Author's response to reviews

Title: Rv1985c, a promising novel antigen for diagnosis of tuberculosis infection from BCG vaccinated controls

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Version: 2 Date: 26 March 2010

Author's response to reviews: see over
Dear Ms Roxane Rajabi:

Thank you very much for your very encouraging decision regarding our manuscript entitled, “Rv1985c, a promising novel antigen for diagnosis of tuberculosis infection from BCG vaccinated controls”. We sincerely appreciate the reviewers’ encouraging and thoughtful comments, which helped further improve the quality of our manuscript. We have now fully addressed all of the comments in a revised manuscript.

We are sorry for the inconvenience about not citing the previous paper in the manuscript. When this manuscript was written and finished, the previous paper was under review and not published yet. After it published, we forgot to revise the introduction and cite it in the manuscript. Now we have gladly added it in the “Introduction” (page 4). Compared with the previous paper, this work concentrated on one different but promising RD2-encoded antigen, Rv1985c. Besides, we investigated the humoral immunodiagnostic potential of it and compared the humoral response with other immunodominant antigens like LAM or 38kDa, which was not performed in the previous paper. Finally, the conclusion is very novel, and especially that humoral response based on Rv1985c achieved 52% sensitivity, which outperformed the sensitivity of PATHOZYME-MYCO kit (34%) in detecting active TB is very interesting and beyond our expectation.

The changes and responses to the comments of the reviewers are described below. The changes are underlined in the text of the revised manuscript:

**Reviewer #1: Kazue Higuchi**

**Major Compulsory Revisions**

1) Although the cut-off value of Rv1985c-ELISPOT appear to be set as the same as T-SPOT.TB, the appropriate cut-off value of Rv1985c-ELISPOT should be determined based on obtained data in this study using ROC analysis. Therefore, authors should analyze the data using ROC to determine the appropriate cut-off value of Rv1985c-ELISPOT, and show the sensitivity and specificity of this assay.

   Response: ROC of Rv1985c-ELISPOT was analyzed (Fig. 4). The cut-off value was revised base on the data of ROC, and the sensitivity and specificity of the assay re-analyzed (Page 3, page 10 and Table 2).

2) The combination of Rv1985c and PATHOZYME-MYCO IgG yielded the promising sensitivity, which is similar to TST. Since the specificity of the combination was superior to TST even in BCG-vaccinated population, this system could be replaced with TST. However authors did not discuss on this point. I would like authors to discuss on this point.

   Response: Discussion on this point was added in the “Discussion” and a reference was also cited (page 14).

3) Although authors concluded that Rv1985c can be used to immunologically
diagnose TB infection, Rv1985c alone cannot be used to immunologically diagnose TB infection based on the data in this study. Thus, authors should alter their conclusion, such as “Rv1985c can be used to immunologically diagnose TB infection along with ESAT-6 and CFP-10”.

Response: The conclusion was rewrote as “Rv1985c can be used to immunologically diagnose TB infection along with other immunodominant antigens among BCG-vaccinated population.”

Minor Essential Revisions
1) RD expresses the region of difference, not the region of deletion (page 2, line 10 and page 4, line 6).
   Response: Expression of RD has been changed to the region of difference (page2 and page4).

2) In background of abstract, insert (TB) (page 2, line 14) after tuberculosis, and use TB afterward in abstract (i.e. page 2, lines 17 and 18).
   Response: (TB) has been added after tuberculosis in the abstract (page2), and TB was used afterward.

3) Insert a hyphen between BCG and vaccinated (page 2, line 14 and page 5, line 1).
   Response: A hyphen has been added between BCG and vaccinated (page 2 and page 5).

4) Add a reference for the sentence “Among people infected with TB bacilli, …. Time during their life” (page 3, line 17).
   Response: A reference [31] has been added after the sentence (page3).

5) Add “by TST” between “BCG-vaccinated individuals” and “is difficult” (page 4, line 3).
   Response: We have added “is difficult” between the “by TST” “BCG-vaccinated individuals” (page 4).

6) Delete a period before reference (page 4, line 8).
   Response: We have deleted a period before the reference (page 4).

