Author's response to reviews

Title: The correlation between the presence of viremia and clinical severity in patients with enterovirus 71 infection: a multi-center cohort study

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Version: 3
Date: 8 June 2014

Author's response to reviews: see over
Dear Editor:

Thank you very much for the editor’s and the reviewer’s comments, which help us improve the quality of our manuscript.

We have responded to each of the reviewers’ comments in a point-by-point fashion in the following section and have revised the manuscript accordingly. We also underline what we have changed and now resubmit this revised manuscript. If you have further questions or concerns, please feel free to contact us at your convenience.

Sincerely Yours,

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Reviewer 1

Reviewer’s report

Title: The correlation between the presence of viremia and clinical severity in patients with enterovirus 71 infection: a multi-center cohort study

Version: 2 Date: 4 March 2014

Reviewer: CHia Yin CHong

Reviewer’s report:

1. Question well-defined? Yes

2. Methods appropriate and well-defined- Not ideal as patient had variable time-frame of doing viral loads and each pat only had 1 viral load performed during the admission. (was noted as a limitation in this study) What % of pats fell into HFMD, herpangina and fever with no obvious focus categories?

Reply:

This % of patients who fell into HFMD, herpangina and fever with no obvious focus is 39% and showed in the column “uncomplicated disease” in Table 1.

How was 2011-2012 considered epidemic years? Do the authors have the denominator for total country’s HFMD notifications for those 2 years?

Reply:

We have the denominator for total country’s enterovirus isolates from patients with enterovirus infections for those 2 years. According to the laboratory-based surveillance system of Taiwan CDC, the baseline proportion of EV71 among all the enterovirus isolates from patients with enterovirus infections was about 2% but the proportion increased sharply between 2011-2012 (shown as the table below) and peaked in 2012 (45%). As a result, we considered the year 2011-2012 were epidemic years for EV71. We added this in the method (Lines 86-89, the first paragraph, Page 5 and ref no. 23).

<table>
<thead>
<tr>
<th>Year</th>
<th>2009</th>
<th>2010</th>
<th>2011</th>
<th>2012</th>
<th>2013</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of enterovirus isolates</td>
<td>2375</td>
<td>3331</td>
<td>3311</td>
<td>2127</td>
<td>1454</td>
</tr>
<tr>
<td>Number of EV71</td>
<td>61</td>
<td>63</td>
<td>365</td>
<td>958</td>
<td>22</td>
</tr>
<tr>
<td>Proportion of EV71 among all the enterovirus isolates</td>
<td>2.6%</td>
<td>1.9%</td>
<td>11.0%</td>
<td>45.0%</td>
<td>1.5%</td>
</tr>
</tbody>
</table>
165 patients had no viremia—was this because they presented late in the illness or were they the febrile patients with no obvious focus? Ideally the patients should also have had antibody testing for preceding EV71 infection.

**Reply:**
The clinical severity of those patients without viremia was demonstrated in Table 2 and their EV71 infection was confirmed either by virus isolation or PCR. They were not only febrile patients with no obvious focus as Table 2 shows. We did not perform the antibody testing. In our previous studies (not published), we found that most patients would have neutralizing antibody on the third day or later of their acute illness, so antibody testing cannot differentiate acute from preceding EV71 infection.

3. Data sound: yes

4. Relevant standards for reporting and data deposition? Yes. Study was approved by review board in National Taiwan University Hospital, how about the other sites?

**Reply:**
Yes. The study was approved by the institutional review board in all study sites.

5. Discussion and conclusions—Table 3: since majority had mild complications—grade 2, was there any statistical difference when comparing mild (grade 1,2) with severe (grade 3,4)?

**Reply:**
We also performed the analysis but the results were not significant, either (p value=0.41).

Were there any deaths in the cohort?

**Reply:**
There were 2 deaths and 3 patients with sequelae in this cohort, and we added their outcome in Table 1.

Was there any difference in the management of patients between the hospitals? What was the outcome of the patients?
Taiwan CDC had published stage-based treatment guidelines of EV71 infections which was extensively conducted in the medical centers in Taiwan [Treatment Guideline for Severe Enterovirus 71 Infection [http://www.cdc.gov.tw/professional/page.aspx?treeid=BEAC9C103DF952C4&nowtreeid=59C07A6A1148235E]]. We believe that there was little difference of the management between the hospitals. In our cohort, there were only 2 fatal cases and 3 patients with neurologic sequelae. Other 219 patients were discharged without sequelae. We show their outcome in Table 1.

