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?

STUDY DESIGN

Diagnostic assay PCR to address Questions 1 and 2:

- **Experimentally infected Kenyan *B. sudanica*:** 16 *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). gDNA was extracted by the Qiagen Blood and Tissue DNA extraction kit. The diagnostic assay for these snails will allow classification of each snail into one of the three outcomes (Figure 5).
- **Markers**: Snail (ND4, ITS1-5.8S-ITS2, GRC) and *S. mansoni* (ND5) specific markers were trialed in duplex and multiplex PCRs. Primers were trialed in equal or skewed concentrations to account for amplification bias during PCR.
- **Master mix**: All PCRs were performed in 10μl reactions using the GoTaq Colorless Master Mix (Promega, Madison, USA) following manufacturers protocols modified for smaller reaction volumes.
- **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* originating from a previous laboratory passage was extracted by the Qiagen Blood and Tissue DNA 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 performed in 10μl reactions using the GoTaq Colorless Master Mix (Promega, Madison, USA) following manufacturers protocols modified for smaller reaction volumes.
- **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.

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 (%)
Full Concentration (n=16)	0.75 μL	ITS1-5.8S-ITS2	19%	0%	0%
		ND4	0%	0%	0%
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 full concentration and 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). GRCB was not performed. ND5 amplified in 38% of samples when multiplexed with ITS1-5.8S-ITS2, and 50% with ND4.
- **Dilution to 1:10 displayed greater amplification for ITS1-5.8S-ITS2 and ND4.**
  - ITS1-5.8S-ITS2 displayed non-specific amplicons ranging in size from 500-600 bp. Bands were large and smeared.
  - GRCB displayed non-specific amplicons ranging in size from 600-900 bp.
  - ND4 showed increased amplification of 19% when diluted to 1:10 and had no 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 amplified in 100% of samples, but bands were large and smeared.
  - ND4 amplified in all negatively infected snails, but no amplification seen in positively infected snails. Primer interference between ND4 and ND5 may be a potential explanation for this observation.
  - ND5 amplified in 100% of positively infected samples.
- Sample 1 (Figure 1 and 2) was a predetermined negatively infected snail (non-shedding at 8 weeks) which amplified ND5 in all 1:10 and 1:100 PCRs, which may indicate detection of missed prepatent infection (Figure 5).
- **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.

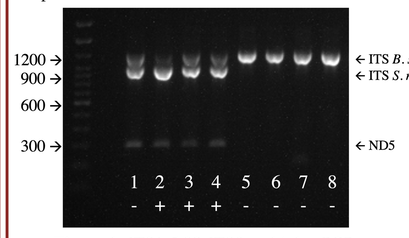

Figure 1: ITS1-5.8S-ITS2 multiplex PCR screening of 8 Kenyan snail samples (3 positive (+), 5 negative (-)) at a 1:100 dilution and 3:1 ITS1-5.8S-ITS2 :ND5 primer concentration.

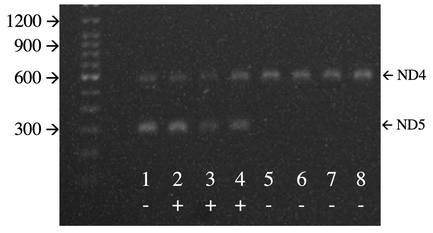

Figure 2: ND4/ND5 multiplex PCR screening of 8 Kenyan snail samples (3 positive (+), 5 negative (-)) at a 1:100 dilution and 3:1 ND4:ND5 primer concentration.

Interpretation:

- Although ITS1-5.8S-ITS2/ND5 at a primer ratio of 3:1 showed 100% amplification, it continued to display large smeared bands (Figure 1) as seen in previous gels (Table 2 Interpretation).
- ND4/ND5 primer ratio adjustment to 3:1 resulted in 100% co-amplification of ND4 with ND5 in predetermined positively infected samples (samples 2-4, Figure 2). Sample 1 continued to amplify ND5 in all multiplex PCRs.
- ND4 amplification was improved 100% relative to the 1:1 ND4/ND5 primer ratio at 1:100 dilution.
- 3:1 GRCB/ND5 primer ratio did not improve co-amplification.
- **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.**

RESULTS – LIMIT OF DETECTION (LOD)

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

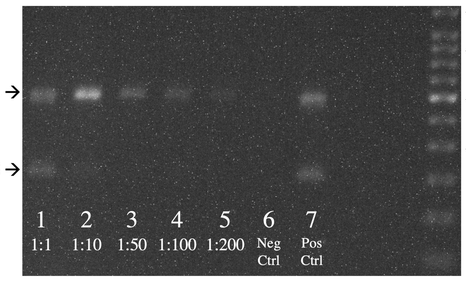

Figure 4: ND4/ND5 multiplex PCR with a 3:1 primer ratio. Template dilution series of a 1:1 mixed gDNA solution (110 snail with S. mansoni adult male).

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.

- ND5 amplification was visible at 1:1 and 1:10 dilutions.
- ND5 amplification was not visible at 1:50 dilution and beyond.

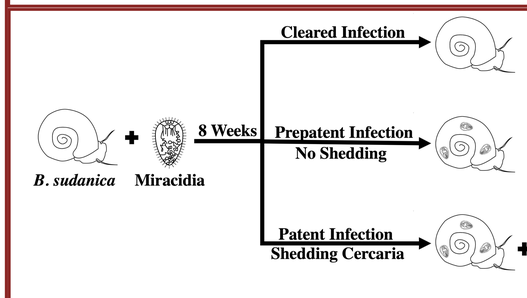

Figure 5: The three predicted outcomes of a *Biomphalaria sudanica* snail being exposed to *Schistosoma mansoni* miracidia. The diagnostic assay is aimed to help determine those with prepatent infections.

CONCLUSION

Although not finalized yet, we have made significant progress in the design of a *S. mansoni* diagnostic assay. We have three 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.
- 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.
- Future directions include trialing new Taq polymerase reagents / protocols that are more resistant to inhibitors and 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 (pre-determined negatively infected snail, no shedding at 8 weeks).
- Moving forward, we want to refine our PCR protocol to improve the limit of detection and will do this using experimentally exposed snails, preserved at varying timepoints (0.5, 3, 24 and 72 hours) post-exposure to *S. mansoni* with the goal of detecting earlier prepatent infections.

ACKNOWLEDGEMENTS

Staff at Kenya Medical Research Institute, Kisumu, Kenya, for assistance in producing schistosome exposed snails.
Dr. Jacob Tennessen (Harvard T.H. Chan School of Public Health) for assistance in GRC primer design.
NIH NAID - R01AI141862

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