Author's response to reviews

Title: Experimental infection of Balb/c nude mice with Hepatitis E virus

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

We would like to resubmit the revised manuscript for your consideration, and we are very appreciating for your considerate suggests. We had edited the manuscript by professional editing service. Revised manuscript conforms to the journal style. The reviewers’ suggests we answered point-by-point as follows:

To Saleem Kamili’s questions:

We had substantially modified the manuscripts according to the three reviewers’ suggestions, and the manuscript was great improved by professional copyediting service.

To Khin SA Myint’s questions:

Major Revision:

Question 1: The authors’ statement that there is no efficient cell culture system to propagate hepatitis E virus (BACKGROUND) is not totally true (References-Takahashi et al, 2007; Tanaka et al, 2007; Lorenzo et al, 2008)

Answer: we added the relevant references in proper position in the third paragraph of BACKGROUND (line 21 of page 3, red color). We corresponding added the three references in Reference 23, 24 and 25 (line 16 of page 16, red color).

Question 2: Inoculation route is mentioned as intradermal in the abstract and intravenous under Materials and Methods. Please clarify.

Answer: It should be [intravenous injection] (line 8 of page 2, red color).

Question 3: The manuscript needs a good proof reading for language.

Answer: we had substantially revised the manuscript by professional editing service.
Question 4: When were blood and fecal samples collected (dpi)? When blood and fecal samples were collected (dpi)? Were the serums samples tested for HEV RNA and if so were they viremic?

Answer: we added relevant information in Detection of HEV RNA by RT-nPCR in RESULTS (line 22 of page 7, red color), summarized briefly in Table1 and discussed in Discussions section (line 21 of page 10; 1, 5, and 11 of page 11, red color).

Minor Revision:

Question 1: Nude mice may serve as a useful model for studying the replication mechanism (ABSTRACT). Suggest changing to studying pathogenesis of HEV.

Answer: we had changed [studying the replication mechanism] into [studying pathogenesis of HEV] in line 5 of page 3, red color.

Question 2: [As we all know, pigs, rodents, cats….in order to protect ourselves against HEV] (BACKGROUND). Suggest deleting these statements.

Answer: we had deleted this sentence.

Question 3: Materials and Methods/ Experimental infection of nude mice with HEV-

The experimental design is confusing. Please clarify the number of mice use for each group/sub-group.

Answer: we had clearly described the experimental design in the section (line 6 of page 5, red color). There were four nude mice for each group.

Question 4: Material and methods- Describe the weight (in range) of the animals used.

[Only seronegative nude mice were used for inoculation] implied that there were
seropositive ones. Suggest changing to [Nude mice confirmed seronegative for HEV infection by ELISA were included in the study].

Answer: we described the weight of nude mouse in Animals of Methods (line 11 of page 4, red color), and changed the sentence you mentioned (line 16 of page 4, red color).

Question 5: Material and methods/Virus- It was not clear how the infectivity titer of swine HEV was determined for nude mice.

Answer: we determined the infectivity titer by Real-Time quantitative PCR, and we rewrote in detail (line 2 of page 5, red color).

Question 6: Material and methods/ELISA determination- how was seroconversion defined?

Answer: The ELISA kit supplied positive and negative controls samples for identification seroconversion, and there is a cutoff value serve as diagnostic criterion. We supplied this information in Methods/ELISA determination (line 1 of page 6, red color).

Question 7: Material and methods/RT-PCR detection- Invitrogen was misspelled.

Answer: we changed the word (line 10 of page 6, red color).

Question 8: Material and methods/RT-PCR detection-[A reverse transcription nested PCR (RT-nPCR) analysis-suggest deleting nested PCR (RT-nPCR)].

Answer: we deleted nested PCR (RT-nPCR).

Question 9: Material and methods/RT-PCR detection-[The expected product of RT-PCR was 348 bp] Spell out bp for the first time.
Answer: we added [base pairs] in line 19 of page 6, red color.

