Author's response to reviews

Title: Genotyping and Drug resistance patterns of M. tuberculosis strains in Pakistan

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Author's response to reviews: see over
REPLIES TO THE EDITOR

Please find below response to each of the comments manuscript that were made by each of the three reviewers. We have made changes accordingly in the manuscript where required. Reviewer’s comments are in italics with answer below.

Reviewer 1; Stefan Niemann

Major revisions:
1. The authors used spoligotyping as a typing method. This has not a sufficient discriminatory power as can be seen in the high cluster rate. Hence, a cluster analysis cannot really be performed and the presentation of the data in this way makes no sense. The authors should use the terminology shared types and orphan types instead of clustered and not clustered. The classification of the strains should be first done according to major genotypes/phylogenetic lineages which are then further divided. It makes no sense to draw special attention to small subgroups e.g CAS1 which belong to a larger principle clade. The authors should follow a strict classification scheme from larger genetic groups to shared or single types observed in Pakistan. The nomenclature in all tables should be changed accordingly.

In agreement with suggestions given by the reviewer, the following changes have been made;

1. The terms “cluster” and “Unique” have been changed to “shared types” and “Orphan types” respectively in the manuscript.
   Page 3, line 63 and 64
   Page 8, line 196
   Page 9, line 198, 203 and 204
   Page 10, line 226, 233 and 242
   Page 11, line 264
   Page 12, line 278, 281 and 294

2. Classification of strains
   Fig 1 and Fig3 has been changed according to the major genogroups. The nomenclature in all tables is also changed accordingly.

2. The MDR rate reported in the study is strikingly high. This needs further classification and reasons and implications should be discussed in detail. The author should present patient classification according to WHO case definition and detailed drug resistance data in an overview table with. This is necessary for a meaningful interpretation of these striking data.
As discussed in methods, page 7, line no.154-155 patient treatment history not available therefore, we are unable to classify on basis of prior treatment or otherwise. A table describing the resistance pattern of the strains tested is included Table 2

3. *Introduction appears very long and redundant and can be shortened.*

Introduction section has been shortened please see attached re-submitted manuscript.

**Reviewer 2; Subhash Parija**

Major revisions:

**Background**

1. *The authors have mentioned Pakistan is surrounded by high TB disease burden countries such as India (rank 1), china (rank 2), Afghanistan (rank 22) and Iran (rank 55) which may further facilitate TB transmission through the region. This doesn’t makes any logic because the global ranking of Pakistan in terms of TB burden is 7th which is very high and it itself has the potential to transmit TB to other regions. This needs to be modified.*

   In accordance with the reviewer suggestion the above mentioned paragraph have been removed.

2. *Authors should mention the recent rate of MDR-TB from Pakistan reported by health surveillances or WHO survey.*

   The data available for the resistance pattern of MTB has been explained in **Page 5, line 109-110** of Introduction section.

3. *what about nucleotide sequencing? Provide brief description of the methodologies followed. Whether sequence analysis was done. Sequence data obtained from Gene Bank, EMBL or DDBJ, authors are requested to mention the source, assesion numbers of the strains submitted.*

   In order to clarify the above mentioned suggestion as the reviewer inferred on genotypic methods, please see the methods section as the results are all phenotypic therefore no data for nucleotide sequencing is available.

4. *Regarding the drug resistance pattern the authors should give a concise description of the sensitivity and resistant pattern on scale. This would be useful information for the researcher working in the field.*

   The resistance pattern of the strains tested is included in Table 2.
Discussion

The discussion could be more enriched by inclusion of more data from Pakistan regarding the M.tuberculosis prevalence and MDR pattern in last 10-15 years.

In accordance to the above suggestion, more information has been added in the manuscript. Please see the revised manuscript.

Methods

1. The authors should mention clearly regarding sample collection and storage conditions under separate heading.

Sample collection and storage conditions are described in “Methods section” at page 6, line 146-148

2. Whether primer validation was done? Authors should mention what have been done to validate the primers for spoligotyping and TbD1 analysis.

Primer validation

Spoligotyping was carried out using a commercially available kit from Isogen Biosciences BV, Maarssen, The Netherlands according to the manufacturer’s instructions. Spoligotyping based on the 43 spacers of the DR region of M. tuberculosis complex was carried out using primers DRa 5’GGTTTTGGGTCTGACGAC3’ and DRb 5’CCGAGAGGGGACGGAAAC 3’ as originally described by Kamerbeek et al[1].

For TbD1 ; PCR analysis was done using the methods described by Brosch et al 2002[2]. The TbD1 contain genes of mmpS6 and mmL6, primer sequence used are available at http://www.pnas.org/cgi/data/052548299/DC1/1[3]

3. A brief description of spoligotyping methodology must be given in the text

A brief description about spoligotyping with reference has been included in method section under the heading of “Molecular methods” in page 8, lines 181-184

4. There is no clear mention of the statistical analysis performed in the work. It would be better if the authors can provide a brief description under separate heading.

