Author's response to reviews

Title: Minisequencing mitochondrial DNA pathogenic mutations

Authors:

Vanesa Alvarez-Iglesias (vaneiml@usc.es)
Francisco Barros (apimlbar@usc.es)
Angel Carracedo (apimlang@usc.es)
Antonio Salas (apimlase@usc.es)

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Author's response to reviews: see over
Dear Editor,

My co-authors and I would like to submit a revised version of our article to BMC Medical Genetics “Minisequencing mitochondrial DNA pathogenic mutations” (authors: Vanesa Álvarez-Iglesias, Francisco Barros, Ángel Carracedo, Antonio Salas)

We present a revised version of our manuscript. We have followed all the suggestions of the reviewers and we thank them for their useful suggestions.

Below are point-by-point the answer to the reviewers suggestions and comments.

We hope the article is now suitable for publication in BMC Medical Genetics,

Sincerely yours,
Antonio Salas

My contact details are

Antonio Salas
Unidad de Genética Forense
Universidad de Santiago de Compostela
Galicia, Spain
Tel: +34–981–582327; Fax: +34–981–580336
E-mail: apimlase@usc.es

REVIEWER 1

1. In the background section, the paragraph which describes the different methods available for mtDNA analysis is not complete. The difference between the methods allowing a complete screening of mtDNA and those which only detect selected mutations is not clearly exhibited. Other techniques than sequencing should be listed.

   We have now extended this section

2. For a better comprehension, a paragraph should be added to give an overview of minisequencing method using SNaPshot kit.

   We have also added a paragraph in order to explain a little bit more the minisequencing approach.

3. The nomenclature is false and need to be corrected (m.11778G>A)

   Done

4. Figure 2, the sequence electrophoregrams corresponding to SNaPshot assays must be added for each pattern. Furthermore, the different percentages of heteroplasmy obtained by mixing 2 types of mtDNA must be indicated.

   Figure 2 contains the different patterns found. We have also added the heteroplasmic proportions in the figure

5. Among the 25 selected mutations, several are frequently identified (m.3243A>G, for example). It should be interesting to show that this method can detect other mutations than m.11778G>A and m.14484T>C variations.
It is a good suggestion, but we have screened all the patients and we did not find other mutations.

6. There are a lot of grammatical and spelling mistakes (minisequencing, minisquencing…)
   **Corrected**

**Minor comments**

1. The pages of the manuscript should be numbered  
   **Done**

2. Page 5, lane 24, Table 1 should be replaced by Table 2  
   **Done**

3. Page 6, lanes 12-13, « primers are sizes (?) between 19 and 67 bps », should be replaced by « the length of primers is between 25 and 76 bp »  
   **Done**

4. Page 6, lanes 14 and 17, Table 2 should be replaced by Table 3  
   **Done**

5. Page 6, lanes 21 and 22, SAP should be replaced by ExoSAP-IT. 
   **There are two different enzymes, the ExoSAP-IT and the SAP. Therefore, this does not need correction in the text.**
   **Done**

6. Page 7 : What do the authors mean by «a clear suspicion of mtDNA disease»?
   **This sentence was deleted**

7. Page 10, lanes 23, 86-197 should be replaced by 89-196 as in Table 2  
   **Done**

**REVIEWER 2**

1. …They have also considered the proximity of two pathologic mutations. However, they have not taken in account the population variation in the mtDNA. Do the target mtDNA sequences for the primers include frequent population polymorphisms? Which is the efficiency of the amplification?
   **We have now commented on this issue.**

2. In this sense, a picture showing the amplicons pattern including the amplification of a rho0 cell, should be included.
   **It would be a good idea. We however do not find very exiting to provide with such a picture, in part because various amplicons have similar sizes each with their own set of SNPs. Below is the picture:**

![Picture of amplicons](image)

3. In the last sentence of the “Subject and SNP selection” section, they claim that all the selected mutations consist of non-synonymous substitutions but this is not true for the 3243 transition.
   **We have now corrected this error**

4. The comments about the 12696 mutation, the founder mutations and the next paragraph are not relevant for this article.
Some points were clarified along the text; for instance the regional provenance of the samples. This could have created some confusion to the reviewer concerning this part of the article. We have now extended this discussion which, to our view, adds interest to the study. We have now genotyped new ('neutral') polymorphisms (Table 5) in order to classify the Galician patients into haplogroups. We have observed that haplogroup J is overrepresented in these patients (as referred in some recent literature). This difference is statistically significant. A new sentence has been added also to the abstract.

5. The percentages of wild type and mutant DNA are not indicated in the mixtures used to check the heteroplasmy levels
   Done

6. FC should be defined in tables 2 and 3.
   Done

7. We here comment on the final reflection of the reviewer. We agree with him in that there could be a lot of mtDNA unknown and known mtDNA or nuclear DNA modifiers of the pathogenic phenotype. These factors however move in an ‘universe’ of probabilities, a field that is not pragmatic in a laboratory of molecular diagnosis and even worst to the eyes of a clinician. Therefore, only those mutations that have a clear cause-effect are general welcome for clinicians. Note moreover that most of these associations do not find replication in the scientific literature and therefore we cannot translate these doubts to the real life. I agree with the referee in that complete genomes should be carried out systematically in all laboratories. However, this is not possible because most of them are not prepared (technically, lab staff, etc). On the other hand, complete genome sequencing is very expensive (e.g. more than a typical \textit{BRCA1/2} screening for breast cancer) a cost that is not acceptable in most of the hospitals for routine work in a molecular diagnosis laboratory.

8. Finally, this is a rapid and cheap protocol but it requires expensive equipment. However, a normal PCR/RFLP can be performed in almost any laboratory.
   The equipment needed is most of the times already present in a laboratory on molecular diagnosis. This is for instance the reality in most of the laboratories of this kind in the Spanish hospitals. The method moreover has other advantages as commented in the text.