Question 10: Material and methods/Immunofluorescence- The authors should describe a brief procedure of IHC since it is one of the key assays in this MS.

Answer: we added the procedure of IHC in line 6 of page 7, red color.

Question 11: Material and methods/Immunofluorescence-[All specimens were tested twice under code with labeled positive and negative controls]. Suggest changing to [All specimens were tested in duplicate with appropriate controls]. Specify the controls used.

Answer: we changed the sentence [All specimens were tested in duplicate with appropriate controls] as you suggested in line 10 of page 7, red color, and added the positive and negative controls in line 10 of page 7, red color.

Question 12: Results/ [There weren’t apparent clinical signs in all the experimental groups]. Suggest changing to [Evidence of apparent clinical disease was not found in any of the experimental groups].

Answer: we changed the sentence as your suggestion in line 19 of page 7, red color.

Question 13: Results/HEV RNA- Suggest adding HEV RNA was not detected in negative control tissues.

Answer: we added the information in line 5 of page 8, red color.

Question 14: Results/ Immunofluorescence- The statement [A large number of HEV antigens were detected in the cytoplasm of hepatocytes] is confusing. Suggest changing to [HEV antigen was diffusely or consistently detected in the cytoplasm].

Answer: we changed the sentence as you suggested (line 7 of page 9, red color).
Question 15: Results/ Immunofluorescence-Clarify the sentence [the number of HEV antigens in spleen was extremely large]. Suggest to add [No signal was observed in any of the negative control tissues].

Answer: we changed the sentence of [The HEV antigen was abounded in the liver; the number of HEV ….] to [The HEV antigen was abounded in the liver and especially in spleen] in line 11 of page 9, red color, and added the sentence [we found no signal in any of the negative control tissues (Figure 3-H)] in line 18 of page 9, red color.

Question 16: Results/ Immunofluorescence-Explain briefly where HEV antigen was located in extrahepatic tissues.

Answer: we found HEV antigens in spleen, kidney, jejunum, ileum and colon, and added the information about the location of HEV antigens in extrahepatic tissues in line 7 of page 9, red color.

Question 17: Results/Histopathology-Briefly mention any histopathological changes in extrahepatic tissues (apart from spleen) of the infected group. Were any changes seen in the negative control group?

Answer: we added more information about histopathological changes in extrahepatic tissues (line 6 of page 10, red color) and identified there was no changes in the negative control group (line 7of page 10, red color).

Question 18: Study results (biochemical, histologic, virologic, serologic data) can be summarized in a table.

Answer: we added a table at the end of Results to summarize biochemical, histologic,
virologic, serologic data in Table 1.

Question 19: Study results-It would be interesting to note whether the findings (virological, serological and histological) are different between the oral and the IV inoculation groups.

Answer: we didn’t see any different in virological, serological and histological between the oral and the IV inoculation group.

Question 20: Discussion- The statement [The inoculated nude mice show subclinical signs] is contradictory to the one mentioned under results [There were no apparent clinical sings].

Answer: It should be [The inoculated nude mice show no clinical signs] in line 13 of page 10, red color.

Question 21: Discussion- Error in stating [This may imply that in jejunum and ileum the HEV particles are too limited to be detectable by RT-nPCR] as PCR is more sensitive than IHC.

Answer: we deleted this sentence.

Question 22: Figure3 (IFA of infected tissues)-some positive signals are not convincing. Suggest using pictures with good resolution.

Answer: we used clearer photographs in Figure3 and supplied positive and negative controls (line 4 in page 20, red color).

Discretionary Revisions:

Materials and Methods/Experimental infection- suggest replacing [nude mice was killed] with humanely euthanized.
Answer: we changed this sentence as your suggestion in line 12 of page 5, red color.