The authors used an age cut-off of 3 yrs, was there a greater difference using an age cut-off of 2 yrs or even 1 yr when comparing risk factors for viremia? Did the authors compare viremia with other characteristics e.g. duration of fever, other clinical symptoms or signs, other laboratory findings e.g. leukocytosis?

Reply:
Thank you every much for this comment. Yes, there was a greater difference using an age cut-off of 1 yr when comparing risk factors for viremia. The cut-off value of 2 yrs showed p-value was 0.15 while 1 yrs was <0.001. We added this result in the abstract (Lines 49 and 55), the results (Lines 173-174, Page 8) and revised Table 2. We also add the analysis of other characteristics in Table 2, and add a new Table 4 to show the results of multivariate analysis.

6. Limitations are described
7. Yes
8. Yes
9. Writing acceptable: typos on page 12- viremia persistence; page 20- statistical analysis performed

Reply:
We have edited it in the revised manuscript.
Reviewer 2
Reviewer’s report
Title: The correlation between the presence of viremia and clinical severity in patients with enterovirus 71 infection: a multi-center cohort study
Version: 2 Date: 21 March 2014
Reviewer: H Rogier van Doorn
Reviewer’s report:
This paper describes the correlation between detection of EV71 by real-time PCR in blood with demographic and clinical data. The questions posed by the authors are well defined and straightforward.

Major compulsory revisions:
The testing methods used require more detail, i.e. there is no reference or description of the real-time PCR for EV71 detection.

Reply:
We have cited the reference of the PCR method we used in the revised manuscript (ref no. 24).

Only in the discussion does it become clear that only one timepoint per patient was used; this should be made clear already in the abstract, described in detail in the methods section (with a table with samples collected per day of illness) and be reflected in the way the results are presented in the abstract and body of text.

Reply:
We added the description in the revised manuscript (Line 42 in the abstract and Lines 109-110 in the methods section).

The authors should also try to describe how these timepoints were chosen, or whether they used residual specimens from the haematology/chemistry labs. This is my main concern with the findings and conclusions of this paper. It is not unlikely that this causes considerable bias in the results, e.g. severe patients more likely to be sampled later (or come to the hospital later, or be transferred to the tertiary centres where the authors enrolled their patients, etc) as they stay in the hospital longer, whereas mild patients would be expected to be discharged earlier. The authors should add a careful multivariate/logistic regression analysis of their findings to correct for this.
Reply:

The time point of blood sampling was mainly on the date of admission and the blood sampling was collected for our study purpose rather than the residual specimens. The sampling time was not affected by whether the patient stayed at hospital longer or was discharged earlier.

Thank you very much for your suggestion. We performed a multivariate analysis to adjust the confounding factors such as fever and other clinical variables and it showed that fever is the most significant risk factor of severe EV71 disease (Lines 144-145 in the method, Lines 181-185 in the results). The result of the multivariate/logistic regression analysis is shown in the new Table 4.

Minor essential revisions:

There are a lot of language and grammatical errors and the manuscript may benefit from additional review for this particular purpose (I have not made any attempts to point these out one by one, as my English is not perfect).

Essential revisions, point by point (most have to do with my major concern stated above)

No line numbering was included, please change for later reviews

As stated above, the results section of the abstract does not properly reflect the sampling method.

A sentence as "two thirds of viremia occurred in within the first three days of infection" only makes sense if there is a denominator and if we know how many samples were collected before and after day 3.

Reply:

We have added the line numbering in the revised manuscripts.

We added the sampling method in the abstract of the revised manuscript (Line 42).

The description “two thirds of viremia occurred in within the first three days of infection” means the denominator is total case number of viremia patients. The proportion (numerator/denominator is also showed) of viremic patients grouped by day of illness is demonstrated in the new Figure 1.

No association between severity and viremia was found, but if severe patients were sampled later and viremia only occurs early in disease than this may have been an artifact. The risk of complications increased if viremia was detected after day 4, but is the chance of sampling after day 4 not also higher in severe patients? A thorough multivariate analysis is required.