7) Once LTBI is used in page 4, line 2, use LTBI afterward (i.e. page 4, lines 17 and 21; page 5 line 13; page 13, line 6; page 14, lines 8 and 12).
   Response: We have used LTBI after the first introduction in the whole paper.

8) Move the full names of ESAT-6 and CFP-10 in page 6 lines 7-8 to page 5 line 16.
   Response: The full names of ESAT-6 and CFP-10 has been moved from page 6 to page 5.

9) In the methods, please add the strain of M. tuberculosis and how M. tuberculosis
was grown for cloning and expression of Rv1985c (page 6).
Response: Method of how M. tuberculosis was grown was added in the “Methods” (page 6).

10) In line 17 of page 7, a degree seems to be a square.
Response: The degree in page 7 has been revised.

11) Please add the full name of SFUs in line 20 of page 7.
Response: The full name of SFUs has been added in page 7.

12) Please describe a type of an automated ELISPOT Reader (page 8, line 2).
Response: The type of the ELISPOT reader was added in page 8.

13) Add (data not shown) after the sentence “……Rv1985c was expressed during the growth in vitro” (page 9, line 14).
Response: We have added “data not shown” after the sentence in page 10.

14) Insert a hyphen between T and cell (page 10, lines 1 and 3).
Response: A hyphen was added between T and cell (page 10).

15) Add p-value after the sentence “However, no statistic ………and LTBI groups” (page 10, line 8).
Response: P-value has been added after the sentence in page 11.

16) In the legend of Figure 3, change “latent TB infection” and “healthy control” to LTBI and HC, respectively. Also change “P-values of SFU....” to “P-value of absorbance....”.
Response: The legend of Figure 3 has been changed in according to the comments.

17) In Table 1, change “Healthy, BCG” to “healthy, BCG-“.
Response: We have changed “Healthy, BCG” to “healthy, BCG-” in table 1.

18) In Table 3, change healthy controls in the title to HC.
Response: We have substituted healthy controls to HC in the title of table 3.

Reviewer #2: Ajit Lalvani
Major compulsory revisions
1) What strain of BCG were the controls vaccinated with? This key information should be stated in the methods.
Response: BCG-Denmark is used in China. The strain was stated in the “Methods” and “Discussion” (page 5 and 15).

2) Discussion on the global distribution of BCG Pasteur-derived strains that lack RD2 is required so readers can assess where Rv1985c would be useful and where
Response: The BCG strains which lack or have RD2 were noted in the last paragraph of “Discussion”.

3) In the Introduction the authors do not adequately contextualise their study of T cell responses to this RD2-encoded antigen by omitting to mention earlier investigations of other RD2-encoded antigens for T cell-based diagnosis of TB, eg Liu et al Infection & Immunity 2004
Response: More information about the previous study on RD2-encoded antigens was added in “Introductions” and two references were cited (35, 36).

4) What is the incidence of TB in this area i.e. how likely are the controls to have been infected because of exposure in the community?
Response: The incidence of new cases TB in China was 98/100 000 population in 2007 based on WTO 2009 report. Currently, there is no data about the rate of LTBI in healthy population in China.

5) The authors make comparisons of the diagnostic sensitivity of Rv1985c with ESAT6 and CFP10 – but they fail to discuss the very large difference in the strength of T cell response to RV1985c compared to ESAT6 and CFP10 as shown in table 2. This difference should be explicitly discussed, especially since several of the active TB and LTBI ‘responders’ has borderline responses to this antigen which would not be considered positive according the cut-off point for appositive result that is mandated by the US FDA for T-SPOT.TB.
Response: The cut-off value of Rv1985c-ELISPOT was revised base on the data of ROC (Fig. 4), and the sensitivity and specificity of the assay re-analyzed (Page 3, page 10 and Table 2). The difference in strength between the antigens was stated in “Results”. The borderline positive responders of Rv1985c-ELISPOT were discussed in the “Discussion”.

6) LTBI was partly defined by a positive T-SPOT.TB. 1985c ELISpot cannot therefore be fairly compared to T-SPOT.TB as there is no objective reference standard against which the 2 tests can be compared. This limitation needs to be acknowledged and discussed.
Response: The limitation of the comparison is acknowledged and discussed in “Discussions”.

