Author's response to reviews

Title: Assessing Genetic Polymorphisms using DNA Extracted from Cells Present in Saliva Samples

Authors:

 Zsofia Nemoda (zsofia.nemoda@eok.sote.hu)
 Maria Horvat-Gordon (mgordon@salimetrics.com)
 Christine K Fortunato (ckf110@psu.edu)
 Emilie K Beltzer (exb33@psu.edu)
 Jessica L Scholl (jscholl@salimetrics.com)
 Douglas A Granger (dgrange2@son.jhmi.edu)

Version: 3 Date: 27 October 2011

Author's response to reviews: see over
Dear BMC Medical Research Methodology Editorial Board,

Please find enclosed our revised manuscript titled as „Assessing Genetic Polymorphisms Using DNA Extracted from Cells Present in Saliva Samples”. We changed the title, as well as other sections in the manuscript according to the reviewers’ suggestions. We also changed the order of authors to reflect the final contribution in preparing our results for publication. All authors agreed to this change.

We hope that our manuscript in its present form is acceptable for publication.

Yours sincerely,
Zsofia Nemoda

Detailed response to the reviewers’ comments:

Reviewer 1:
We thank the reviewer for his remarks and suggestions. We made the following changes in the manuscript accordingly:

1. Background; Third paragraph; Third to last sentence.
A more detailed genomic location of the 5-HTTLPR would be beneficial (e.g. located in the promoter region of the serotonin transporter gene (SLC6A4)).
We added the suggested “promoter region” to the indicated sentence to specify the localization of this polymorphism within the gene.

A description of the methods used to obtain the reference COMT and 5HTTLPR genotypes for the sample participants in order to compare concordances with the experimental genotypes is warranted.
We added a sentence at the end of the method section: “In each study the reference genotypes of the participants were obtained from the .50 ml whole saliva samples collected by the passive drool technique and stored under the control condition, i.e., frozen within half an hour of the collection and thawed only once on the day of DNA extraction.”

3. Results and Discussion; Study 2; DNA Quality.
An additional T-test to determine if the Sarstedt pledget produces a statistically significant higher A260/A280 ratio compared to whole saliva would be relevant.
We thank the reviewer for pointing out that we did not report the level of significance at the 260/280 nm ratio of the Sarstedt pledget; we have conducted the t-tests to compare the whole saliva and each devices, but did not report the values when the difference was not significant. In the revised manuscript we added the t-values and “ns” at the p-values for the Sarstedt pledget filtrate (t (9) = -2.20, p = .055), and to filtrates from cotton rope (t (9) = .96, p = .362) and Salimetrics synthetic swab (t (9) = 1.63, p = .138).

4. Conclusions; Second Paragraph; Last Sentence
There are two periods at the end of the last sentence in the second paragraph of the conclusions section.
We thank the reviewer for pointing out this mistyping.

Reviewer 2:
We thank the reviewer for his constructive comments. We made the following changes in the manuscript according his suggestions:

1) The number of participants should be clearly stated in the abstract.
The number of participants is stated in the abstract as “Saliva samples were collected from ten adults in each study.”

2a) An important element of the design of this study is that DNA is extracted from a saliva pellet obtained after centrifugation of the saliva (for the adhered cell extraction, lysis solution was applied directly to the collection device after prior centrifugation). This raises the issue of how the study is titled and described. The study examines the quantity and quality of DNA extracted from cell pellets from oral fluids and should be described in this way, rather than using terms such as oral fluids, or whole saliva, without providing the context. The title is misleading.

We changed the title to “Assessing Genetic Polymorphisms Using DNA Extracted from Cells Present in Saliva Samples” to indicate that DNA can be isolated from the cell pellets or from the collection devices used to obtain saliva.

2b) The context of this approach to DNA extraction, as opposed to the literature on DNA extraction from whole saliva, or on DNA extraction from cell-free supernatant, should be discussed. E.g. are the striking results from the first part of substudy 2 concordant with the amounts of DNA extracted from cell free saliva, as might be expected?

We added a paragraph in the Conclusions about comparing our results with other groups’ findings. Most of the research groups used Oragene kit or other immediate addition of lysis buffer procedure. We found only two papers reporting DNA amount extracted from saliva collected with an absorbent device (Salivette cotton rope or swab) using PubMed search with “DNA extraction or isolation” and “saliva” keywords. One of them used the pellets to extract DNA (Keijzer et al., 2010), the other used the cotton rope itself (Etter et al., 2005). We found only one paper reporting DNA extraction from saliva supernatant (Jiang et al., 2009), however, the amount of DNA was not reported. Since we wanted to check the possibility that the same saliva sample can be used for parallel analyses of hormones, cytokines or other biomarkers from the supernatant and for genetic analyses using the cell pellet, we did not carry out experiments using the cell-free supernatant. Therefore, we compared the reported DNA amounts derived from whole saliva samples using cell lysis buffer at the time of collection to the DNA amounts of our immediately frozen samples (which does not allow bacterial growth) as a most probable comparative condition.

“Our main goal was to show that sufficient amount of DNA can be obtained from saliva samples without immediate addition of cell lysis buffer, which is used by the most widespread saliva DNA collection techniques, opening up the possibilities to use saliva samples for genetic analyses without interfering with hormone, cytokine or other biomarker analyses. When comparing the average amount of DNA per 1 ml saliva calculated from the samples we stored under the control condition (i.e., frozen within half an hour of the collection) to published data of other research groups using whole saliva mixed with cell lysis buffer at the time of collection, our results (ranging from 11.99 µg/ml at Study 1 to 26.67 µg/ml at Study 5) represented the lower end of the range, as the reported average DNA amounts in pilot studies vary from 11.4 µg/ml [2] to 77.4 µg/ml [3], whereas in large scale studies the calculated total DNA per 1 ml saliva is between 23-42 µg/ml [4,34,35]. It is interesting to note that the total amount of DNA obtained from saliva samples shows huge variability both under experimental laboratory settings (e.g., 155 ± 103 µg per 2 ml saliva [3]) and in epidemiological studies (e.g., 92 ± 74 µg per 4 ml saliva from a sample of 555 adults [34]).”

3) For most studies, independent variables had little effect on the ability to extract sufficient DNA for most applications, although two results deserve more attention to enhance the impact of this study and to suggest additional limitations of certain approaches.
3a) The first result is the phenomenon of the range of DNA quantity extracted from the smaller volumes in the 10 samples, with the low end of the range sufficiently low to suggest that in a larger laboratory or field study, there will some saliva samples that yield insufficient DNA for some methods of analysis. This possibility, a function of the normal distribution, should be highlighted in the discussion.

We agree with reviewer, and at the discussion part of Study 1 we added a sentence “However, we have to note that the lowest DNA concentration was 9.9 ng/µl (total DNA amount .50 µg) among the 100 µl samples, hence this sample can be used only for 25-50 genotyping reactions.”

3b) The second result is the suggestion that DNA quality as measured by the 260/280 ratio increases with time at room temperature (Study 3) and that DNA quantity (albeit only the quadratic contrast) and DNA quality increase number of freeze thaw cycles (Study 4). How these results might inform the investigators and the reader of the effects of such sample handling and storage, e.g., whether the effects are due to degradation or digestion of proteins, and what implication this has for the study of other components of saliva should be discussed.

We thank the reviewer for pointing out these findings; however, we refrain to discuss these observations in details, because we feel that we can not make clear statements, only probable speculations, based on 10 samples. At Study 3 it is probable that much of the proteins are degraded after a couple of days at room temperature (see paper by Jiang et al., 2009), but due to the proteinase K treatment in every sample, we can not be sure if this is the reason for the observed 260/280 nm ratio differences. At Study 4 we removed the quadratic contrast statistics, since it was not reported at any of the other sections either, and added an explanation that the difference came from the extracted immediately samples vs samples exposed to one or two freeze-thaw cycles.

“However, the linear contrast for DNA quantity was not significant; the main difference came from the lower DNA concentration of the extracted immediately samples compared to the samples exposed to one or two freeze-thaw cycles (but not to the 4 or 6 freeze-thaw cycles samples, see Figure 2A).”

We also made a few changes at the discussion part of Study 4, to make it clear that the lower 260/280 nm ratio and the lower DNA concentration of the immediately extracted samples were probably due to losing DNA at the decantation step.

“The lower 260/280 nm ratio and the lower DNA yield of the samples extracted immediately compared to that of the frozen samples were probably due to the pellet formation after centrifugation, because many times the pellets in unfrozen saliva were very loose, and the supernatant was very viscous and thick.”

As for the implication of other components of saliva, we put additional sentences in the Background at Study 3 and 4 to show the stability of different steroid hormones and proteins at room temperature storage and during freeze-thaw cycles.

“For example, salivary cortisol or alpha-amylase levels were shown to be stable at room temperature (RT) for up to 5 days [25,26].”

“Levels of certain salivary steroid hormones (e.g., cortisol, progesterone) and protein biomarkers (e.g., alpha-amylase, immunoglobulin A) show stability over the repeated freeze-thaw cycles in pilot studies [25-28].”

References not added to the manuscript:
