Author's response to reviews

Title: Optimization of simultaneous screening of the main mutations involved in non-syndromic deafness using TaqMan(R) OpenArrayTM Genotyping Platform

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Author's response to reviews: see over
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Cover Letter
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Authors are thankful to the reviewers (prof. Viviana and prof. Camila). We appreciated the suggestions made by them, and their comments definitely helped to improve the quality of the manuscript.

To better understanding of this cover letter:

- The revisions and the response to each concern were separated according the reviewer. Firstly, were answered the notes related to prof. Viviana Dalamon. After, were commented the prof. Camila Oliveira revisions.

- The suggestion of the reviewers are written with red color and the font size is 10.

- The comments of the authors are written with black color and font size 12.

- There were some writing changes that the reviewers suggested, and some questions about the manuscript. The structural changes were obeyed, aiming to make easier the reading and understanding of the manuscript. The questions about the manuscript were analyzed and answered below.

Fábio Tadeu Arrojo Martins
PhD Student
Reviewer: Viviana Dalamon

Viviana suggested the following structural and English writing changes:

1) "0,27% of 1000...", should be changed to "....2,7:1000"....

2) "0,35% of 1000...", should be changed to "....3,5:1000"....

3) "So far already aware of 150 loci and 64 genes involved in hearing loss.", should be change to "To date nearly 150 loci and 64 genes have been described in hearing loss".

4) "The gene has a higher number of changes is the GJB2, encoding connexin 26. Only this gene has over 302 changes confirmed so far, and the main gene-related hearing loss of genetic origin.", should be changed to "Mutations in the GJB2 gene, which encodes connexin 26, constitute the main genetic cause of hearing loss. So far, more than 300 genetic variations have been described."  

5) The names of Mitochondrial genes should be changed: "12S rRNA" should be named "MT-RNR1", and "MT-TR1" should be change to "MT-TS1".

Introduction is too long and redundant, I strongly suggest to shorten it.

6) " restricting the spoken communication of the deficient..." should be changed to "...restricting the spoken skills of patients..."

7) bibliographic citation [1] is updated in World Health Organization homepage, so First paragraph should be corrected in manuscript according to new data. According to 2013 data, 360 million people worldwide have disabling hearing loss greater than 40dB in adults or 30dB in children.

8) "In developed countries, one in every 500 children born carrier deafness bilateral (both ears) which is deep or severe (pre-lingual)", should be changed to "...one in every 500 children have congenital moderate bilateral deafness".

9) The sentence " In less severe cases of unilateral profound deafness..." has no sense, since profound deafness is the most severe form of deafness.

10) " three to six children in a thousand", should be changed to “3- 6:1000...”, “two to four in a thousand children will be deaf before adulthood (post-lingual), is redundant since it has already been mentioned above.

12) Fourth paragraph is too long, and should be changed to: "hearing loss can be classified as conductive or sensorineural according to malfunction of external or inner ear structures."

13) Fourth paragraph, lane 9: "deep"...should be replaced to “profound”.

14) Fourth paragraph, lane 11: “Another classification is according to the time that the hearing loss is manifested...”, should be changed to “Another classification takes into consideration the onset...”

15) The sentence “The causes congenital represent individuals who are born with or acquire the loss shortly after his birth.” It is redundant and should be deleted.

17) The sentence “Of these 60% cases of hearing loss of genetic origin, 70% occurs in isolated way, being called Non-Syndromic Hearing Loss (NSHL), where a person has
hearing loss only in isolated way in their phenotype. The remaining 30% represent the Syndromic Hearing Loss (SHL), with deafness associated with other symptoms or abnormalities, such as malformations, vision problems, among others [10,11]. Should be changed to "Of these 60% cases of hearing loss of genetic origin, 70% are Non-Syndromic Hearing Loss (NSHL), with hearing loss as isolated symptom. The remaining 30% represent the Syndromic Hearing Loss (SHL), which is associated with other symptoms or abnormalities in addition to hearing loss, such as ear malformations, vision problems, renal, thyroid, cardiac dysfunction, among others [10,11]."

The sentence “The direct sequencing was widely used in the discovery of new genes and changes....” Should be shortened and corrected to: "The direct sequencing is widely used but has high cost, is time consuming and has low yield for sequencing large fragments”.