Reply:
If the patient had severe disease later but the viremia had subsided much earlier, we could not say that the viremia was associated with more severe disease. This might not be an artifact in our analysis because most EV71 patients might have a viremia phase with variable duration or magnitude. If the severe patient did not have viremia found in earlier phase, it was less likely to develop a viremia later and cause severe disease. As a result, the chance of sampling after day 4 is higher or lower did not actually have influence on our analysis. A multivariate analysis for complicated EV71 infections is added as your suggestion (Table 4).

The background section can be more to the point, e.g. in third and fourth paragraph: 'the professionals', 'several health policies', 'made some success', 'still did not identify', 'have been investigated for a long time, but no conclusion', 'still unclear'. Better to describe well what we know, and what assessment of viremia may add.

Reply:
We have done some revision in the manuscript (Lines 71-74, the third paragraph, Page 4). We also described what assessment of viremia may add in the statement of our study purpose.

In the methods section 'patients and clinical data collection', 'sampling and laboratory testing' should be grouped together for clarity. Explain that 1 blood sample per patient was collected for viremia assessments and how these timepoints were chosen. Similar for swabs: Was EV71 detection by PCR/culture done on throat swabs taken on admission? Were multiple swabs taken?

Reply:
We have done the revision in the manuscript (Lines 109-110, the first paragraph, Page 6). Yes, we took throat swab once on admission. No multiple swabs were taken.

Reference the grading system used.

Reply:
We have added a reference in the revised manuscript (ref no.15).

In the results section:
Age is better expressed as median, than mean.

Reply:
Yes, we have revised in the manuscript (Line 151, the first paragraph, Page 8).
Classify the patients by grade first and then further explain in more detail, particularly for the grade 3 and 4 patients (as now only grade 1 and 2 are further detailed)

**Reply:**

It has been described in the ‘Methods” section (Lines 102-108, the second paragraph, Page 5):

Grade 3 included those with severe CNS involvement, such as encephalitis, encephalomyelitis or polio-like syndrome, but without cardiopulmonary failure while Grade 4 included those with severe CNS involvement listed in grade 3 but also complicated by cardiopulmonary failure.

Define ‘acute stage’, HFMD patients usually progress to severe disease within 72 hours, but samples up to day 7 are included in this study.

Include how many samples were negative per sampling day (e.g. in a table).

**Reply:**

HFMD patients usually progress to severe disease between 3 and 5 days but sometimes on the 7th days of their illness. Generally speaking, “acute stage” indicate the periods during which the patient had acute illness and it mainly accounts for the hospitalization in our study because patients included in our cohort were all hospitalized patients.

Thanks to your suggestion, we put a new Figure 1 to show how many samples were negative per sampling day in the revised manuscript.

The sentence “The peak... rapidly afterwards” cannot be understood in the context of single sampling timepoints and without knowing the numbers of negatives. Similar for the last paragraph on page 8, which should first be corrected for severity, day of illness etc. I have commented on the conclusion of the paragraph of page 9 in the abstract section.

The discussion and conclusion should be rewritten entirely after the extra analyses have been done. The authors should take care to choose their wording so as not to suggest longitudinal sampling and kinetics thus not use terms as “peak of viremia”, “at day 4, viremia halved” etc.

We do not have information on how many patients were sampled at day 7 and therefore cannot interpret what it means that no viremia was detected.

**Reply:**
We added a new Figure 1 to show the proportion of viremic cases according to the day of illness and it might provide more information.

Some revision of the discussion and conclusion was done in the manuscript (Lines 209-211, 215-217, 245-249, and 267-268).

The authors discuss the value of viremia in EBV/CMV vs dengue/noro/hanta viral infections and state that EV71 behaves more like the latter, which basically is a description of the difference between chronic and acute viral infection.

**Reply:**

Yes. What we would like to denote is the EV71 infection was more like an acute viral infection and thus the status of viremia or viral kinetic, which is usually helpful in chronic and systemic infection such as EBV, might not be applied in EV71 infection.

The authors do not describe the possible pathophysiological role of viremia/haematogenous seeding or retrograde axonal transport in spread to the CNS.

