Optimization of a Diagnostic Assay for the Detection of *Schistosoma mansoni* in Biomphalaria sudanica

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INTRODUCTION

Schistosoma mansoni is the causative agent for intestinal schistosomiasis, a neglected tropical disease responsible for severe morbidity in regions of Sub-Saharan Africa. Humans become infected when contacting contaminated water infested by cercariae shed by freshwater snail intermediate hosts (1). Detecting snail infections using microscopy is a critical component of transmission site monitoring, but the development time of schistosomes in snails is variable (6-22 weeks) and thus, delayed infections may be missed using microscopy. There is a need for a PCR-based diagnostic assay to detect *S. mansoni* in African snail vector species, *Biomphalaria sudanica*, to provide the capacity for detecting infections in both field-collected and laboratory-bred snails that are not displaying patent schistosome infections.

OBJECTIVE

Problem: Routine diagnostics for snail infection with schistosomes is to visualize parasites released from the snails via microscopy. Parasites take 6-22 weeks to develop, and thus many infections are missed with this method.

Our overall goal is to develop a highly sensitive method for detecting early *Schistosoma mansoni* infection in field obtained and experimentally infected *Biomphalaria sudanica* snails.

Uses: Surveillance of transmission sites, assessing control program efficacy, assessing infection status in experimentally challenged snails to ensure accuracy and efficiency of results when investigating resistance.

Strategy: Develop a multiplex PCR assay that amplifies a specific genomic region of the snail (internal control) and a specific genomic region of *S. mansoni* (diagnostic). We will address the following:

- **Question 1:** What test sample gDNA dilution provides the best multiplex PCR amplification?
- **Question 2:** Will adjustment of the primer ratio improve co-amplification of our multiplex PCR?
- **Question 3:** What is the limit of detection of our most promising multiplex candidate protocol?
- **Question 4:** Will an inhibitor resistant polymerase improve amplification with reduced sample dilution?

STUDY DESIGN

Diagnostic assay PCR to address Questions 1 and 2:

- **Experimentally infected Kenyan *B. sudanica*:** *B. sudanica* snails from Kenya were exposed to *S. mansoni* miracidia and left to reach patency for 8 weeks were either deemed to be positive (n=7) or negative (n=9) for schistosome infection by microscopy (cercarial shedding). These snails are being used for a genome wide association study (GWAS) comparing allele frequencies of infected and uninfected snails. gDNA was extracted by the Qiagen Blood and Tissue DNA extraction kit.
- **Markers**: Snail and *S. mansoni* specific markers were trialed in duplex and multiplex PCRs (Table 1). Primers were trialed in equal or skewed concentrations to account for amplification bias during PCR.
- **Master mix**: All PCRs were performed using the GoTaq Colorless Master Mix (Promega, Madison, USA)
- **gDNA:** To control for the effect of PCR inhibitors in extracted snail gDNA samples (2), PCRs were performed using extracted snail gDNA that had been diluted 1:10 and 1:100 with nuclease free water.

Table 1: PCR primer targets and the expected length of amplification products

Marker	Snail or parasite	Region	Expected Length
ND5	Parasite	NADH dehydrogenase 5	302 BP
ITS	Parasite and Snail	Internal Transcribed Spacer Region	Parasite: ~1200 BP Snail: ~1000 BP
GRCB	Snail	Guadeloupe Resistance Complex	567 BP
ND4	Snail	NADH dehydrogenase 4	~400 BP

Limit-of-detection (LOD) assay PCR to address Question 3:

- **Laboratory inbred unexposed *B. sudanica*:** 1 snail of an inbred *B. sudanica* laboratory strain (110) with no previous exposure to schistosomes, or other parasites, was extracted by a modified Cetyl Trimethylammonium Bromide (CTAB) extraction protocol (5).
- ***Schistosoma mansoni* adult worm DNA:** A single male adult *S. mansoni* was extracted by the Qiagen extraction kit. gDNA extract concentration were measured using the NanoDrop Spectrophotometer.
- **Markers:** Snail (ND4) and *S. mansoni* (ND5) specific markers were trialed in multiplex PCR reactions. Primers were run at a 3:1 ratio (ND4:ND5).
- **Master mix**: All PCRs were using the GoTaq Colorless Master Mix (Promega, Madison, USA)
- **gDNA:** 110 snail gDNA was mixed at a 1:1 ratio with male adult *S. mansoni* gDNA. This solution was diluted to 1:1, 1:10, 1:50, 1:100, and 1:1000 with nuclease free water to determine the limit of detection.

Utilization of Phire polymerase to address Question 4:

- **Laboratory inbred unexposed *B. sudanica*:** Unexposed inbred *B. sudanica* snails (same snails utilized for LOD assay) were extracted using Qiagen DNA extraction kit.
- ***Schistosoma mansoni* miracidia DNA:** *S. mansoni* miracidia were extracted by Qiagen DNA extraction kit.
- **Markers:** Snail (ND4) and *S. mansoni* (ND5) specific markers were trialed at a 1:1 ratio in multiplex PCR reactions. Preliminary testing showed little benefit from skewed primer ratio.
- **Master mix:** All PCRs were performed using Phire Hot Start II DNA Polymerase (ThermoFisher Scientific).
- **gDNA:** 1 *B. sudanica* gDNA was mixed with the gDNA of 1, 2, 5, or 10 *S. mansoni* miracidia. The solutions were diluted to 1:2 or 1:10 using nuclease free water.
- **Application to GWAS snail samples:** The experimentally infected Kenyan *B. sudanica* snails being used for the GWAS study are describe above (Diagnostic assay PCR to address **Questions 1 and 2**).