“...The arising of technologies high-throughput makes it...” should be changed to “The emergence of high-throughput technologies...”

“These technologies are new and has as main characteristic...” should be changed to "These technologies are new and have as main...

“After choosing the OpenArrayTM plate layout, were performed a study of"... should be changed to "...a study was performed..."

Discussion: Paragraph 1: Must be moved to results.

My position about the revisions:

• The suggestions above were analyzed and obeyed. They were changes in the English writing that should be done to let the sentence clearer to the readers.
• Some structural changes were realized too, as the move of the first paragraph of the discussion to the results, because it was wrongly positioned.

Viviana asked some points of my work. This points and my explanation is following below.

11) citation [5] regarding patients in Brazil is from 1992 and should be replaced by a recent one.

This work realized in 1992 is the only one that analyzed the hearing loss causes in Brazilian population. It is necessary a new study.

16) In the sentence “Studies show that in developed countries, about 60% of the problems related to congenital deafness are hereditary, 30% are acquired and 10% idiopathic etiology. It is estimated that 2/3 of hearing loss of genetic origin be prelingual [9].” the authors claim that most of the congenital deafness are of genetic origin (hereditary), and that most of them are prelingual (75%). This data is confusing and is usually presented in bibliography: Hearing loss is caused by several environmental and genetic factors and the proportion attributed to inherited causes is assumed in at least 50%. Approximately 70% are non-syndromic. The pattern of inheritance of non-syndromic cases is autosomal recessive in about 80% [Morton,
This sentence was really confused. The wrong data was repaired and the sentence improved to better understanding.

The sentence... "to better classify them..." is too long and the acronym “DFN” is not used throughout the. The whole sentence should be deleted. The introduction is too long. A short sentence should be add with short information about genes SLC64A5, TMC1, GJB6, OTOF, and others that were selected as target in the array.

This sentence was really long, so it was reduced. About the acronym “DFN”, it still in use to classify the types of hearing loss related of hearing loss. The reviewer suggests deleting the sentence, but inserting information of the studied genes. I believe that addiction of these information will let the introduction longer than it is. Thinking about this, was add a brief overview of the genes.

Why were they chosen? How is the frequency of mutations worldwide and in Brazil? Were they usually studied in the lab before the array? Why are those specific GJB2 mutations studied? What is the frequency in Brazil? Is the population similar to Europe? Those mutations seem to be selected for Mediterranean or European population, is ethnic background in Brazil similar?

These genes were chosen according to the frequency perceived in our laboratory and our wide samples. Those changes were previously studied in our laboratory, and some of the positive cases were used in this work as probes control.

The GJB2 mutations studied were selected according the samples that we had here in the laboratory. So, I selected them to check the OpenArray technique, verifying the usability of the same. Mutations in this gene represent the main cause of genetic hearing loss, being the c.35delG the most common. The others have not so high frequency as the deletion above cited, but they were find in the population that we studied, being this the parameter to include them in the layout to be analyzed.

Were studied mutations present in Asians, Europeans and Mediterranean populations. These mutations were found in our population and this may be explained by the fact of the Brazilian population be very heterogeneous.

Other important reason to check the technique was the use of positive controls to these alterations to test the probes with the mutant sequence. If we did not test these cases of heterozygous or mutant homozygous would be unable to notice if the probes designed were working.

To explain the frequency of genes and alterations involved in hearing loss of Brazilian population is used the argument that Brazilian standard of alterations related to hearing loss still unclear. It is necessary a multicenter study to try define the mutations bases of the Brazilian population.

Number of table 1 is wrong and should be changed to table 2.
had authors claim to have analyzed 376 patients (282 with deafness and 94 listener controls), if that is so... 376 are samples, not patients. On the other hand, if 9 samples had bad purity or concentration, then they should have been excluded from the study 282 were patients (with hearing loss) and 86 had already been studied with identified mutations. I understand that the rest of them were genotyped manually to check the accuracy of the array? Was there any other variation detected which was not searched for in the array? In this regard, more than 300 variations have been described in the GJB2 gene, and only 32 were analyzed using TaqMan array.

