Author's response to reviews

Title: Potential immunomodulatory effects of latent toxoplasmosis in humans

Authors:

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Author's response to reviews: see over
Dear Dr. Marshall

Please, find enclosed corrected version of our paper entitled “Potential immunomodulatory effects of latent toxoplasmosis in humans”.

We have made all changes suggested by the section editor and reviewer. All changes have been highlighted in the new version of manuscript and commented in the List of changes - below).

I would like to thank the referees and especially the Section Editor for useful and inspiring suggestions and I hope that the new version of the papers is suitable for publication in BMC Infect. Dis.

Yours Sincerely
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List of changes

Comments from the Section Editor:

1. The wider literature should be cited regarding toxoplasma and the hygiene hypothesis (see e.g. Fernandes et al Clinical Immunology (2010) 136; 148-156.
We would like to thank to the Section Editor for bringing to our attention this very important paper, which is extremely relevant to the topic of our study. Actually, we feel ashamed to had missed this important paper that confirmed our (until now a little bit speculative) conclusions on possible negative correlation between toxoplasmosis and atopic disease). We included three new references and also following paragraph into the Discussion section of the paper: Decreased frequency of *Toxoplasma*-positive subjects in patients with atopic disease has already reported in several studies [32-34]. It must be remind, however, the low frequency of *Toxoplasma* infected men as well as the statistically significant associations between the *Toxoplasma* infection and the cell counts were observed mostly in subjects with immunodeficiencies (D.89.9) not in patients with allergies (J.30.1-4) in our study.

2. What was the definition of allergy (subjectively and objectively defined) in the patients who were seen in the clinic? How did different categories of allergy relate to seropositivity for toxoplasma?
The diagnosis of allergy was based on characteristic clinical symptoms together with either elevated serum concentrations of IgE/ specific IgE and/or positivity of skin prick test. Again, we have to thank to the Section Editor for inspiring us to return to the analysis of our data. We reanalysed our data to see whether different categories of patients differ in the associations between toxoplasmosis and the cell counts. We found that the subpopulation of patients with immunodeficiencies (D89.9), not the subpopulation of patients with allergies, was responsible for most of observed effects. We included these very important results into the corrected version of the manuscript. The reanalyses confirmed the effect of sex on the risk of
**Toxoplasma** infection but did not reveal any significant effect of the category of disease on the risk of **Toxoplasma** infection.

It is implied, but not stated, that all the patients in a particular year were studied. However, the patient flow as seen in clinic, and those entered into this study, should be made explicit in a flow chart.

We explicitly stated that “the clinical data of all immunology outpatients of the Institute of Clinical and Experimental Medicine in Prague from 2008-2009 were included into the study.” We included following information into the new version of the manuscript:

Diagnosis (ICD-10) of most subjects (N=250) was D89.9 (disorder involving the immune mechanism, unspecified) and J30.1-3 seasonal allergic rhinitis (N=117). Other diagnoses observed in our analysed population were A69.2 (2×), B009 (3×), B007 (2×), D80.0 (1×), D80.2 (1×), D80.6 (1×), D81.9 (10×), D83.9 (2×), D89.8, E06.3 (2×), F48.0 (3×), H10.1 (1×), H10.9 (1×), J01.0 (1×), J06.8 (1×), J06.9 (1×), J30.0 (1×), J30.3 (12×), J45.0 (11×), J45.9 (10×), K30 (1×), K50.9 (6×), M05.9 (1×), M35.0 (1×), N76.1 (2×), O26.9 (1×), T63.4 (1×), T78.4 (1×). We analysed the whole population of patients and also two subpopulations separately: patients with immunodeficiencies D89.9 and patients with allergies, J30.0-4, J45.0, J45.9.

There is still causality implied in the paper, for what is a simple, small correlative study (see for example, pg 6 line 4, where it has not been shown that toxoplasmosis ‘influences several haematological (sic) and cytometric parameters,...), simply that there might be an association between these variables.

We checked the manuscript again for the formulations that can (mis)imply the causality relation.

We changed toxoplasmosis influences several haematological and cytometric parameters, namely the leukocyte, CD19, CD16 + 56 and monocyte counts, mostly shifting the size of immune cell subpopulations in opposite directions in men and women (Table 1).

Toxoplasmosis correlates with several haematological and cytometric parameters, namely the leukocyte, CD19, CD16 + 56 and monocyte counts, mostly shifting the size of immune cell subpopulations in opposite directions in men and women (Table 1).

The numbers of patients contributing to each category shown in the figures should be included.

We included following information into the legend of Fig. 1:

Number of patients with titres 64, 32, 16, 8, 4 and >4 was 4, 14, 37, 45, 45 and 295, respectively.

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**Reviewer's report**

**Title:** Potential immunomodulatory effects of latent toxoplasmosis in humans

**Version:** 1 Date: 18 February 2011

**Reviewer:** Rodrigo Da Silva

**Reviewer's report:**

This paper presents an excellent contribution to toxoplasmosis in human beings. It can be accepted after Minor Revision.

