Author’s response to reviews

Title: External decontamination of wild leeches with hypochloric acid

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Dear Editor,

We received the reviewers’ comments regarding our manuscript which was titled as "practical guides fot the utilization of ordinary eeches". Taking the critiques and suggestions into consideration, the following changes were made in the manuscript.

Revisions made to the manuscript:

* The title is changed, and a new author, Serdar Tuncer, is added.
* The whole manuscript, especially with respect to English is revised
* The discussion is shortened
* Four new references are added
* The methodology section is revised. More specific bacterial identification with biochemical studies and a new table demonstrating the results (Table 2) are added. We tried to put special emphasis on the revision of the nomenclature and sampling. To clarify the sampling method, a new figure (Figure 5) is introduced.

Responses to each of the reviewer’s comments as follow,

Response to Dr. Drancourt’s Review:

Major Compulsory Revisions:

Results: the study aims the prevention of infections caused by Aeromonas. For the two most common Aeromonas species isolated from cultures, A. hydro/caviae and A.sobria, further biochemical studies were carried out, definitive identification was made. As a result of this, A.hydro/caviae was in fact A.hydrophila, and A. sobria was in fact A.veronii biovar sobria. A table in the manuscript describes these microorganisms. MIC values could have been studied, but we tried to emphasize the concentration of hydrochloric acid.

Discussion: The detailed description of disinfectants have been removed from the discussion. As Aeromonas are similar to Pseudomonas, we cited the study by William and David, who studied the effect of hypochloride on various microorganisms and took this study as a guide.

Minor essential revision:

Latin names are in italics
BioMerieux is corrected
Betadine is corrected as alcoholic solution of povidine-iodine.

Response to Dr. Graf’s Review:
General:
1. The title is changed to "External decontamination of wild leeches with hypochloric acid". "Ordinary" is replaced by "wild" in the description of non-farm raised leeches.
2. In this study, we stressed external decontamination of the leech flora and transport fluid. We believe that the new title corresponds to the message of the text. As we do not aim to give guidelines on the use of leeches, we did not cite such articles.
3. We aimed to examine the effect of hypochloric acid on bacterial load in the leech flora and environment, and showed its efficacy in the targeted end points. Whether this positive effect has any clinical benefits needs to be investigated in new clinical studies.

Major Compulsory Revisions:
1. This study aimed to investigate whether selective decontamination using hypochloric acid is effective in reducing the bacteria in transport fluid and oral flora of leeches. The expected clinical effect of this reduction is decreased infection rates. However, randomized controlled studies are still required for demonstrating such an effect, if there really is. Such studies as well as studies comparing antibiotics with external decontamination with respect to efficacy, cost and practicality may be the next step.
2. The study by Abbott and Janda was not cited in the first manuscript. As suggested, it was added as a reference. This study was especially used in the methodology section of our manuscript for the definitive identification of bacteria by utilizing the biochemical characteristics described in the study.
3. Eroglu et al. (reference 14) performed a similar experimental study on medical leeches, and investigated the flora of Hirudo medicinalis. They also used alcoholic solution of povidone-iodine for preparation of ventral surface cleaning prior to intestinal sampling. Guided by their methods, we carried out the experiment with the same solution.

Crop or intestinal content sampling was after dissecting the ventral surface of the animal. The details of dissection and the anatomy are shown in figure 5, which is added during this revision. This diagram also shows the relation between the intestines and the urinary system. The samples collected from the midline, whereas the urinary system is located laterally. In addition the intestinal content is green - brown colored, and occasionally blood, taken as another guide for sampling.

Samples collected with a cotton swab were incubated at 37o C, for 24-48 hours. At the end of a 24 hour period, those agar plates without colony formation or those having small colonies were reevaluated at 48 hours.

4. In the original study, we used Analytical Profile Index 32 GN (BioMerieux, France) for bacterial identification. This system, as stated by the reviewers, is inadequate for the definitive identification of A. hydrophila, A.caviae, A. sobria and A.veronii biovar sobria. Therefore, plates from the original study that were kept at -70 o C were studied again and this time additional biochemical parameters were studied. Thus, we came up with new results and identifications, which are shown in a new table (Table 2) in the revised text. A. hydrophila and A.veronii biovar sobria were the definitive identification.