**Reply:**

We added the discussion about the pathophysiological role of viremia and hematogenous seeding comparing to previous pathogenesis study in the animal model in the revised manuscript (Lines 245-249, the first paragraph, Page 12).
Reviewer 3

Reviewer’s report

Title: The correlation between the presence of viremia and clinical severity in patients with enterovirus 71 infection: a multi-center cohort study

Version: 2 Date: 29 March 2014

Reviewer: Kulkanya Chokephaibulkit

Reviewer’s report:

This study is looking for the relationship of viremia of EV71 and disease severity. The results may lead to a better understanding of the pathogenesis. The results suggested that presence and magnitude of viremia are not correlated with disease severity. But for those who had viremia detected after day 4 of illness had a higher proportion of severe disease.

There are some major issues need to clarify.

1. The study design for single sample collection makes it unable to firmly conclude the viremia with the severity. The disease severity and viremia are both dynamic. As there are some uncomplicated patients who were not hospitalized, how can the author be sure that the patients who were not severe at the time of blood drawn did not progress to a more severe disease in a few days later with the higher viral load. Could it be possible that the patients visited other health care institutions later when the disease become more severe?

Reply:

The study in animal model also suggested the viremia precedes severe EV71 disease. That is to days, if viremia did was correlated to severe disease, a patient with severe disease late might have lower viral load earlier rather than absence of viremia. As a result, the result of our study would not be influenced.

All the medical centers participated in our study are all tertiary hospital in their local areas, and all our enrolled patients were followed up till they were discharged with final clinical outcomes as Table 1 shows, so we could define their clinical severity. That is, all the enrolled patients did not visit other health care institutions later when the disease become more severe.

2. The method was unclear whether the quantitative PCR was performed real time for was done in batch of stored samples. If the samples were stored, the duration of storage need to be clarify and discuss whether it could reduced the detection of viremia or reduce the viral load detected.
**Reply:**
The quantitative PCR was performed real time and we did not store the samples long time before we performed the test.

3. It would be helpful to understand the nature of viremia if the author can generate the figure or table to show the proportion of detectable viremia by the day of illness, according to the day that the samples were collected. For example, the viral load on day 1-2 may be found in the higher proportion or higher peak than day 3. How many samples were available by each day of illness.

**Reply:**
Thanks for your great suggestion, we added new Figure 1 to demonstrate the proportion of detectable viremia and the sample number by the day of illness.

4. As the viral load in a single time point cannot be determined whether it is the peak, the correlation of the viral load and severity should be compared in the very same day of illness.

**Reply:**
Because patients were not hospitalized on the very same day of illness, it’s very difficult for us to have the sample on the very same day of illness. We only collected the blood sample on the day of admission. For the young children, blood sampling is difficult and painful, so we did not do multiple blood sampling to have the sample on the same day of illness.

5. It should be clarified that the severity grading was at the time of blood collection or at the hospital discharge.

**Reply:**
The diagnosis and severity grading was at the hospital discharge and clarified in the method (Line 102, Page 5).

6. The author found a higher proportion of severe diseases in the patients who had viral load detected after day 4 of illness. On the other hand, it is important to understand the reasons for more severe patients to show up late, and that was why the samples was collected in the later days. Only if author can get the serial samples of patients in various severity before the conclusion
that prolonged viremia correlate with the severity can be drawn. The conclusion that “viremia persistent for four days or after the onset of disease correlate with more severe disease” may be misleading.

**Reply:**

We agree with that it is important to understand the reasons for more severe patients to show up late and it needs a further study to investigate the social and behavior factors of the reasons for more severe patients to show up late.

For the young children, blood sampling is difficult and painful, so we did not have serial blood samples to compare.

The viremia persisted beyond fourth day of the disease might be correlated to more severe disease is a subgroup analysis limited in viremic patients. The evidence might be not very solid and we had described in the limitations of our study. However, we believe this observation might not be misleading since it is compatible to clinical experience demonstrated in a previous study (Chang LY, Lin TY, Hsu KH, et al. Clinical features and risk factors of pulmonary oedema after enterovirus 71-related hand, foot, and mouth disease. Lancet 1999;354:1682-6), which reported that prolonger fever was a risk factor of more severe EV71 infection.

6. Although the study seems to be the very first report of viremia and severity in human, there were studies in animal. The one published by Ying Zhang et al (2011) in monkey suggested that the peak viremia correlate with route of inoculation and the clinical illness. