Author's response to reviews

Title: Evaluation of real-time PCR assay for detecting Schistosoma mansoni infections in a low endemic setting

Authors:

Maria Cristina Espirito-Santo (cristinasanto@usp.br)
Mónica Viviana Alvorado-Mora (monica.viviana@usp.br)
Emmanuel Dias-Neto (emmanuel@usp.br)
Lívia Botelho-Lima (liviabotelhoimt@gmail.com)
João P Moreira (joaopaulomoreirapr@gmail.com)
Maria G Amorim (mamorim22@yahoo.com)
Pedro Luiz S Pinto (pedro.luiz44@terra.com.br)
Ashley R Heath (ashley.heath@sial.com)
Vera Lúcia P Castilho (vera.castilho@hc.fm.usp.br)
Elenice M.N Gonçalves (elenice.goncalves@hc.fm.usp.br)
Expedito José A Luna (eluna@usp.br)
Flair José Carrilho (fjcarril@usp.br)
João Renato R Pinho (jrrpinho@usp.br)
Ronaldo Cesar Borges Gryschek (rcbgry@usp.br)

Version: 4 Date: 4 September 2014

Author's response to reviews: see over
POINT BY POINT REPLY TO THE REVIEWERS

First, we would like to thank the reviewers for their careful evaluation of our work, for all the important suggestions and edits, and for their positive overall evaluation of our manuscript entitled “Evaluation of real-time PCR assay for detecting *Schistosoma mansoni* infections in a low endemic setting”.

Please find below a point-by-point reply to all criticisms that were raised.

**Replies to the Reviewer 1: Dr. Ricardo Igreja**

This referee requested only two minor essential revisions, as follows:

1) **“Table 2 is not in the text”**

**Answer:** We completed the information in the text: Table 2 shows the characteristics of the five subjects with positive results for the KK and/or HH techniques. It should be noted that four individuals are male and, older than 20 years. The qPCR technique feces was able to detect four positive results regarding the parasitological techniques, while the qPCR technique-serum was positive in only one of five positive individuals in the parasitological techniques.

2) **“Many references (10) are not numbered”**

**Answer:** We removed three references and added seven references to the text. All of them are now numbered. Thank you for the careful evaluation of our work.

**Replies to the reviewer 2: Dr. Hamed Farag**

**Major Compulsory Revisions:**

“The data introduced by the authors are in need to more graphs or charts (may be one) to illustrate real time PCR results to clarify Ct i.e. clarify +ve and –ve Ct of different cases. I think that the authors have these charts and can add it to the manuscript. Adding these chart(s) will make the manuscript more adherent to the scientific relevant standards”.

**Answer:** Thanks for this suggestion. These charts have been added to the new version of the manuscript.

**Minor Essential Revisions:**

**Line 144 & line 145: “Time elapsed after samples obtained to be tested”**

**Answer:** We correct the sentence, as suggested by the reviewer. The elapsed time after the samples were tested was of around six months. This is now informed in the text (Line 143)

**Discretionary Revisions:**
"DNA of hamsters (without data)"

**Answer:** We deleted the word “hamster”, drafted a new sentence and added a reference that explains the procedure. The phrase now reads: “DNA extraction from the serum was performed using the guanidine isothiocyanate-phenol-chloroform (GT) method [14, 24]. DNA was stored at -20°C after extraction [23].”

"Omit the letter a".

**Authors answer:** We deleted the letter a.

"Purified DNA sample (has been mentioned in the same line)"

**Answer:** We correct the sentence, as suggested. The phrase now reads: “TaqMan® Real-Time PCR was performed in a final volume of 20 µL containing: 10 µL of TaqMan® Universal PCR Master Mix 2X; 20 pmol of primers F2 and R2, 5 pmol of the FAM-labelled PO2 probe (Sigma Custom Products), and 2 µL of purified DNA. For each sample, another reaction was performed in parallel using the TaqMan® Reagents Exogenous Internal Positive Control (IPC) in the final volume of 21 µL, containing the following: 10 µL of TaqMan® Universal PCR Master Mix 2X, 5 µL of 10X exogenous IPC mix, 1 µL of 50X Exo IPC, and 5 µL of the purified DNA sample.”

**Table 4 & 5:** The mean values written in the tables should be proofed by a chart

**Answer:** This is now given in the new Tables 4 & 5.

**Replies to the Reviewer 3: Nicholas Midzi**

**Comments on the methods:**

The authors should explain how the sample size was calculated

**Answer:** We apologize for this oversight. This information is now given in the text as follows: “The sample size was calculated assuming a prevalence of 1%. An increment of 30% was made to compensate for follow-up losses. The estimated sample size was 650 individuals residing in the neighborhoods described above”.

