Author's response to reviews

Title: Mycobacterium tuberculosis ecology in Venezuela: Epidemiologic correlates of common spoligotypes and a large clonal cluster defined by MIRU-VNTR-24

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Version: 2 Date: 29 March 2009

Author's response to reviews:

29 March 2009

Editors,
BMC Infectious Disease

Dear Editors,

On behalf of all the authors, I hereby return the revised manuscript entitled “Mycobacterium tuberculosis ecology in Venezuela: Epidemiologic correlates of common spoligotypes and a large clonal cluster defined by MIRU-VNTR-24”. The referees comments are addressed point-by-point below. The figures have been corrected, the text has been revised, and all of the statistical tables have been redone to make proposed associations easier to appreciate. We hope that the manuscript is now acceptable for publication.

Thank you again for considering our manuscript,

Sincerely,

Howard Takiff, corresponding author
IVIC, Caracas Venezuela
Reviewer's report
Title: Mycobacterium tuberculosis ecology in Venezuela: Epidemiologic correlates of common spoligotypes and a large clonal cluster defined by MIRU-VNTR-24
Version: 1 Date: 17 February 2009
Reviewer: Viviana Ritacco

Reviewer's report:
- Discretionary Revisions (which are recommendations for improvement but which the author can choose to ignore)

1. ABSTRACT. There is certain ambiguity in the attributes ascribed to SIT 605. The statement that this SIT is “infrequent in regions other than Valencia” seems to disagree with the previous one saying that “all six SITs were the most common in almost all regions” and the conclusion stating that SIT 605 “appears to be spreading throughout the country”.
   Changed

2. ABSTRACT. The statement “Six SIT’s cause 49% of cases of tuberculosis in Venezuela and are the most common in all regions” in the Conclusions, is a repetition of Results.
   Changed

3. ABSTRACT. Again in Conclusions, a repetition of Results is found in the first clause of the sentence “Patients with SIT 53 were older and more commonly smear negative, suggesting that…”. This could be simplified as follows “SIT 53 appears to be less virulent and associated with reactivation of past infection in older people.
   Changed

4. ABSTRACT. Only 3 of the 6 most common SITs are commented in the abstract. Any comment on the other 3, in particular SIT 93, the second most frequently found in the study?
   Nothing notable was found for these strains, even SIT93. While this itself is interesting, given the lower age for SIT17, this lack of distinguishing characteristics is hard to address in the abstract.

5. BACKGROUND. Were the genotypes differently distributed through time in the 10 yr period of study? Is there any temporal evidence of some genotypes emerging and others disappearing as suggested in the last paragraph of this
The principal SITs have been analyzed for time trends, but none are apparent so far.

6. DISCUSSION: Please, fundament the assertion “SIT 605…. appears to be spreading around the country” in page 11, last 2 lines, which is reiterated in the abstract.
This is an hypothesis that has been taken out of the abst.

7. DISCUSSION. (page 12) According to supplementary data, all 5 strains of the Beijing family were isolated in Caracas, the most cosmopolitan of the areas in this study. There is some evidence of Peru being a South American reservoir of Beijing strains (see Aristimuño JMM 2007 & Ritacco MIOC 2008). It would therefore be of interest to know if patients with Beijing strains in this study were born in Venezuela, in other South American country or overseas.
As now noted in the mss, at least one Beijing patient was a Peruvian national.

8. DISCUSSION. (page 12, line 10) How would drug resistance as such explain the extensive local transmission of a genotype?
A drug resistant strain would not be controlled by first line therapy and might be transmitted until adequate therapy is instituted, which could take several months as resistance testing is not routine.

9. Results and Discussion are lucid, fluent and stimulating but there is some overlapping and some information is presented for the first time in the discussion. Perhaps both sections of the manuscript could be merged under a joint heading “Results and Discussion”.