Gel: Amplified DNA of all snail and schistosome PCRs were analyzed using 1.5% agarose gel electrophoresis.

RESULTS – DIAGNOSTIC ASSAY

Question 1: What test sample gDNA dilution provides the best multiplex PCR amplification?

Snail Dilutions	ND5 Concentration	Snail Primer Concentration (All 0.75 µL)	Amplification (%)	Nonspecific Amplification(%)	No Amplification (%)
1:10 (n=16)	0.75 µL	ITS1-5.8S-ITS2	31%	38%	31%
		GRCB	50%	25%	25%
		ND 4	19%	0%	81%
1:100 (n=8)	0.75 µL	ITS1-5.8S-ITS2	100%	0%	0%
		GRCB	25%	25%	50%
		ND 4	50%	0%	50%

Table 2: Multiplex PCR screening of 16 Kenyan snail samples (7 positive, 9 negative) at 1:10 dilutions. The 1:100 dilution screened 8 Kenyan snail samples (3 positive, 5 negative). All PCR screenings were performed with a 1:1 primer ratio (snail marker:ND5). Red numbers denote samples with non-specific amplification.

Interpretation:

- **Full concentration test sample gDNA resulted in poor amplification of ITS1-5.8S-ITS2 and ND4.**
  - May be due to the presence of PCR inhibitors (2).
- **Dilution to 1:10 displayed greater amplification for ITS1-5.8S-ITS2 and ND4.**
  - ITS1-5.8S-ITS2 and GRCB displayed non-specific amplification. ND5 amplified in 100% of positively infected
  - samples.
- **Dilution to 1:100 further improved amplification for ITS1-5.8S-ITS2 and ND4, but not GRCB.**
  - ITS1-5.8S-ITS2 bands were large and smeared. ND4 amplified in all negatively infected snails, but no amplification seen in positively infected snails. ND5 amplified in 100% of positively infected samples.
  - Primer interference between ND4 and ND5 may be a potential explanation for this observation.
- **Overall, 1:100 dilution of Kenyan snail samples improved amplification of ITS1-5.8S-ITS2 and ND4 markers.**

Question 2: Will adjustment of the primer ratio improve co-amplification of our multiplex PCR reaction?

Snail Dilutions	ND5 Concentration	Snail Primer Concentration (All 0.75 µL)	Amplification (%)	Nonspecific Amplification(%)	No Amplification (%)
1:100 (n=8)	0.25 µL	ITS1-5.8S-ITS2	100%	0%	0%
		GRC	50%	25%	25%
		ND 4	100%	0%	0%

Table 3: Multiplex PCR screening of 8 Kenyan snail samples (3 positive, 5 negative) at a 1:100 dilution. All PCR screenings were performed with a 3:1 primer ratio (snail marker:ND5). Red numbers denote conditions with 100% amplification.

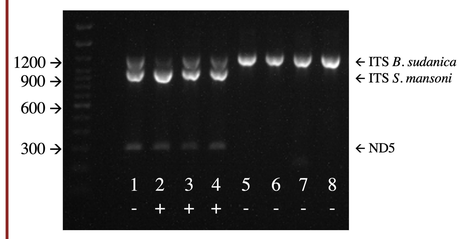

Figure 1: ITS1-5.8S-ITS2 multiplex PCR screening of 8 Kenyan snail samples (3 positive (+), 5 negative (-)) at a 1:100 dilution and 3:1 ITS1-5.8S-ITS2 :ND5 primer concentration.

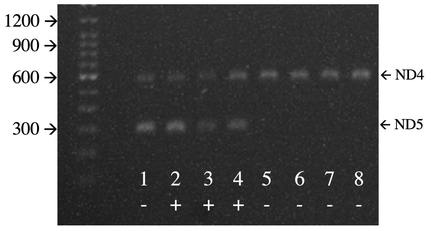

Figure 2: ND4/ND5 multiplex PCR screening of 8 Kenyan snail samples (3 positive (+), 5 negative (-)) at a 1:100 dilution and 3:1 ND4:ND5 primer concentration.

Interpretation:

- ITS1-5.8S-ITS2/ND5 continued to display large smeared bands (Figure 1) as seen in previous gels.
- ND4/ND5 primer ratio adjustment to 3:1 resulted in 100% co-amplification in positively infected samples (samples 2-4, Figure 2).
- **Overall, ND4/ND5 with at 3:1 primer ratio is the most promising multiplex candidate for infection screening of field obtained, and laboratory exposed, snails.**

Question 3: What is the limit of detection of our ND4/ND5 multiplex PCR?

