistical significance level fixed at 5%.

## 3. RESULTS

Participation to the study was proposed to 30 consecutive eligible patients: they all accepted and supplied both stool and urine samples (Figure 1).

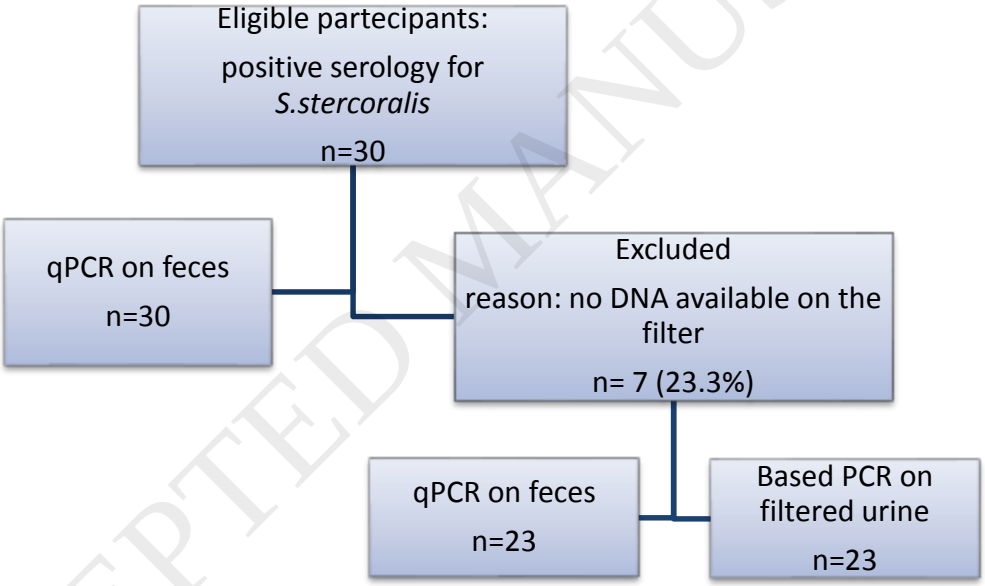


**Figure 1. Flow diagram for patients selection and samples analyzed.**

Of these 30 patients tested, 9 (30%) were female and 21 (70%) male; the median age was 30.5 (IQR 25 to 53). Three patients were from South-America, 5 from Italy, and 22 from Africa. Seven samples were excluded from the analysis of the conventional PCR on urine and also from the tests concordance analysis, as there was no human detectable DNA on the filters. In the full dataset 19/30 patients (63.3%)

were positive at the qPCR on feces. Considering the 23 patients dataset 15 subjects (65.2%) were positive at the qPCR on feces, and 4 (17.4%) were positive at conventional PCR on urine. The two tests measures of accuracy are presented in Table 1.

**Table 1. qPCR on feces and conventional PCR on filtered urine measures of accuracy. Se = Sensitivity, FNP = False Negative Probability. * We compared the results including both the 23 and 30 samples.**

| Positive Serology (n=30) | | | |
|---|---|---|---|
| | qPCR on feces | | Conventional PCR on filtered urine |
| **Number of samples tested** | 30* | 23* | 23 |
| **Positive samples** | 19 | 15 | 4 |
| **Se (95% CI)** | 63.3% (43.9-80.1) | 65.2% (42.7-83.6) | 17.4% (5-38.8) |
| **FNP (95% CI)** | 36.7% (19.9-56.1) | 34.8% (16.4-57.3) | 82.6% (61.2-95.1) |

The concordance between the two tests, considering the 23 patients datasetis presented in Table 2. The overall, positive and negative agreements are 52.2% (CI 31.8 to 72.6), 26.7% (CI 7.8 to 55.1) and 100.0% (CI 64.1 to 100.0), respectively. We further assessed the agreement between the qPCR and PCR tests sensitivities through McNemar's test, exact p-value of 0.001, and the kappa coefficient of 0.2019 (CI -0.0034 to 0.4072).