5. In stage 2 of the study, leeches that had bacterial reproduction in either the transport fluid or oral flora samples, or both, were taken into the study. Following hypochloric acid application, we did not observe any bacterial reproduction in transport fluid or oral flora samples. Therefore the number of bacterial colonies before the application of hypochloric acid were not reported in the original text. On the other hand, due to the endosymbiosis of Aeromonas and Hirudo medicinalis, we do not aim to eradicate all Aeromonas from the gut, but rather from the oral flora and transport fluid. Therefore the results of the reproduction in the gut were not reported. If we mention these intestinal suppression results: 12.5 ppm concentration had a significant suppression of gut flora when compared with the control group (p < 0.05, F= 30.1, ANOVA). The suppression in the 6.25 ppm group was insignificant compared to the control group. These intestinal suppression results are reported in a new table (Table 3) in the revised text.
Table 4), which quantitatively states the number of colonies in the oral flora, transport fluid and intestine.

Due to physiological regurgitation, the intestinal digest may be transmitted to the oral flora and hence to the transport fluid. Therefore, in the following days, bacteria will possibly regrow in these latter media. In clinical application, we do not consider reutilization of leeches, therefore we did not examine whether the bacteria reappeared or not.

6. Due to the death of leeches at higher hypochloric acid concentrations (100, 50, 25 ppm) quantification was reported only in the 6.25 and 12.5 ppm groups. We agree with the comment that the number of bacteria is important in determining the risk of infection. Therefore quantitative analysis was added to the study (details given above).

7. We removed the list of members of the Aeromonadaceae family from the text, as the taxonomy we used was unnecessary and outdated as stated. On the other hand, separation of A. hydrophila, A. caviae, A. sobria and A. veronii biovar sobria were performed, since these are important pathogens for humans.

8. Richerson et al. (reference 10) used the term "denaturation" in their article. As written in their study, "Aeromonas hydrophila denatures the haemoglobin, the haem is utilized by the Aeromonas organism while the globin provides a food source for the leech."

9. Eroglu et al. also used wild leeches in their study. They isolated A. hydrophila, Ochrobacter antropia, nonfermentating gram (-) rods, Acinetobacter Iwofii and A. sobria most commonly. The most commonly isolated organism in our study was A. hydrophila, followed by A. veronii biovar sobria. The leeches in their study were taken from the Black Sea region, which is in northern Turkey. We used leeches taken from Istanbul, located in northwestern part of the country. The difference between the microorganisms in these two studies are written in the discussion.

10. The statements regarding the use of chlorhexidine or antibiotic solutions to disinfect the guts of leeches, and their failure was taken from our second reference, by Sartor et al. We took Sartor et al.’s statements as a guide, who also referred to Lucht et al.’s study. We had not cited Lucht's letter. Lucht et al., did not, as you wrote, examine the gut flora.

11. The effect of hypochloric acid on digestive bacteria at 6.25 and 12.5 ppm were studied in our study, and compared with the control group. The details are given both in the text (results) and also in this letter in the answer to question 5.

12. When placed on the tissues, we believe that direct inoculation occurs from the surface (transport fluid and skin) and oral flora. In the literature, we did not come across any studies which separate the two possibilities.

Minor Essential Revisions:
1. The sentence "we studied bacterial identification" is corrected as: "In this research, results of identification of bacteria in the transport fluid is reported, oral and intestinal floras and the antibiograms of the identified microorganisms are investigated."
2. The term "like us" is deleted, leaving only "in many countries"
3. The spelling of BioMerieux is corrected.

Response to Dr. Lineaweaver's Review:
The decontamination of medicinal leeches offers an opportunity to avoid the use of wide spectrum antibiotics, especially if this is possible without adversely affecting the function of the leeches. In countries where leeches are not obtained from medical farms or where patients and antibiotic costs
are not covered by health insurance, this may serve as a cheap and effective alternative.

Following these revisions, we now would like to resubmit our article, currently titled "Extends decontamination of wild leeches with hypochloric acid". On behalf of all co-authors, I would like to thank you for your attention.

Sincerely Yours
Atakan Aydin, MD