To Volker Thiel’s questions: (The revision was labeled in red)

Major concerns:

Question 1: The presented data are based on a total number of 12 mice that were divided into three groups (3 mice each): uninfected control-HEV infected-contact-exposured mice. In none of the experiments it is clear at which time points the analyses were done and how many samples/mice were used. Therefore, there are also no statistics provided to support the main conclusions. Some experiments show only little effect of virus infection (as compared to non-infected mice) which makes it even more difficult to draw conclusions.

Answer: we supplied information about the time points of when HEV RNA detected in feces and sera (line 22 of page 7, red color) and the time points of when the HEV IgG elevated in Figuer1. The number of nude mice in each group and the experimental design was detailed in line 6 of page 5, red color, and all data was provided with statistic analysis by SAS system software (line 3 of page 6, blue color, and line 11 of page 8, blue color).

Question 2: RT-PCR analysis: Please provide more information on when and where HEV is detectable. To get an idea on how HEV can replicate and spread in Balb/c nude mice it is inevitable to analyze HEV replication by RT-PCR frequently at several time points post infection. When is the virus first detectable, when is maximal spread and when is the virus cleared?
Answer: we supplied the information about when and where HEV RNA was detected in Results/Detection of HEV RNA by RT-nPCR in line 22 of page 7, red color, and we detected HEV RNA everyday in feces and 4, 7, 14 and 21 days post-inoculation in tissues and sera. HEV RNA was detected from 4 days post-inoculation in feces in inoculation group, 4, 7 and 14 days post-inoculation in tissues and 4, and 7 days post-inoculation in sera. All the information was supplied in Results/Detection of HEV RNA by RT-nPCR (in line 22 of page 7, red color) and Discussion (line 21 of page 10; 1, 5 and 11 of page 11, red color).

Question 3: Figure1 and corresponding results section: it is not clear what the putative differences in OD\textsubscript{450} measurements between serum from infected mice and non-infected mice mean. Assuming that each measurement has only been done once, this experiment is not convincing. Furthermore, the figure legend describes the experiment (this should be in the results section) and not relevant facts to describe the figure.

Answer: The ELISA kit we used supplied positive and negative control samples (line 1 of page 6, red color), and there is a cutoff value serve as diagnostic criterion (line 3 of page 6, blue color). We repeated each sample three times and data were analysis by software SAS system software (line 11 of page 8, blue color). All the information was supplied in Material and methods/ELISA determination (line 1 of page 6) and Figure1.

Question 4: Figure2 and corresponding results section: When was the analysis done, how many mice were analyzed, are the presented data the mean of several
experiments and if yes provide statistics and error bars. How is kinetics of liver enzyme values at different time points post infection?

Answer: All procedures were performed in triplicate and data are expressed as means (±S.D.) (line 22 of page 8, blue color). We supplied the standard deviation and error bars in Figure2-A and kinetics of liver enzyme values at different time points post infection as well Figure2-B.

Question 5: Figure3 and corresponding results section: Please provide evidence that the used antibody is specifically recognizing HEV antigens- show control panels. Are presented data derived from an experimentally infected mouse of from a mouse of the contact-exposed group? At which time point has the analysis been performed?

Answer: The specific HEV antibody is purchased from Biodesign (America, line 4 of page 7, blue color), the recombinant protein is completely conservational in four genotypes (I-IV) and there is only one serotype. Figure3 is presented data derived from the inoculated group 4 days post-inoculation. We supplied the positive control (liver of HEV infected rat) and negative control (liver of nude mice in negative control group) in line 4 of page 20, red color.

Question 6: Figure4: Again no control panels and no indication when the analysis was done.

Answer: we supplied corresponding tissues of nude mice in negative control group in Figure4 and all the pictures were took on day 21 in line 16 of page 20, blue color.

Question 7: General comment: the manuscript needs spell check.
Answer: we had substantially modified the manuscripts by professional copyediting service.

We believe that the revised manuscript is substantially improved. So, we wish to be considered for publication. Thank you very much for your attention and consideration.

We are look forward to receiving your letter. Best Regards.

Sincerely yours,

Xiuguo Hua