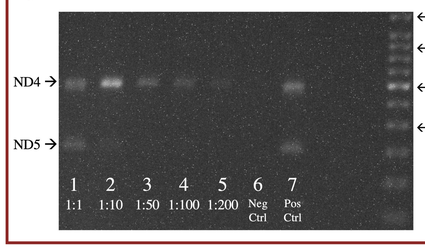

Figure 3: ND4/ND5 multiplex PCR with a 3:1 primer ratio. Template dilution series of a 1:1 mixed gDNA solution (110 snail with S. mansoni adult male).

Interpretation:
The limit of detection for ND5 was at a 1:10 dilution which contained **0.36-0.39 ng** of *S. mansoni* adult male gDNA.

RESULTS – PHIRE POLYMERASE AND APPLICATION

Question 4: Will an inhibitor resistant polymerase improve amplification with reduced sample dilution?

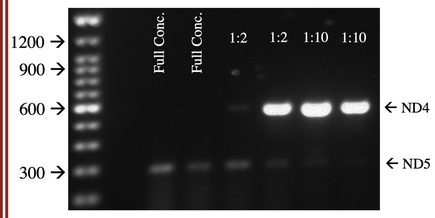

Figure 4: Phire polymerase ND4/ND5 (1:1) multiplex PCR of 2 laboratory inbred snail samples spiked with 5 miracidia each. gDNA was run at full concentration or diluted 1:2 and 1:10.

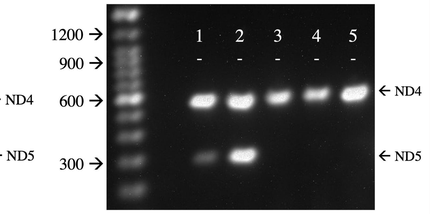

Figure 5: Phire polymerase ND4/ND5 (1:1) multiplex PCR screening of experimentally infected Kenyan *B. sudanica* snails (5 negative (-)). Samples were diluted to 1:10 with water.

Interpretation:

- **Dilution to 1:10 displayed 100% ND4 amplification (n=32) in laboratory strain *B. sudanica* snail gDNA extract spiked with miracidia gDNA (Figure 4).**
  - ND4 failed to amplify at full concentration gDNA extract. ND4 amplification increased to 60% when diluted to 1:2.
- **Application of method was able to pick up prepatent infection in challenged first generation offspring of wild caught Kenyan snails who were non-shedding at 8 weeks (GWAS snail samples; Figure 5, lanes 1 and 2).**
- **When applied to our GWAS snail samples, 11% failed to amplify ND4 at the 1:10 gDNA dilution. All GWAS samples retested at 1:100 gDNA dilution displayed proper amplification of ND4.**

CONCLUSION

Although not finalized yet, we have made significant progress in the design of a *S. mansoni* diagnostic assay. We have four main conclusions that we are using to move forward with an improved assay:

1) Full concentration snail DNA contained inhibitors that interfered with our PCR amplification.

- Snail gDNA sample dilutions of 1:100 were necessary to remove PCR inhibitors present in snail extracts when using the GoTaq system.
- Sample dilution poses an issue by reducing the concentration of schistosome gDNA in test samples which could affect the limit of detection of our diagnostic assay.
- Trialing new Taq polymerase reagents / protocols that are more resistant to inhibitors will help avoid unnecessary, and potentially detrimental, test sample dilution.

2) The ND4/ND5 multiplex PCR (1:100 dilution, 3:1 primer ratio) worked best when identifying infection status in field obtained snail samples.

- When the ND4/ND5 primer ratio was adjusted from 1:1 to 3:1 to account for amplification bias, ND4 and ND5 bands were consistent and easily distinguishable on the same agarose gel.
- The GRC B and ITS1-5.8S-ITS2 primers displayed amplification variability which may make interpretation of PCR results more difficult. The ITS1-5.8S-ITS2 primer also displayed large widened amplicons.

3) The limit of detection of the ND5 *S. manoni* marker multiplexed with ND4 was not as high as expected.

- Previous publications report a limit of detection (LOD) of 0.1 fg of *S. mansoni* gDNA using a similar PCR protocol with the ND5 primer alone (3)
- The LOD for DNA from a single miracidia in snail extract diluted to 1:100 would be 0.72-0.75 pg.
- While our limit of detection may make it difficult to identify early prepatent infection, we managed to identify a prepatent infection in sample 1 (Figure 1 and 2, pre-determined negatively infected snail, no shedding at 8 weeks).

4) Utilization of inhibitor resistant Phire Polymerase reduced the degree of dilution necessary to amplify ND4 when multiplexed with ND5.

- Reduction of our sample dilution from 1:100 to 1:10 avoids unnecessary reduction of assay sensitivity.
- Although not completely optimized, application of this method to snail samples being utilized for our GWAS study successfully identified prepatent infection in predetermined negatively infected snails (Figure 5, samples 1 and 2, non-shedding via microscopy).
- Moving forward, we are exploring alternative reaction condition to improve the amplification of our control marker (ND4) without the need for further test sample dilution.

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