Author’s response to reviews

Title: Comparison of Two DNA Targets for the Diagnosis of Toxoplasmosis by Real-Time PCR using Fluorescence Resonance Energy Transfer Hybridization Probes

Authors:

Dr Udo Reischl (udo.reischl@klinik.uni-regensburg.de)
Stephane Bretagne (bretagne@univ-Paris12.fr)
Dominique Krueger (kruegerD@rki.de)
Pauline Ernault (pauline.ernault@ahparis.org)
Jean-Marc Costa (jean-marc.costa@ahparis.org)

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To

Sophy McHugh
Assistant Editor

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Submitted by: Udo Reischl (Udo.Reischl@klinik.uni-regensburg.de)

here: Revision letter and "point by point response"

Dear Ms. McHugh,

Enclosed please find a point by point response to the reviewers comments and a carefully revised version of our manuscript entitled "Comparison of Two DNA Targets for the Diagnosis of Toxoplasmosis by Real-Time PCR using Fluorescence Resonance Energy Transfer Hybridization Probes".

Our detailed list of responses to the the comments of reviewer #1 are summarized below:

**Point 1:**
The authors agree with the reviewer in the fact that a high proportion of the population is susceptible to *T. gondii* and a high prevalence of anti-toxoplasma antibodies is observed in the European population. It is already stated in the "Abstract/Background" that the presence of Toxo-IgM does not necessarily indicate recent infection. To document the diagnostic advantage of PCR testing over serology, we included a corresponding statement in the first paragraph of the "Background" section in the revised version of the manuscript. Furthermore it is now stated in the first paragraph of the Methods section, that about 50% of the patients (from which the "160 clinical specimens of various types" came from) were seropositive for *Toxoplasma* but did not show clinical symptoms of acute disease. Since all of the various clinical specimens from seropositive patients tested negative with the proposed new PCR protocol, the following sentence was added at the end of the Discussion section: "Consistently negative PCR results were observed with specimens of various types originating from individuals who had anti-toxoplasma antibodies but no clinical symptoms of acute toxoplasmosis."

We have also added a reference (new Ref. [25]; Toxoplasma PCR on blood samples of immunocompromised patients) which may further support our statements. We very much hope that you and the reviewer will find these changes sufficient to clarify this important aspect (advantage) of PCR testing for toxoplasmosis.

**Point 2:**
To clarify this point, we have added the following sentence to the first paragraph of the "Background" section: "Both the damage for the fetus (the sooner, the more deleterious) and
the frequency of trans-placental transmission (the later, the more frequent) is usually depending on the stage of gestation." We have not included such a statement in the original version of the manuscript since this information can be found in other sources (see Refs. 3 and 11) and it was not considered as absolutely relevant for the present work.

**Points 3 and 4:**
As already discussed (point 1), we have added a paragraph at the end of the Discussion section dealing with the investigation of body fluids and tissues by Toxoplasma-PCR. The revised version of the manuscript also contains an additional reference (new Ref. [26]; Lack of *Toxoplasma gondii* DNA in muscles of patients with inflammatory myopathy and increased anti Toxoplasma antibodies) to support our statements. We have tested a lot of different tissues so far and we do have experience with this issue. As stated in the paragraph added at the end of the Discussion section, the problem is not the sensitivity of the PCR process itself, but the DNA extraction protocol and the non-homogenous distribution of the toxoplasmosis cysts in tissues (including lymphatic tissue).

Biopsies of lymph nodes for the diagnosis of acute toxoplasmosis in immunocompetent individuals are not routinely performed in our institutions (elsewhere, we don't know). To the best of our knowledge, the only clear indication is a suspected hematological disease. So we have not addressed this issue.

**Point 5:**
We agree with the reviewer that determination of the intragenic copy number of the novel target gene is an important issue of this study. The statement "between 200 and 300 copies" originates from the original publication of Homan et al. and is now classified as such in the revised version of the manuscript (Reference number added to the statement in the last paragraph of the Background section).

As stated several times in the manuscript, our quantitative PCR results clearly indicate that that the actual number of 529-bp repeat elements within the genome of *T. gondii* is at least tenfold higher than the 35-fold repeated B1 gene. This led to the statement in the "Conclusions" section, that "the 529-bp repeat element of *T. gondii* is repeated more than 300-fold". From the scientific (or academic) point of view, we have been highly interested in clarifying this aspect. We have performed RFLP experiments with Pvull-digested genomic DNA and hybridized Southern blots with a target-specific probe to count the actual number of "bands". Although this strategy worked fine with multicopy target genes of other organisms showing copy numbers in the range of 1 to approx. 50 per genome (Reischl et al., *J. Clin. Microbiol.* 2001, p.1963-1966 ), we were not able to count or even to distinguish the high numbers of 529-bp repeat element-bands per lane. Like the reviewer, we have also asked ourselves: "What is the right number?", but it seems that we have to rely on the results of quantitative PCR: > 300 copies.

With respect to the second question in point 5: yes, there is an argument which at least indicates that all (the investigated) *Toxoplasma* strains have such a high numbers of target copies in their respective genomes. All of the *T. gondii* reference strains and the 51 positive amniotic fluids were examined with both PCR-protocols (B1 and the novel 529-bp target genes). With the "novel target gene" we observed a continuous gain in LightCycler crossing point values of 3 to 4 cycles with all of the various samples investigated in the course of this study. We must concede that there is no definitive argument making sure that ALL *Toxoplasma* strains will have such a high numbers of target copies. However, that is what we have observed with the reference strains tested and all the samples tested positive so far.

**Point 6:**
To clarify this point, we have added the following sentence to the first paragraph of the Methods section: "DNA preparations from 51 amniotic fluid samples, which were received by the Hôpital américain de Paris between 1996 to 2001 and tested *T. gondii* positive using a previously published PCR assay, were used as PCR templates." The samples originated from metropolitan France (Caribbean, Tahiti, etc. excluded), but the latter aspect should not have an influence on the scientific aspect of the present study.

**Point 7:**
With respect this point, we have checked the GenBank again for recent sequence entries showing potential homologies to the 529-bp *T. gondii*-specific target sequence. Again, only a few and some very small regions of the target sequence showing partial homology with other GenBank entries. So the sequence of the novel target gene can still be considered as specific and cross-reactions or false-positive PCR-results due to the presence of DNA from other organisms are still very unlikely. It is now stated in the "Conclusions" section that no significant sequence homologies with other GenBank entries are observed for this novel target gene.

We hope the revised manuscript satisfactorily will meet the reviewers’ concerns and is acceptable in form for publication in the *BMC Infectious Diseases*.

My colleagues and I look forward to hearing from you soon.

With best personal regards,

Udo Reischl