- Minor Essential Revisions (such as missing labels on figures, or the wrong use of a term, which the author can be trusted to correct)
1. TITLE PAGE. Affiliation of corresponding author is camouflaged because an enter is missing before it
2. TITLE PAGE. What is the contribution to the work of authors marked with symbols other than “*”? These appear to have been problems with the transfer to pdf format
3. TITLE PAGE. The initial E for the author named Howard Takiff is either missing in the authors list or redundant in the list of emails. Corrected.
4. ABSTRACT. Mycobacterium tuberculosis is abbreviated the first time it is
mentioned in the abstract and written in full the second time. Please invert this order.
Corrected

5. THROUGHOUT BODYTEXT. Different abbreviations (“ST” and “SIT”, singular; “SITs” and “SIT’s”, plural) are used for the same concept; when mentioned individually, some SIT numbers are preceded by a space and others are not (“SIT605” and “SIT 605”). Please, unity style.
The style has been unified.

6. THROUGHOUT BODYTEXT. Some spaces are missing, particularly before references in brackets.

7. INTRODUCTION. page 5, line 4: replace “100.000 (WHO Report 2.006) by “100,000 (WHO Report 2006)”;
Changed.
line 15: replace “Laboratory” by “Laboratorio”.
Changed

8. BACKGROUND. First paragraph. Mycobacterium tuberculosis should be written in full the first time it is mentioned in the bodytext.
Changed

9. BACKGROUND. page 4, last paragraph: Suggested modification: “The more recent technique of microsatellite analysis (34), MIRU-VNTR 24 loci, is less technically demanding than the IS6110 RFLP typing technique, can discriminate at strain level and (in place of but) is also promising for use in phylogenetic studies.”
Changed

10. BACKGROUND. Page 5, line 15. Please, clarify the expression “…have been working in national networks with the National TB Control Program …”(does it mean “in collaboration with”?)
Changed

11. DISCUSSION: page 11, line 3: Replace “Camaroon” by “Cameroon”.
Corrected

12. CONCLUSIONS: (page 14, line 19: “…of the Venezuela..” (delete “the” and duplicated full stop).
Changed.
“The only two strains with ST605 described outside of
Venezuela were isolated in New York in patients from Colombia, and (suggested modification: …"were confirmed by MIRU-VNTR to belong to the Valencia”… instead of “ST 605”) cluster.”

13. METHODS: Could you please make clear if every strain corresponded to a different patient?

The Methods now begins, “The 1298 clinical isolates in this report were obtained from an equal number of different patients…”

14. METHODS (Page 15, line 12): The statement “The strains were obtained by culturing sputa or non-pulmonary clinical specimens that were positive for Acid Fast Bacilli by microscopy.” is not congruent with results of correlation of clustering with epidemiological analysis (page 9) where only 75% of the patients were found to produce AFB positive sputa and “…patients with SIT 53 were less likely to have bacilli seen in their sputa compared to patients with SIT 17 and SIT 605”. Also, the abbreviation AFB appears several times in the bodytext without previous decoding.

15. METHODS (Page 16, line 12): Please clarify the statement “For some of the patients whose isolates were included in the study, the information on these parameters was not available” in the light of this other in RESULTS (Page 8, line 5) “…for several parameters the information was recorded for 25% to 50% of the patients”. The validity of results on correlation with epidemiological parameters can be argued if the study sample is not clearly described.

As in response to this point by other referees: although information was available for only 25% in some cases, this still represents more than 300 patients. The epidemiologic information was recorded, or not recorded, prior to spoligotyping, so should not be subject to bias. Possible selection or recording bias based could have occurred if there were regional differences in data entry or patient admission that skewed the data for dominant local strains. We have attempted to eliminate this possibility by stratifying analyses by region, as presented in the new table.

16. METHODS (page 18) & RESULTS (page 7) the description of SNP method is exiguous, compared with the description of methods more widely used, like spoligotyping and MIRU-VNTR genotyping.

The SNP analysis is described only very briefly in the manuscript and the methods are detailed in the references cited.
Likewise, readers would welcome some clarification on SNPs analysis and also information on how many/which isolates with SIT 605 were analyzed, given that some MIRU-VNTR variation within this SIT is observed in the study.

