Author’s response to reviews

Title: Early detection of novel Leishmania species DNA in the saliva of two HIV-infected patients

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Author’s response to reviews:

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Dear Editors:

We would like to submit the revised manuscript entitled (INFD-D-15-00411) “Early Detection of novel Leishmania species DNA in the Saliva of HIV-infected Patients”. We would like to thank the reviewers for their comments and suggestions. Based on the comments we received from the reviewers, we have made changes to the manuscript. All changes have been track changes and detailed below. English style and formatting have been revised via a Wiley Editing Services (Certificate Verification Key: 50A9-CF67-A08C-4EDC-22CD). The material in this study has not been published in any other journal nor has it been submitted elsewhere.

Response to the reviewers’ comments
Reviewer #1:

1) The objective as outlined by the authors is (last sentence of introduction): "This study's objectives are to determine the prevalence of Leishmania infection in HIV-infected Thai patients from southern Thailand through PCR analysis of saliva and blood samples." Yet, this objective is not reflected by the title, nor by the conclusions of the paper. The authors start their study from an observation stated in the introduction: "Saliva has been shown to be a good source for the detection of the new Leishmania species DNA". Hence it was not their goal to determine the usefulness of the saliva PCR, and in fact the 3 cases they have observed are by far insufficient to draw any conclusions regarding the potential higher sensitivity of saliva PCR relative to blood or buffy coat PCR. In contrast, the authors do not answer their actual objective in any of the conclusions.

As the reviewer suggests, we added a sentence “The prevalence of Leishmania infection in HIV infected patients within this study is 0.95%.” in the conclusion. With regards to the sensitivity of PCR in saliva as with the reviewers concerns, we are also aware that our study was not designed to determine the sensitivity and the number of positive samples were only 3 of the 316 tested. Therefore we have never used “sensitivity” in our manuscript, but we used “early detection” throughout the manuscript.

2) One of the conclusions drawn by the authors is: "This could result in earlier treatment and lead to the outcome of decreased mortality rates." Are the authors suggesting that asymptomatic saliva positive HIV patients should be treated with anti-leishmanials? The one individual tested remained asymptomatic during the study, it would be unacceptable to start treatment in such case. A clinical study on many co-infected individuals would be required to draw any such conclusion.

We actually meant treatment in symptomatic leishmaniasis, however for a more clear description we changed the sentence to "This could result in the closer follow up of asymptomatic infected patients and for earlier treatment of symptomatic leishmaniasis. This could then lead to the outcome of decreased morbidity and mortality rates."

3) Another conclusion is: "Moreover, early diagnosis can be a tool for disease surveillance, especially in asymptomatic subjects, and can enable better design of control strategies." In fact we have not a single idea on the sensitivity of the PCR for detecting asymptomatic infection, the authors could have missed 99% of them without knowing it. Again, to draw the conclusion that
saliva PCR is superior would require demonstration in many HIV patients that blood/buffy coat PCR is negative while saliva PCR is positive.

As mentioned in the previous comments, we were never analysing “sensitivity” of our test in the manuscript because our positive PCR samples in the study were not enough to determine sensitivity.

As the reviewer comments, we are also concerned that most of healthy individuals who have Leishmania infection can be self-limited. However, for immunocompromised hosts, especially AIDS patients, leishmaniasis can be a cause of morbidity and mortality therefore in this study we focused on HIV infected patients. Our study just determined Leishmania infection in HIV patients and described our finding that PCR in saliva was positive prior to blood and buffy coat. For fully determination of sensitivity of the test we will collected more clinical samples (saliva, blood and buffy coat) from HIV infected patients.

4) Based on these findings, my recommendation is a major revision of the way the paper is written. Maybe it would be more appropriately presented as case studies. In any case the objectives, title and conclusions should be consequent and clearly stated, and the limitations from only 3 observations should be clearly discussed, and over-interpretation avoided. Personally I am not convinced that the findings are of enough weight for publication in BMC infectious diseases, but I leave this decision to the editor.

The actual objective of this work was to determine the prevalence of Leishmania infection in HIV infected patients in an endemic area of Thailand using saliva and blood samples (the idea of the study was based on the report by Phumee et al., 2013). The discovery of early presentation of Leishmania DNA in saliva prior to buffy coat was by accident, we just described this as early detection and have never used “sensitivity” of the technique in the manuscript.

For the title, we believe "Early Detection of novel Leishmania species DNA in the Saliva of HIV-infected Patients" is clear and engaging, however we can change the title to “Prevalence of Leishmania martiniquensis infection among HIV infected patients in southern Thailand” if the editor suggests.

5) I have the following specific comments: "Primers that are anneal to human DNA (UNFOR403: 5′-TGA GGA CAA ATA TCA TTC TGA GG-3′ and UNREV1025: 5′-GGT TGT CCT CCA ATT CAT GTT A-3′) were used to determine template DNA". I have no idea what
this means. These primers are from a paper that describes blood meal analysis from mosquitos, what do the authors mean by "determine template DNA"? Also, the sentence is grammatically incorrect.

These primers were designed to anneal specifically to human DNA and have been used to detect human DNA through the mosquito blood meal. The reasons we chose to use this primer set was due to the fact that it will anneal specifically to human DNA and is available in our lab due to previous research on mosquito blood meals in Thailand.

DNA samples in this report were extracted from clinical specimens. Therefore, to demonstrate that our extraction process was appropriate, as all clinically-extracted DNA should contain human DNA, we used the primer to ensure that the template DNA was appropriate. For a more clear description we can use “to maintain that the template DNA had been extracted properly”.