- **MINOR ESSENTIAL REVISIONS** -

  - **ABSTRACT**
    - substitute “About 30% of the population are infected with the protozoan parasite Toxoplasma gondii worldwide.” To “About 30% of the population are worldwide infected with the protozoan parasite Toxoplasma gondii.”

Corrected.

- the sentence “For example, the immunosuppression hypothesis explains the higher probability of the birth of male offspring observed in toxoplasma-infected humans and mice by the protection of the (more immunogenic) male embryos against abortion.” is a good example for the main objective of the study but it is
We changed
Latent toxoplasmosis has many specific behavioral and physiological effects on the human organism. Modified reactivity of the immune system has been suggested to play a key role in many of these effects. For example, the immunosuppression hypothesis explains the higher probability of the birth of male offspring observed in Toxoplasma-infected humans and mice by the protection of the (more immunogenic) male embryos against abortion.

To
Latent toxoplasmosis has many specific behavioral and physiological effects on the human organism, including an increase of length of pregnancy and a shift in sex ratio in humans.

- the authors can insert the type of epidemiological study (cohort study) in the Methods section, not in Results section.

We moved following information from the Results to Methods section:
The experimental design of present study was a prospective cohort study.

- in the first line of the Results section, change “Our cohort study” to “Our study”.
  Done.

- what’s the difference between latent toxoplasmosis in male and Toxoplasma-infected female? Both are infected, but both latent infection? Need to clarify even in abstract (Methods)

There is no difference between latent toxoplasmosis (both in men and women) and long-term infection by Toxoplasma. To avoid possible misunderstanding we substituted Toxoplasma-infected with Toxoplasma-positive everywhere.

We also explicitly explained the new terminology by substituting:
In the present cohort study, we searched for indices of immunomodulation in humans with latent toxoplasmosis by comparing the available clinical records, namely the flow cytometry and haematological data, in Toxoplasma-infected (positive) and Toxoplasma-free (negative) outpatients undergoing routine immunological tests at the Institute of Clinical and Experimental Medicine in Prague.

With
In the present cohort study, we searched for indices of immunomodulation in humans with latent toxoplasmosis by comparing the available clinical records, namely the flow cytometry and haematological data, in Toxoplasma-positive (infected) and Toxoplasma-negative (noninfected) outpatients undergoing routine immunological tests at the Institute of Clinical and Experimental Medicine in Prague.

- change “specific anti-Toxoplasma antibody titre” to “specific Toxoplasma antibody titer”.
  Corrected.

- change “with normal prevalence of toxoplasmosis in 312 female” to “with normal prevalence in 312 female”.
  Changed.

- in the Conclusion section substitute “some classes of immunology disease” to “some infectious diseases”
  Corrected.

BACKGROUND
- in the fourth line, the expression “anti” already express reaction against something. In this case, change “anti-Toxoplasma IgG antibodies” to “Toxoplasma IgG antibodies”.
Done.
- twenty can be expressed by number (20)
Done.
- the sentence “For example, the sex ratio of human embryos decreases from about 1.64 to 1.05 from pregnancy week 5-7 to delivery [17,18]. The prevalence of toxoplasmosis in mothers of children with Down syndrome was 84 % compared to about 30 % in fathers of these children and in the general population [15].” can be moved to Discussion section.
Done.
- in the second paragraph, remove “, for review see”
Removed.

METHODS
- in the fourth and fifth lines of Subjects subsection change “anamnestic titres of anti-Toxoplasma antibodies” to “anamnestic titers of Toxoplasma antibodies”
Done.
- the authors should insert the register number of the study in Institutional Review Board, of the Faculty of Science, Charles University.
Done.
- change the subsection “Immunological tests for toxoplasmosis” to “Serological tests for toxoplasmosis”.
Done.
- in the third line of the same subsection change remove “anti-“
Done.
- change “, which is more reliable in established (“old”) T. gondii infections, as decrease in CFT titres is more regular [23]. Toxoplasma antibody titers in the sera were measured at dilutions between 1 : 4 and 1 : 1024.” to “[23], at dilutions between 1 : 4 and 1 : 1024.”
Done.
- in the ninth line of this subsection remove “testing”
Done.

RESULTS
- in some parts of the paper (Results and Discussion sections), the authors express P value as “P” but in other parts as “p”. The authors should to standardize. The correct form is “P”.
Corrected.
- change “The study population” to “The studied population”.
Done.
- the authors informed that the studied population was 312 women and 228 men, totalizing 540, not 440.
We would like to apology for this error. The number of males was 128, not 228. We carefully checked the whole manuscript whether all tests were performed with the correct number of male subjects (they were). We corrected 228 to 128 and frequency of Toxoplasma-positive men from 6.1 to 10.9 % everywhere.
- in the text, the authors named the Tables as 1 and 2, but in supplement material both tables are named 1a and 1b. The authors should standardize. Corrected.

- in Table 1, change “p” to “P”, “L” to “L” Done.

- legends of tables 1 and 2 express “eta2”, but in the body of the table the authors express “eta”. The authors should to standardize, changing “eta” to “eta2” or “eta2” to “eta”. We included more information into the tables 1 and 2, therefore, we have to omit the eta to make the tables intelligible.