A brief description of statistical analysis has been mention under separate heading (page 9, lines 205-208)

Minor revisions

Abstract

1. In the abstract section the M.tuberculosis should be written as Mycobacterium tuberculosis in the first mention. Abbreviation such as CAS, T1, EAI, CAS_DEHLI, U, MANU, TbD1, NWFP should be mentioned in full.
Background

1. In the background section instead of using symbol (>) use more than in the text. References should be appropriately mentioned under reference section and in text.

Editorial changes

All editorial changes have been made such as abbreviations; symbol and insertion of reference numbers have been changed.

Abstract

Page 3, line 54 – *M.tuberculosis* use *Mycobacterium tuberculosis*

Page 3, line 65-68 abbreviations mention in full form and spelling

Background

Page 5, line 101 – instead of using > symbol use more than

Page 5, line 102 – indicated as reference number in text instead of using (WHO 2007)

Results

1. There is repetition of the data in text and illustrations (Fig 1 and 2). Author is advised to avoid the repetition. Also Fig 1 is not clear and resolving therefore the UPGMA data (dendrogram) cannot be appreciated well.

In agreement with suggestion the repetitive text and data have been removed. Please see the re-submitted manuscript.

Discussion

1. In page no. 12 the sentence (prevalence of Beijing strains in our study at 3% (n=25) compares well with data from Dehli, where 8% of 105 isolates are thought to be of Beijing family) should be written as (prevalence of Beijing strains in our study at 3% (n=25) compares well with data from Dehli, where 8% of 105 isolates are reported to be of Beijing family.

As the manuscript has been formatted according to BMC Infectious disease the page no. has been changed.

Page 12, line 291 changed as required.

2. In page no. 12 the sentence the reference ([http://www.pnas.org/cgi/doi/10.1073/pnas.052548299](http://www.pnas.org/cgi/doi/10.1073/pnas.052548299)) should be appropriately mentioned in the reference section and should be numbered in the text accordingly. The same should be followed in other cases as this has been repeated on many occasions

Appropriate reference numbers

The web sites described in particular text have been appropriately numbered in the text as well as in the reference section.

Page 8, line 192 website [http://www.pnas.org/cgi/data/052548299/DC1/1](http://www.pnas.org/cgi/data/052548299/DC1/1) [3]
Methods

1. This section is not structured properly. It should be structured with suitable subheadings to make the presentation clear.

The following subheadings have been added;

Page 6, line 141– **Mycobacterial strain collection**
Page 7, line 158– **Mycobacterial cultures and antibiotic susceptibility testing**
Page 9, line 205– **Statistical analysis**

**Reviewer 3; Richard Huard**

Comments: the authors provide a survey of the population structure of M.tuberculosis strains causing tuberculosis in Pakistan along with resistance patterns and other demographic comparisons. The work is novel and important, as well as complete and well-presented. Although the quality of written English is satisfactory, there are small errors throughout the manuscript that a second proof-reader could catch and correct. In the future, the authors should remember include line numbers in draft manuscripts in order to aid the reviewer in making remarks.

Minor revisions:

Abstract:

1. **Use of term unique in place of non-clustered with respect to spoligotyping is confusing.**

   The terms “cluster” and “Unique” have been changed to “**shared types**” and **Orphan types** respectively in the manuscript.

Page 3, line 63 and 64
Page 8, line 196
Page 9, line 198, 203 and 204
Page 10, line 226, 233 and 242
Page 11, line 264
Page 12, line 278, 281 and 294

2. **The use of term homologous is nonconfirming to accepted nomenclature and lacks specificity when variants or derivatives of a major spoligotype is more clear.**

   The term homologous/homology have been changed to “**similarity**” as the information given in detail in results section is based on the similarity index pattern.

Page 10, line 228 and 230….. **similarity**
Page 13, line 321……. **similarity**
3. The conclusion statement beginning with a (Lack of association) does not make sense. Lack of MDR on CAS1 strains would suggest that appropriate therapy is delivered. More appropriate conclusions are required

As required the conclusion statement has been changed.

Page 4, line 78-79

Introduction:

1. Overly long with content that is not relevant to the study data and somewhat illogical order of presentation. Efforts in addition to the following suggestions should be made to streamline it better.

   In agreement with above suggestion the manuscript has been revised. Please see the re-submitted manuscript

2. Paragraph 1 is OK except that MDR should be defined i.e resistance to rifampicin and isoniazid

   Page 5, line 105 – MDR has been defined

3. Paragraph 2 should be cut, it is superfluous.

   In accordance with the reviewer’s suggestions the changes have been made in the Introduction section

4. Paragraph 3 should be moved back in introduction. EAI strains are introduced before spoligotyping which defines EAI. The definition of TbD1 is confusing. TbD1 is present in modern MTB and intact in ancestral MTB strains. The final statement starting with (it is further suggested ) is logically flawed. There are few SNPs known that define specific MTB lineages and none of these are resistance related, perhaps excepting the pncA mutation in M.bovis conferring PZA resistance. No restricted geographical localization has been found for the major SNPs conferring resistance to Rif or INH. This sentence should be cut.