The term “pacients”, wrongly used, was changed for “Samples”.

As I was trying to show optimization of the OpenArray platform, I believe that all the steps to optimization of an able layout must be show and be clear to the understanding. That is the reason that I imagine be better to keep the 9 samples with bad purity and concentration in the total of samples, showing the accuracy of the platform.

The samples that had not previous results were validated after, using the methods presents in the Table 3 into the manuscript.

There were others mutations detected during the validation using direct sequencing, as the case of p.R143W, presented in heterozygosis in two individuals, and the p.T86R, present in heterozygosis in other individual.

Were analyzed only 32 mutations due the cost of the kits. As this work was a test of the technique, we had the idea to increase the number of mutations to be studied, buying a new powerful OpenArray kit after checking the accuracy of the plates and assays of this work.

"Other 33 reactions (0.28%) showed different results compared to the results of the pre-established techniques that were used to validate the plates." Were the duplicates consistent with the differences?

Yes, the differences were consistent including in the duplicates. This may be explaining due the sample and dilution be the same.

Cost of molecular test: As mentioned in the last paragraph, the cost per sample does not take into account the cost, parts and maintance of the equipment, nor the reagents and skilled labor. A sentence in discussion must specify pros and cons, but is meaningless in results.

This sentence was excluded, being changed for other that explain a little about the cost of the OpenArray plates, without to compare the routine tests applied in our lab, that had calculated the value of the others spending.

The decision of using rs11843171 in the CRYL1 gene for the detection of del(GJB6-D13S1830) and del(GJB6-D13S1854) should be explained in detail in the section “Material and Methods”. Are the patients supposed to be heterozygous for those SNPs if they are not deleted?

Exactly. The SNPs selected to detect the GJB6 deletions could not detect the large deletions. The only thing that this SNPs assay could show is, if the individual is heterozygous for the SNP, so you can have sure that the
Table 4:
What is the meaning of the + in the second lane?

The + signal means the individuals are compound heterozygote/homozygous.
Reviewer: Camila Oliveira

- Major Compulsory Revisions

1- In Abstract (Results): the authors have to describe the error rate (in percentage) that occurred during the validation of the results using the TaqMan® Open ArrayTM genotyping platform. Likewise in the results section (eighth paragraph).

This alteration is already done in the parts that the reviewer requested.

2- In Discussion (third paragraph): The phrase "These assays were not excluded from the selected layout, but will need, in new genotyping reactions, be validated by other techniques, in this case by direct sequencing" is confusing and needs to be rewritten. The authors state that the false positives mutations were validated by direct sequencing. Then they say that in new genotyping reactions will be validated by other techniques, the same direct sequencing.

The paragraph cited above was confused as the reviewer had noted. So it was rewritten.

3- Conclusion: The section has to describe concisely and specifically the findings (and limitations) found in this study and do not redundantly repeat those that have been mentioned in the above sections.

This alteration is already done in the parts that the reviewer requested.

4- In authors’ contribution is expected to find the contribution of the each author’s manuscript and not only the first author. Therefore, this section should include the contribution of all the authors.

The relevant contributions of each author were noted in the specific part of the manuscript.

- Minor Essential Revisions

1- In Abstract (Results): Remove the dot of the number 11656.

2- In Background remove (Intro).

3- Background (2nd paragraph): the term (both ears) is not necessary.

4- The authors could write the introduction of the manuscript more briefly. I suggest removing the fourth and the ninth paragraph.

5- A lower space between each symbol (>) on name of the mutations has to be uniformly applied in the results and casuistic.

6- Results (1st paragraph): the number of the Table is 2.

7- Results (third paragraph): The information described in this paragraph has already
been cited in casuistry.

8- Results (last paragraph): Replace the dot by comma in etc. 9- The entire discussion is without reference.

10- In Materials and Methods (Sample Preparation - 2nd paragraph): enter a space between the number and the word rpm.

11- Figures: in legend of Figure 1 replace 48 nano-wells by 64. 12- Table 1: Replace pós-natal by pos-natal or postnatal.

13- In table 3 (1st column, rows 4, 5 and 6): the authors should use the name of the genes and do not the name of the mutations.

All minor essentials revisions were obeyed.