**Ethical aspects - Ethical aspects of animal experiment**

(2) (Geneva, 1995) - For consistence, please revert to numbering your references in text rather than changing to text

**Answer:** This was corrected in the new text.
(3) In order to avoid putting too many subheadings that are unnecessary the subheadings on ethics should not be two. Only one subheading (ethical aspect) should be written. The two sections on ethics should form two paragraphs

Answer: We accepted your suggestion and have now merged these two blocks regarding ethical issues.

Method of laboratory diagnosis

The subheading should read specimen collection

Answer: We accepted your suggestion and added the suggested subheading.

The two references here about Kato Katz should be numbered

Answer: We accepted your suggestion and drafted the numeric references Kato-Katz technique.

In brief the authors should explain how the Helm Test Kit was used to perform the Kato Katz technique

Authors answer: We have now inserted the following paragraph (Lines 15-160:
“The Kit Helm Test® Bio-Manguinhos is a qualitative-quantitative test for parasitological detection in stool. It allows the detection of all helminth eggs that are usually found in the stool: Ascaris lumbricoides, Schistosoma mansoni, Hookworm, Trichuris, Taenia and, more rarely, Enterobius and Strongyloides. The method comprises: a screen that filters the material to be examined, retaining debris that would hinder or prevent the visualization of helminth eggs; a coverslip to be pre-colored in diaphanizing fixative solution, allowing the conservation of eggs and clearing the smear, and a specially designed perforated plate. This implies that always the same amount of feces is examined, enabling standardization and excellent observation of a sufficient sample amount that is easily prepared and may be examined after a short time (approximately 1 hour) or stored for several months. The number of eggs S. mansoni in each slide is counted through optical microscopy, multiplied by the factor 24 and the result is released in eggs per gram of faces (epg).”

This subheading is already under the main heading “Methods”. It should therefore not have other subheadings such as “faecal sample preparation and standardizing the detection of S. mansoni DNA by qPCR” under it. The two sub-section should form two paragraphs just to show flow of ideas

Answer: We have accepted this suggestion and removed the subheading.

All the references here should be numbered as in the background

Answer: We numbered all references as in background, as suggested.
The following is not a full sentence “Egg-derived DNA and 170 DNA derived from a solution of 500 mg mixed with 200 S. mansoni eggs”. It should be revised

**Answer:** Thank you for pointing this out.
The new phrase now reads: "For each batch of reactions, two positive controls were used for qPCR-feces and qPCR-serum, respectively: DNA extracted from human feces marked with 0.9% saline solution containing 200 S. mansoni eggs and DNA extracted from saline 0.9% S. mansoni containing 200 eggs, employing the same standardization [23]."

**Standardizing the detection of S. mansoni DNA by qPCR**

**All references to be numbered as in the background**

**Authors answer:** We have corrected this in the new version of the manuscript.

**The subheading should read DNA extraction from the serum of hamsters. Purification of DNA extracted from faecal and sérum**

**Authors answer:** We accepted your suggestion and reorganized the whole paragraph “Standardizing the detection of S. mansoni DNA by qPCR”, according to the suggestions.

**Primers and probes**

**All references to be numbered as in the background**

**Authors answer:** We have corrected this in the new version of the manuscript.

**Results**

**The majority of subjects in the study population were females, with an average age of 40-41 years**

**Authors answer:** We accepted this suggestion and this phrase was incorporated (Line 243).

**If table 2 is not referred in the text then it is a redundant table and should be removed**

**Authors answer:** Thank you for observation. Table has now been indicated in the text (Line 249). However, as we believe this table is important to highlight the results, we opted to keep it for the clarity of the manuscript.

**Discussion**

**This section is well discussed with reference being made to the results and what others have found in the same field of study. However, authors**
needed to point out limitations in their study. For example, they took a single day stool when they are aware that the sensitivity of the KK test is improved by testing samples collected on two to 3 successive days to cater for daily variability egg production by an infected person. Overall, the paper is well written and provides useful information. The paper can be accepted when minor revisions indicated in this review are addressed.

Authors answer: We accepted your suggestion and we add a paragraph about the limitation of the study in the discussion: “A limitation of this study was the collection of feces in a single day per individual. The sensitivity could have been improved, as in the case of KK test, by feces collection in two or three consecutive days, followed by testing, to meet daily egg production variability in an infected individual. However this was not done here, mainly because of the high number of individuals enrolled.” (Lines 302-305).