   The following changes have been made
   Page 5, line 114-116  SpolDB4 Information has been added before genotypic information.
   Page 8, line 181-184  TbD1 defined with relevant reference. While the text mentioned for drug targets has been removed.

5. Paragraph 4 MIRU is not synonymous with IS6110 RFLP. SpolDB4.0 is not defined The sentence starting with (studies reporting strain diversity ) is out of place and should be moved somewhere else. The BCG information is not necessary to understand the current study and should be cut
1. As the Introduction section has been revised the above mentioned paragraph has been changed. Please see the re-submitted manuscript.

2. Page 9, line 202-204 SpolDB4 has been defined appropriately in Method section under the subheading of Data analysis

6. Paragraph 5 and 6 should be merged and reorganized significantly. The authors are in factual error with regard to the prevalence of the Beijing family. The prototype Beijing spoligotype is the single predominant signature in SpolDB4 but Beijing-family account for a smaller proportion of TB cases worldwide than T, LAM, and Haarlem

In agreement with the comments more information is included with reference.
Page 6, line 124-125 information included

7. Paragraph 7 the definition of XDR is incorrect. The correct definition of MDR plus resistance to any fluorquinolone and resistance to at least one second-line injectable drug (amikacin, capreomycin, or kanamycin).

We had only tested for MDR plus resistance to ciprofloxacin and capreomycin. Amikacin and kanamycin were not tested. We have therefore, removed XDR information from the manuscript.

8. Paragraph 8 ‘ancestral’ not ‘ancient’

Page 11, line 259 the word “ancient” has been changed to “ancestral”

Result section:

1. Paragraph 1: extrapulmonary (n=76) is stated twice. Spelling of Balochistan.

   Why so few strains from Balochistan? Five strains is hardly a representative sample and authors should temper their conclusion with respect to this province, a note in discussion

Editorial changes
Page 9, line 216 “A total of 76 isolate” changed to “out of seventy six isolates”
Page 11, line 251 - spelling of “Baluchistan” has been changed to “Balochistan”

The same observation (few strains from Balochistan) was made by the authors as well; therefore, strains were selected by stratified random sampling method to overcome this limitation

2. Paragraph 4: stating % homology is noninformative and unnecessary

Authors are in the opinion that the information (regarding % similarity) highlights the prevalence of a similar type of strains in the study therefore, it needs to be addressed

3. Paragraph 6: all ST information is present in Fig 2 and should be cut from the text. Use of homology should be altered.

In accordance all the ST information from the text has been removed, please see the re-submitted manuscript.
The term homologous/homology have been changed to “similarity” as the information given in detail in results section is based on the similarity index pattern.

Page 10, line 228 and 230….. similarity
Page 13, line 321…… similarity

4. Paragraph 7: same with unique strains also confusing with U spoligotype designation. The term “Unique” is also changed to “Orphan types” in the manuscript
The designation for “U spoligotype” and “MANU1” have been added in the Abstract section
Page 3, line 66-68 description for U type and MANU1 added

5. Final paragraph: definition of XDR correctly applied? The XDR data should be in a separate paragraph from other first line drugs. The p value foe MANU1 is different in the abstract. The breakdown of the number of strains in Pak, U, Unique and MANU1 is not given. The relevance of statistical association.

As mentioned above the XDR information has been removed from the manuscript.

Major revisions:
Discussion:
1. Paragraph 2: the authors should temper their enthusiasm for the idea that unique strains causing extrapulmonary disease are more virulent and so more genetically variable. To the contrary, the body of literature to date supports that unique strains are less virulent. They are certainly less transmissible.
In agreement to suggestion given the required text is modified.
Page 12, line 293-297

2. Paragraph 3: SST? The authors have also fallen prey to the disregard of certain other authors to previously established nomenclature. So called TbD1-intact EAI strains are not related to TbD1 deleted EAI at all. In fact they are CAS strains. the authors should modify their discussion accordingly.
As discussion section has also been modified. Please see the revised manuscript.

3. Fig 2 data would be more comprehensible and associations easier to make if the spoligos were arranged according to Binary rather than class (eg group Pak 1-12 and CAS). Classes are missing (such as LAM9, LAM6, and Pak 34-36) should be included
   The data in Fig 1 has been arranged as suggested

4. legend should overly say that nonclustered strains are not include
   As the information for orphan types not included in Fig 1, therefore the legend only describes the information for shared types.
References