Of the 23 strains tested there were 4 SIT 605, 4 SIT17 and 4 SIT93 isolates. All of these, and all the other LAM patterns gave the same SNP pattern, SCG V. Only the Haarlem strains included gave a distinct pattern. The analysis with 45 SNPs is not sufficiently discriminative to distinguish amongst LAM strains. This is briefly stated in the mss.

17. METHODS (Page 15, line 7): Replace “capitol” by ”capital”.

Changed

18. METHODS (Page 16, line 18): Replace “(31, 32, 34) (33)” by “(31-34)”

This is no longer present

19. METHODS. Page 17, line 1: replace “Megative” by “Negative”; line 7: replace “dendograms” by “dendrograms”

Changed

20. REFERENCES. The list of references included under this heading does not fit citation numbers in the bodytext. Please replace it by the alphabetical list (without heading) found misplaced in pages 30-34.

There is now one list of references.

21. TABLE 1. The table appears truncated at SIT ranked 27 while, according to its heading, it should present up to SIT ranked 36, the last SIT with a frequency of 5.

This appears to be related to the transfer to pdf

22. FIGURE 1. In captions for Caracas and Sucre, years are truncated and illegible.

Corrected

23. FIGURE 2. Graphics look elongated

This figure was redone

24. FIGURE 3. Two slightly different headings are found for the same figure, one in figure list and the other in the figure itself.

There is now only a heading in the figure list.

The box including SIT 605 strains is displaced downwards.

Corrected
Reviewer's report
Title: Mycobacterium tuberculosis ecology in Venezuela: Epidemiologic correlates of common spoligotypes and a large clonal cluster defined by MIRU-VNTR-24
Version: 1 Date: 3 February 2009
Reviewer: Igor Mokrousov
Reviewer's report:
Major Compulsory Revisions
1. Definition of a new genotype cluster as “Valencia” is misleading. There is more than one city with this name while the most known is Spanish city. In other countries there are:
   Valencia, Córdoba, Colombia
   Valencia, Lahore, Pakistan
   Valencia, Bohol, Philippines
   Valencia, Negros Oriental, Philippines
   Valencia, Trinidad and Tobago
   Valencia, Santa Clarita, California, U.S.
   Valencia, New Mexico, U.S.
   Valencia, Pennsylvania, U.S.
   Accordingly, authors should propose another name for this genotype.
   The name was changed to “Carabobo” the state of which Valencia is the capital
2. Please check References style. According to the Instructions for this journal “All references must be numbered consecutively, in square brackets, in the order in which they are cited in the text”
   At present, you have two reference lists in this submission:
   In order of appearance in the text (pages 19-25).
   In alphabetical order at the very end of the ms (pages 30-34) – this one you actually used for citation in the text.
   So you should renumber all your citations in the text based on your correct list in pages 19-25 (and remember to delete the list in pages 30-34).
   Pay special attention to correct association between reference number in text and Reference list.
   Apologies. There is now one set of references, formatted by ENDNOTE for BMC
Inf. Dis.

Minor Essential Revisions
Page 3, Background subsection: “analyzed ….. with spoligotyping and 24 loci …”
Corrected

Page 4, the VNTR-MIRU are MINIsatellites, not MICROsatellites.
Corrected

Discretionary Revisions
Page 10, last line: “originated” not “began”
Corrected

Page 11, line 3: Cameroon
Corrected

Page 13, line 8. p=0.04 is significant but at borderline level
While the regional comparisons are only just above or below the 0.05 level of significance, when all patients are analyzed together as a single cohort the P value is more significant. However, the borderline significance in different regional comparisons shows that the trend is not based on regional selection or recording bias.

Page 13, 2nd paragraph. ST53 is global type and actually I do not see much significance in its 6th place among your spoligotypes, 4% of strains is a very minor proportion.
While 4% is not that high, it is the sixth most common of 300 spoligotypes, which would not be expected if it were really less virulent. These are observations that seem worth mentioning, and doubts about their validity and importance, and mechanistic explanations will hopefully be addressed in subsequent studies.