**Table 2. qPCR and PCR tests agreement.**

| | PCR on filtered urine | | |
|---|---|---|---|
| **qPCR on feces** | + | - | Total |
| + | 4 | 11 | 15 |

| | | | |
|---|---|---|---|
| - | 0 | 8 | 8 |
| Total | 4 | 19 | 23 |

The results of qPCR and PCR were classified according to IFAT titer (Table 3). The overall, positive and negative agreement derived from these results are 60% (CI 14.7 to 94.7), 50% (CI 6.7 to 93.2) and 100% (CI 2.5 to 100), respectively for titer less than 160. For titers bigger or equal 160 instead, the overall, positive and negative agreement estimates are 50% (CI 26.0 to 73.9), 18.2% (CI 2.3 to 51.8) and 100% (CI 59.0 to 100), respectively.

**Table 3. qPCR and PCR tests agreement stratified for antibody titer**

| **qPCR on feces** | **PCR on filtered urine** | | | **Serology titer** |
|---|---|---|---|---|
| | + | - | Total | |
| + | 2 | 2 | 4 | < 160 |
| - | 0 | 1 | 1 | |
| Total | 2 | 3 | 5 | |
| + | 2 | 9 | 11 | **≥160** |
| - | 0 | 7 | 7 | |
| Total | 2 | 16 | 18 | |

## 4. DISCUSSION

While the sensitivity of the qPCR resulted in line with previous results (Buonfrate et al., 2017; Buonfrate et al., 2018), the sensitivity of conventional PCR on urine appeared in contrast with previous findings (Lodh et al., 2016b). In particular, the agreement between the two techniques resulted low to poor when

comparing the overall and positive agreement probabilities and also when further assessing the Cohen's Kappa coefficient and McNemar's test of the difference between sensitivities. Moreover the dataset presented 7 samples where no DNA was detectable, probably due to a problem during the DNA extraction process as confirmed by the analysis at Nanodrop. It would be important to further understand whether a certain amount of patients would undergo a second round tests. In terms of results, if the "non-DNA cases" were to be considered negatives, the conventional PCR sensitivity would drop to a sensitivity as low as 13.3% (CI 3.8 to 30.7). There are some reasons that might have caused the discrepancy in the results of the two PCR techniques. First, the test against which the conventional PCR was compared with: although Baermann method and Harada Mori culture have a higher sensitivity than stool microscopy for the diagnosis of *S. stercoralis* infection (Requena-Mendez et al., 2013), they still miss a high proportion of infections, misclassifying some positive results as negative. This tends to cause an overestimation of the sensitivity of the test evaluated. On the other hand, serology is currently the diagnostic method with the highest sensitivity (Requena-Mendez et al., 2013) for the diagnosis of strongyloidiasis, thus finding a higher proportion of positive cases in a population compared to any other method so far available. Other factors might have influenced the results, for instance the different settings, in particular in relation to two different aspects: i) the patients enrolled in Argentina (Lodh et al., 2016b) might have had a higher level of infection, entailing higher levels of parasite DNA detectable in urine; this is plausible also considering that those patients were enrolled on the basis of positive fecal tests; and ii) the possible presence of different *S. stercoralis* genotypes, not all of them necessarily detected by the conventional PCR in urine. Although at the moment there is no evidence supporting the existence of different genotypes of *S. stercoralis* with different geographical origin, it might be possible that different PCR primers identify only some genotypes. The authors acknowledge the limited sample size considered in this preliminary study; indeed further studies need to be done in order to evaluate the

potential use and implementation of the PCR on specimens other than stool. In particular, the advantage of a molecular method on urine is that filtration is carried out in the field and urine can be obtained simply, filtered and dried within 2-3 hours, and when the specimen is dry, it can be put in a plastic bag with desiccant and transported with little cost to a centre for analysis.

## 5. CONCLUSION

Our preliminary results showed an unsatisfactory sensitivity and high FNP of the conventional PCR on urine. Even though this new technique on urine has several practical advantages, especially for use in the field, further improvements are needed to consider it a reliable alternative method for the diagnosis of *S. stercoralis* infection.

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