- the space among lines should be standardized. Done.

- in the third paragraph, second line, remove “anti-“. Done.

- two tables were submitted in this paper but only the title of the Figure 1 is present, and registering “a)” for women and “b)” for men. Table 1 and 2 did not present any “a” or “b” that explains this. The authors should include the title of the Figure 2 too, and inform where “a” and “b” are inserted. In the corrected version of our manuscript, the parts a and b have been arranged into one figure.

DISCUSSION
- in the first paragraph, second line, remove “anti-“. Done.

- the authors need to standardize the space between the lines Done.

- in the second paragraph, line 15, remove “anti-“ Done.

- remove “For example, in humans, the sex ratio decreases from about 2.6 in the first weeks of pregnancy to about 1.06 at the time of delivery [17,18].” In the present version of the manuscript, we omitted this redundant information in the section Introduction. However, we consider necessary to keep this information in the Discussion section of the manuscript (as has been recommended by the referee above).

- change “The main limitation of the present study is the absence” to “The main limitation of the present study was the absence” Done.

- in the third paragraph, line 17, change “Toxoplasma infected men” to “Toxoplasma-infected men”. Done.

- remove “It must be stressed, however, that this potential limitation only increases the risk of missing some effects of toxoplasmosis, i.e. the risk of finding false negative but not false positive results.” Done.

CONCLUSION
- this section is a brief sentence based on the obtained results and data
discussed. The authors should exclude “Latent toxoplasmosis is a very common parasitic disease and about 30% of the population of both developed and developing countries are likely to be infected with this protozoan parasite. Given the high prevalence of latent toxoplasmosis, its possible immunosuppressive effects, although relatively weak in individual patients, might have a considerable impact on the health of the world population. Our results, i.e., the observed effects of latent toxoplasmosis on the immune cell counts,”
- change “might be a point of departure for further studies on healthy subjects to search for more specific symptoms of immunosuppression” to “Thus, further studies on healthy subjects are necessary to search for more specific symptoms of immunosuppression.”

The section Conclusions was considerably shortened in the new version of the manuscript.

REFERENCES
- Authors need to standardize all references using surname and the initials of the first names. In the reference 21, change “Costa da Silva R” to “Silva RC”.

Reviewer's report:
Overall a straightforward paper that describes the analysis of data collected at an allergy clinic using the filter of T. gondii seropositivity. It is intriguing that only 6% of men in the clinic were seropositive compared to the expected 20 to 30% seropositivity in the general population which fit that of women in the clinic (24% seropositive). The theory that this represents immune suppression that limits allergy symptoms in men is intriguing.

Major issues: none

Minor issues (Essential):
1. Methods. Why do you expect that WBC would not affect cell counts in peripheral blood. CD4 and CD8 are obtained as a percentage of total cells, so if the total falls the absolute number will fall, even if the percentages remain the same.

Please, excuse our wrong formulation. The proper meaning should be that the standard medication does not affect the proportions of blood cells. We have corrected this sentence in the revised manuscript.

2. Results: The sample of men who are seropositive is small (14) and statistically this limits the data. This should be discussed.

Now we say in the first paragraph of the Limitations section:
The most obvious problem of our study was related to low number of Toxoplasma-positive men in our population. Such highly unbalanced design can result in false negative result – i.e. in missing some effects of toxoplasmosis. It must be stressed, however, that it cannot lead in false positive results, i.e. in detection of non-existent effects of toxoplasmosis.

3. Ideally a control of non allergy clinic T. gondii positive patients and negative patients should be examined; however, I understand that the data is not available on that group (as they have no reason to have immune profiles done)

We hope that our results would provoke other students to try to repeat our study on different more suitable populations, e.g. on the blood donors population.

4. While testosterone can explain differences between men and women on cell counts in general; what is the data (in animal models) that this has an effect on the immune changes caused by T. gondii (or other immune stimuli). A more detailed discussion of this would strengthen this paper.

We included following paragraph into the discussion:
It must be remind, however, that experiments performed with artificially infected laboratory mice demonstrated decreased level of testosterone in both males and females. Moreover, we did not found any significant differences in the concentration of testosterone between Toxoplasma-infected and Toxoplasma-free solders (nonpublished results). It is critically needed to search for changes of cell counts in the Toxoplasma-infected mice and also to search for possible correlation between concentration of testosterone and blood cell count in our patients.

5. The data on return of counts is weak and given the number of comparisons made I do not think it is well supported by the data set presented. The discussion of this point in the manuscript, however, is very reasonable. We agree that it is critically needed to repeat our study on different population.

6. I agree that a major limitation is the lack of data on immune cell subpopulations. I also think the limitations of the data should be discussed in the abstract as it is in the last paragraph of this manuscript. We included following paragraph into the Abstract:
The main limitation of the present study was the absence of the data on the immunoreactivity of immune cells subpopulations. Therefore, further studies are needed to search for indices of immunosuppression in human using more specific markers.