Page 17.
UPGMA - ................. method of averages.
Corrected

Page 19. I think “doctors” should be abbreviated as Drs (not Dra)
Corrected (Dra. Is the form for female Drs. in Spanish)

References
Page 19. ... Brudey ...
The references were downloaded and inserted by the program Endnote. I don’t understand the correction required.

Page 21, page 33. - Valcheva et al. – give complete citation, volume and pages
The reference has been updated with volume and page.#.

Figure 2. It would be nicer to show circle, not ellipse.
This manuscript describes the spoligotypes of 1,298 Mycobacterium tuberculosis complex strains isolated from patients at different localities in Venezuela from 1997 to 2006, including urban and indigenous areas. A subgroup of strains representing the major spoligotype clusters were submitted to additional genotyping by analysis of MIRU-VNTR 24 loci. The authors associated the spoligotypes with epidemiological parameters in an attempt to track the dynamic of Mycobacterium tuberculosis strains with particular genotypes in Venezuela.

Major comments

1) This manuscript is an extension of previous reports by the same authors showing the population structure of M. tuberculosis strains in diverse areas of Venezuela. A significant part of data from the two Amerindian population have been already published by the authors (ref 24-in press-, Delta Amacuro; ref 25, Amazonas, 41 isolates), as well as data from the city of Valencia (ref 28, 317 strains) describing the predominant local clone.

The Article from the Amazonas has yet to appear, and will come out in a Spanish language journal with limited diffusion. The Article on Valencia is in Spanish in a Chilean journal of Infectious Disease, and is unlikely to be widely available.

The current article compares the results from all these areas and presents a broader picture, something not done in previous reports.

Although the present manuscript includes a larger collection of strains, the genotyping data do not represent a novelty as there are not new findings, and the population structure of M. tuberculosis strains in Venezuela has already been described in the frame of a nation wide study performed by others (reference 5).

Compared with the previous study, this study shows:

a) That the most common strains are the most common in diverse regions of the country, even two poorly accessible indigenous areas.
b) That the third most common, SIT 605, is concentrated geographically.
c) MIRU data showing that while SIT 605 is largely clonal, the other common spoligotypes are not.
d) That SIT 605 strains found in New York belong to this clonal group.
e) Patients with SIT 17 appear to be of a younger age group and more AFB positive.
f) SIT 53 may be in an older patient population.

The previous study simply showed the spoligotypes present in the country, without any concept of true clustering, due to the relatively limited discriminative power of spoligotyping. The addition of the MIRU, geographic and epidemiologic information in our study give a much more complete picture the ecology of TB strains in the country.

2) Abstract, background, lines 1-2: could the authors modify this phrase. Please note that the situation they describe (endemic TB with few highly transmitted M. tuberculosis genotypes) is not the rule but just one of the multiple situations found in the world.
This has been changed.

3) Table 1 shows that 70 out of 467 strains in Valencia had genotype SIT605. This corresponds to 15%, and not to 75% as indicated in the text Page 11, line 24.
This has been corrected

4) The authors invested many efforts to determine the genetic family of the M. tuberculosis strains. However their classification relies only on spoligotypes, except for an indeterminate number of strains with SIT605 which were additionally analysed for the presence of 45 SNPs. Could I suggest to use their available MIRU-VNTR data to determine and/or confirm the assignment of strains to a particular family, as described in reference 3.
This had been done, and is now mentioned.

5) This paper has a clearly defined epidemiological objective, that makes its originality. However, it does not offer sufficient evidence for the conclusions:
5.1 ) It is confuse how the patients were selected for the study. Is it possible that the criteria differed between the different localities? For instances, did the collections comprised only smear positive, culture positive specimens, or all the culture positive specimens? There were only adults included in some local surveys, as the patients were > 15 year-old? If the selection criteria were not
harmonized, it would not be valid to merge patients from different localities for correlation of genotypes with epidemiological data.

Differences in patient admission criteria are mentioned. Results are analyzed by region.