7) Ultimately, the value of any MTB antigen for diagnosis of latent TB hinges on the prognostic power of T cell responses to the antigen for predicting subsequent development of active TB disease (eg Diel at al Am J Resp Crit Care Med 2008 and Bakir et al Ann Intern Med 2008). The authors should recognise that such longitudinal data will be required to confirm the true diagnostic utility of Rv1985c
Response: Longitudinal data will be required to confirm the true diagnostic utility of Rv1985c is acknowledged and discussed in Discussions and two references were cited
8) Why would *M. tuberculosis*-specific antigens be a potential source of antigens for vaccine development?
Response: Antigens of *M. tuberculosis* with strong immunogenicity can be potential candidate for vaccine development. TB specific antigens like ESAT6, MPT64 are good examples of how they are used to enhance the protective efficacy of TB vaccine (Ref 13, 14). However, we are not talking about vaccine development in this paper; thus, we have deleted some statement about vaccine development in the “Introduction”.

9) Why was the T cell assay not done on all the participants that had the antibody tests?
Response: T-cell assays cost more than the antibody tests. Due to limitation of expense and resources, only part of participants did the T-cell assay.

**Discretionary revisions**
1) Was Rv1985c antigen used rather than peptides in the ELISpot? Why was this antigen chosen?
Response: Recombinant protein Rv1985c rather than peptides was used in the ELISpot. Previously we found some RD2-encoded proteins were potential T-cell antigens and could be used in TB diagnosis (Ref 36). Then we began to consequently screen other RD2-encoded proteins, which included Rv1985c antigen.

2) The recently determined incremental diagnostic sensitivity of certain other antigens when used in combination with ESAT6 and CFP10 in ELISA and ELISpot assays is not mentioned, eg TB 7.7 in Quantiferon TB Gold In-tube and Rv3879c in ELISpot (Dosanjh et al, 2008)
Response: More information on antigens combination used in IGRAs was added in “Discussion” and two references were also cited (38, 39).

3) The following text in the last para of the discussion is confusing: ‘Furthermore, we also observed one T-SPOT TB-negative subject had positive ELISPOT response’. Are the authors referring to a result in the LTBI group? And what do they mean by ‘ELISPOT response’?
Response: The last paragraph of discussion was rewrote to make it understandable.

**Reviewer #3: SANG NAE CHO**
Major revision:
The authors recruited tuberculosis (TB) patients and their contacts (LTBI) from three regions of China, Chongqing, Jinan, and Suzhou, but healthy controls from Fudan University, which is located in Shanghai. In addition, the mean ages of TB and LTBI subjects were older by more than 10 years than that of healthy controls. Therefore, the extent of exposure to *M. tuberculosis*, non-tuberculosis mycobacteria,
and other infectious agents would be markedly varied resulting in difference in background immune responses to any antigen including the Rv1985c. Difficulty in recruiting healthy control subjects in the same areas as the TB and LTBI subjects would be understandable, but such limitations and potential confounding factors should be mentioned in the discussion section. If healthy subjects in the same areas and in similar age groups were recruited, the cut-off levels for T cell and antibody responses might have been elevated, which in turn affected the sensitivity and specificity of the assays using Rv1985c.

Response: The same ethnics but different areas and age groups which we recruited could affect the sensitivity and specificity of the assays. We acknowledged and discussed in the “Discussion”.

Minor revisions:
Table 2: the title of Table 2 contains numbers of subjects of TB, LTBI, and Healthy controls. Like in Table 3, the numbers need to be indicated in the first row of the Table 2 instead.
Response: We have changed the title of Table 2, and moved the numbers to the first row of the Table 2.

Table 3: If LTBI subjects were not tested using the PATHOZYME-MYCO IgG kits, it would be better to indicated as “ND: not done: or NT: not tested” instead of “-“.
One may confuse the “-“ as all negative.
Response: “NT” in stead of “-” was now used to indicate the tests were not done in Table 3.

We hope we have now fully and appropriately addressed all of the reviewers’ concerns in a satisfactory manner. However, we would be happy to further address any concerns the reviewers and the editor may have.

Thank you very much for your further consideration.

Sincerely yours,

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