Author's response to reviews

Title: Cynomolgus monkeys (Macaca fascicularis) inoculated with Brazilian and Dutch swine HEV strains are successfully infected and exhibit hematological changes

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Author’s response to reviews: see over
Dear referees,

Thank you for your important suggestions; all of them were accepted by our research group. The responses are typed in red here, and they have been included in the body of the article.

First reviewer:
1 - More detailed data about lymphopenia and monocytosis have been included in Figure 1, and additional graphics have been added as Figures 3B and 3E. OK.

Major Compulsory Revisions
1. I would like to see more detail about the lymphopenia, and in particular I would like to see a figure which shows the lymphocyte count over time post exposure. OK; this revision has been included in the discussion.
2. Some of the paragraphs extend to more than one page. They are too long, and need to be made shorter. This will make the paper easier to read. We concur with the reviewer and have changed this section.
3. There is too much emphasis in the results section on the fact that the macaques get subclinical hepatitis. We already know this, so the authors should consider shortening this section of the results and conclusions, and expanding the bit about lymphopenia. These suggestions was accepted.
4. I would like to see a bit more discussion and speculation in the discussion section about the lymphopenia observed. It is a really interesting observation in my view. Additional informations about haematology were included in discussion.
5. The quality of the English language used is OK, but sometimes not quite up to the standard one might expect in a major international journal. Having said this, I could not even contemplate trying to write a paper in Portuguese. Perhaps the authors might like to ask a colleague who's first language is English to help them improve this aspect. The English was improved by American Journal Experts.

Second reviewer:
1- OK, the presentation of results were shorted in order to became more clear. This suggestions was accepted.
2- Though HEV can be transmitted intravenously to humans by transfusion of plasma or blood derivatives, it would be merely accidental in comparison with the ingestion of contaminated water or food. Therefore, the haematological findings described by the authors might be an artifact from the route of inoculation chosen for the experimental infection that perhaps does not happen during the natural infection. In addition, the ability of these swine and human HEV-3 strains to overcome the species barrier and infect cynomolagus macaques might also be different after ingestion than after intravenous
inoculation. These limitations of the study must be recognized in the Discussion section.

Based on our previous experience in producing infections with enterically transmitted viral hepatitis in different NHP species, the intravenous method represents a suitable route of virus inoculation. The only difference between oral and intravenous inoculation is the period of virus incubation, which is generally shorter for intravenous inoculation.

3- Two of the eight subjects of the experiment (F3 and J3) did not display detectable virus shedding during the study. For subject F3, the anti-HEV secondary-type response observed suggests an unnoticed, pre-existing immune memory against HEV that would have controlled efficiently the experimental infection. However, the subject displayed biochemical alterations and focal inflammation areas in the liver tissue, which the authors thought due to an “immunomediated response”. Do the authors suggest that reinfection by HEV in a subject with specific immunological memory can lead to liver damage? What means exactly the expression “immunomediated response” in regard to such damage?

The liver injury has been attributed to either a dose-dependent mechanism or to specific cytotoxic T lymphocytes (CTL), and the antibody activities correspond to those described for other viral hepatitis infections. In subject F3, the peak ALT level was observed at 45 dpi and was not associated with either viremia or viral shedding. The observed hepatocyte damage can only be explained by immunomediated mechanisms.

4- For subject J3, even the anti-HEV response was limited to IgG antibody, but, again, biochemical and histological alterations were observed. The authors propose either virus neutralization (by antibody present in the inoculum) or virus inactivation (due to a long storage time) to explain the findings. Though the rising of anti-HEV IgG would not be totally incompatible with these explanations, how to explain the liver damage if the inoculum contained just neutralized or inactivated HEV particles?

Regarding J3, we believe that HEV neutralization/inactivation in the inoculum may have prevented the infection. Therefore, the liver lesion score of 1 and the discrete ALT elevations observed could be explained by an unexpected, abnormal immune response against the hepatocytes, or even the lymphocytes, as has been observed in other inoculated animals. One reference about “molecular mimicry” (Mizukawa et al 2000) has been included in the article.

5- There are similarities between the pattern of experimental infection recorded in subject I3 and the observations from persistent infections by HEV-3 among immunocompromised patients. However, the lack of biochemical alterations in this macaque on follow-up is a difference worth to be commented. Subject I3 did exhibit biochemical changes: ALT, AST and GGT levels were poorly elevated (as depicted in Table 2) in comparison with the baseline levels obtained before inoculation (ALT and AST below 20 IU/dL and GGT below 200 IU/dL). However, similar results have also been described by other authors in both immunocompromised and immunocompetent patients. (Two new citations have been included.)
**minor points:**

1. Experimental design. If “changes of general behavior -such as anorexia- and well-being were verified by the veterinary staff, daily”, a comment on the results from such verifications should be added to the Results section. Alternatively, the sentence can be removed. **OK; this sentence has been removed.**

2-ELISA tests. Was anti-HEV IgG tested by two different EIA tests (MP and DiaCheck) on all samples? **Yes, we performed two different assays.** Is the MP Biomedicals test designed to test samples from cynomolgus macaques? Was it modified by the authors? **No, this test is not specific to cynomolgus; however, the cross-reaction between human and macaque IgG is well-known among researchers in this field. When we performed assays for IgM and IgG after HEV inoculation, the anti-human IgG was replaced by anti-macaque IgG, as there is poor cross-reactivity between human and macaque IgM (Pages 148-150).** Please, clarify how the anti-HEV IgG results from Table 2 and Figure 1 were obtained. **OK; this explanation has been included in the footnote of Table 2.**

3- Real-Time PCR. Authors should provide a reference for the full details of the method used for quantifying HEV RNA by R-T PCR, or provide in other case these details in the text of the manuscript. **OK; this reference has been included.**

**Discretionary revisions**

1. I suggest replacing references 3-5 in the first paragraph of Introduction by “Journal of Medical Virology 2013, 85:1037-1045”, since it offers a full updated review of cases of acute hepatitis E reported from Latin America. **OK; the article has been included in the body of the paper. However, the authors would like to keep the original references for the reports of sporadic cases of HEV infection in South America.**

**Regards,**

**Marcelo Alves Pinto, MV., PhD.**