5.2) It is unclear which and how many epidemiological data were available for each local group of patients. These records should be presented in a general Table. Correlation of these data with particular spoligotypes should only be done if available data are representative.

As suggested, the analysis was stratified by region to eliminate potential patient selection or recording biases. As the spoligotyping was done after the epidemiologic data was collected, it is hard to identify possible additional bias within each region. The new table includes the number of patients with data that were included in the statistical calculations.

5.3) The multivariate analyse indicated that none patient’s data was associated with any specific spoligotype.

The statistical analyses with the two tailed T test comparison assuming equal variance in all regional populations suggest that there are robust differences that reach statistical significance when combined. We report these observations and statistical probabilities because they appear interesting and relatively novel, and we have been unable to invalidate them by identifying any apparent bias. Similar trends have been shown for the Beijing family strains. The reference to multivariate analysis has been deleted.

6) The authors speculate on the less virulence of SIT53. Please note that strains with this spoligotype are not a compact group but may show high genetic heterogeneity when they are analyzed using other markers, due to the lower discriminatory power of spoligotyping. This is precisely the result obtained by the authors in this manuscript, as showed in figure 3.

The argument of spoligotype heterogeneity as shown by MIRU for ST17, was addressed for both SIT17 and SIT53 in the last paragraph of the discussion.

Minor comments
1) Results, clustering analysis: please indicate “clustering analysis of spoligotypes”.
   The subtitle has been changed
2) The authors classed strains with ST605 either in “LAM” or in “U” families in the Figures. Please review the Figures and check the corresponding percents in the text.
Figure 2 A reflects SpolDB4 classification of SIT 605 as U. Fig. 2B is based on SPOTCLUST classification of spoligo patterns not appearing in spolDB4.

3) Methods, spoligotyping and MIRU-VNTR typing: please indicate in the text only modifications to standard methodology already described in the included references.

These sections have been shortened as suggested.

Level of interest: An article of limited interest

Quality of written English: Needs some language corrections before being published

Statistical review: No, the manuscript does not need to be seen by a statistician.

Declaration of competing interests:
'I declare that I have no competing interests'

Reviewer's report

Title: Mycobacterium tuberculosis ecology in Venezuela: Epidemiologic correlates of common spoligotypes and a large clonal cluster defined by MIRU-VNTR-24

Version: 1 Date: 2 February 2009

Reviewer: Yong-Jiang Sun

Reviewer's report:
In this study the authors analysed a convenient sample of 1298 MTB isolates collected in Venezuela between 1997 and 2006 using spoligotyping, while 24-loci MIRU-VNTR typing was used to further discriminate the isolates with identical spoligotypes. The study sample was fairly large and collected from multiple centres in Venezuela, the spoligotyping data could therefore well reflect the MTB population structure in the country. But unfortunately it does not provide much more information than does the previous study (Aristimuño et al., 2006).

The following comments were also presented in answer to similar comments by another reviewer:

Compared with the previous study, this study shows:
g) That the most common strains are the most common in diverse regions of the country, even two poorly accessible indigenous areas.
h) That the third most common, ST605, is concentrated geographically.
i) MIRU data showing that while ST605 is largely clonal, the other common spoligotypes are not.
j) That ST605 strains found in New York belong to this clonal group.
k) Patients with ST17 appear to be of a younger age group.

The previous study simply showed the spoligotypes present in the country, without any concept of true clustering, because the relatively low discriminative power of this technique. The addition of the MIRU, geographic and epidemiologic information in our study give a much more complete picture the ecology of TB strains in the country.

Molecular characterisation of Mycobacterium tuberculosis isolates in the First National Survey of Anti-tuberculosis Drug Resistance from Venezuela. BMC Microbiol. 6:90) in this aspect. On the other hand, the data used for phenotype-genotype relationship analyses appeared too weak to draw any sound conclusions.

The tables of epidemiologic correlations has been redone in a different format, and the associations appear to be statistically significant. Although the relationships are not overwhelmingly strong, they appear interesting and either obtain or approach significance at the 0.05 level. Of course this means that one in 20 times this result will occur by chance, and some undiscovered bias could have occurred. However, as the reviewer suggests no possible source of bias to invalidate the relationships, which persist after stratification by region, they would appear to be reportable.