6) "Cloning and sequencing": Why did the authors go through the cumbersome cloning procedure? There is no need, as PCR amplicons can be sequenced directly.

Direct sequencing requires at least 30-50 ng/µl, but our PCR showed only faints bands, especially in the saliva of asymptomatic infections. Therefore, we decided to use cloning for the sequencing.

In addition, these ITS1 primers can amplify closely of L. martiniquensis and L. siamensis at 371 and 379 bps, respectively. Therefore, we think that the most effective method was to clone our gene.

7) "100 mg/ml of streptomycin": Is this concentration correct?

We changed to 100 µg/ml of streptomycin.

8) "The nucleotide sequencing of all PCR-positive samples were 100% identical to L. martiniquensis (data not shown)" (results) and "Sequence analysis of amplified PCR products were 100% identical to L. martiniquensis (Accession no KM677931)" (discussion): I have performed a BLAST analysis of accession KM677931 against GenBank nucleotide sequences, and found 5 identical sequences, 3 of which were listed as L. siamensis. Based on this sequence,
it is hence impossible to discriminate L. martiniquensis from L. siamensis. How can the authors claim that the infections they documented were L. martiniquensis?

Before December 2014, it was suspected that two L. siamensis lineages were found in Thailand. Therefore, sequences found before that time were reported as L. siamensis (as discussed in the discussion). However, in December 2014, Pothirat et al., reported that one lineage was L. martiniquensis (also in the discussion). This primer set can amplify both L. martiniquensis and L. siamensis. PCR amplicons were 371 bp and 379 bp for L. martiniquensis and L. siamensis respectively. Moreover, a sequence comparison of both species gave different alignment and a phylogenetic tree of these two species was constructed.

9) Last sentences of results: "PCR annealed specifically to human DNA. DNA controls were positive for all clinical samples (Figure 1A and 1B)." What are these controls? What are they for? I have no idea.

In the case that the PCR for Leishmania gives a positive result for saliva and a negative result for blood and buffy coat, the readers may question whether there is DNA in the samples. The sample DNA was extracted from clinical infections and so we used human DNA as a control template. This just demonstrated that the DNA extracted was appropriate by amplifying human DNA in the samples (As described in 6).

We changed the sentence to "PCR annealed specifically to human DNA gave positive results for all clinical samples (Figure 2A and 2B). This showed that all extracted DNA from clinical samples were appropriate for PCR amplifications."

10) "In other parts of the world such as CU1 isolates (Accession no JQ001751), isolates from cows in Switzerland (Accession no GQ281282), a horse in Germany (Accession no GQ281278) and a horse in the USA (Accession no JQ617283)19-21 may be L. martiniquensis." This sentence is semantically incorrect: CU1 isolates is not a part of the world.

We have changed the sentences to "They also reiterated that most cases of leishmaniasis are caused by L. martiniquensis in Thailand such as; CU1 isolates was taken from a patient from southern Thailand (Accession no JQ001751). This is similar to our result of this study, all cases were infected by L. martiniquensis. In other parts of the world, isolates from cows in Switzerland
(Accession no GQ281282), a horse in Germany (Accession no GQ281278) and a horse in the USA (Accession no JQ617283)19-21 may be L. martiniquensis."

11) The first 4 paragraphs of the discussion (up to "Moreover, the collection of saliva is non-invasive, requires no special equipment, and is suitable for children and elders") are in fact not a discussion of the results, but an introduction to the paper.

We have added more data and figures in the manuscript and discussed according to our results in these paragraphs.

12) "This study identified Leishmania and HIV co-infected patients using saliva and blood samples for PCR in an endemic area of Thailand." Incorrect, this study did not identify HIV patients.

The sentence was changed to "This study identified Leishmania co-infections in HIV patients using saliva and blood samples for PCR within an endemic area of Thailand."

13) "Interestingly, Leishmania DNA was detected in saliva prior to appearing in buffy coat in two of our cases." I see only one such case in Table 1.

We rewrote the sentence to "Interestingly, Leishmania DNA was detected in saliva prior to appearing in buffy coat in patient two and was also detected only in saliva for patient three."

14) "However, we demonstrated in this study that DNA was detected in saliva two months prior to buffy coat." in one patient, this is irrelevant.

We changed the sentence to "However, in this study we found that Leishmania DNA was detected in saliva two months prior to buffy coat in a patient presenting nodular leishmaniasis. Leishmania DNA was also detected only in the saliva of an asymptomatic patient."
15) "Liautaud et al. (2015) reported the first case of visceral leishmaniasis caused by L. martiniquensis in an AIDS patient in the Caribbean region by detecting ribosomal an 18S RNA locus.": semantically incorrect sentence.

We changed to "Liautaud et al. (2015) reported the first case of visceral leishmaniasis caused by L. martiniquensis. This was detected in an AIDS patient living in the Caribbean region."

16) "This study also demonstrated the first asymptomatic leishmaniasis case caused by L. martiniquensis in Thailand.": If it is asymptomatic, it is not leishmaniasis. Leishmaniasis is a disease, not an infection.

We changed to "This study also demonstrated the first asymptomatic L. martiniquensis infection in Thailand."

Reviewer #2: The article could be improved by adding clinical pictures of the patients diagnosed with leishmaniasis.

We added Figure 1 in the manuscript, “Figure 2. Cutaneous leishmaniasis lesions of patient 1”