In addition, the data is not well presented and the manuscript is not well prepared.

Some of the data, such as the statistical analysis, has been redone. The manuscript has been corrected to reflect the specific comments of the other referees.

Major Compulsory Revisions

1. Epidemiological data were available for only 25% to 50% of the total subjects of the convenient sample, such a low availability of epidemiological information could easily lead to bias. For example, it looks too high that 75% of subjects had cavitations; in addition, in the Delta Amacuro region, patients were younger and less males compared to other areas, how about the general population in this region compared to other regions? This should be considered when make such comparisons.

While data is available for only 25 – 50% of cases, given the total of 1298 TB patients in the study, this represents a minimum of over 300 cases. The percentage or number of isolates with data is now shown in the tables. A previous study published in BMC Inf Dis proposing that the Beijing engendered distinct characteristics was based on a total of less than 50 patients, with only
about 20 infected with Beijing strains. As the data recording was done before the spoligotyping, it is hard to see where the bias would enter, unless the recording was more complete in regions with a higher percentage of a particular spoligotype. To check for this possibility, the results were analyzed by region (see new tables). Although in some regions the differences were slightly above the 0.05% significance level, they all confirmed the tendency and the P values for regional differences on SIT 17 were almost all below 0.1.

As for the age and male to female ratio in the Delta Amacuro region, the differences as compared to the other regions are striking, and while they could reflect a younger population, they are still noteworthy. There is no accurate and recent demographic data for this population, but it is difficult to imagine that the male:female ratio in the population is so different from other regions as to account for the nearly equal M/F ratio in the Delta TB population. Of course the suggestion of extensive transmission in this population is only an hypothesis, but it is supported by the fact that this is the region of the country with the highest TB incidence.

2. The authors found that the patients with SIT 605 were more likely to have pulmonary cavities (when compared to all other genotypes) and more likely have AFB positive sputa (when compared to SIT 53). All these comparisons are inappropriate because most of the ST 605 isolates (~70%) were from Valencia and it might also involved in transmissions among inmates in a prison and most of the ST53 isolates (~55%) were from Caracas.

While the regional distribution of these strains is skewed, it is not clear why this makes comparisons inappropriate, except for a possible reporting bias. Nonetheless, the comparisons have also been performed within the regional cohorts and again display the same tendencies, as shown in the new tables. The Xray correlations to SIT 605, have been deleted, as the significance level did not warrant conclusions. The AFB negative sputa for SIT 53 is noted, but the absence of statistical significance is emphasized, and it is stated that it “may” have less AFB positivity.

3. The last paragraphs in the Background should not be here, they should be in the Results or Discussion sections.

This seems to be a style issue, as many articles end the introduction with a short foretelling of the results.

4. Since the spoligotypes and MIRU-VNTR types all suggest that ST605 was a sublineage of LAM, then it should be named as LAMx instead of a new name “Valencia”.

There don’t seem to be any clear rules for this nomenclature, but clonal outbreak strains are often named. The 605 spoligotype could be given a LAM designation, but MIRU shows that it is more a genotype than a spoligotype. As pointed out by
another reviewer, there are many cities with the name “Valencia”, so we have changed the name to “Carabobo”, the state where it predominates.

5. Table 1 is exactly the same as the upper part of the first page of the supplementary Table 2.
   This is correct. Supl. table 2 is a listing of all the spoligotypes, including, for convenience, the ones that appear in Table 1. These could be eliminated, and it could just be the continuation of Table 1 if this is the editors’ preference.

6. The discussion is unnecessary lengthy, it can be more succinct.
   This was not noted by the other reviewers.

7. There are two sets of references in the manuscript. Reviewers have to guess which is the correct one.
   Apologies. This has been corrected.

Level of interest: An article of limited interest
Quality of written English: Acceptable
Statistical review: No, the manuscript does not need to be seen by a statistician.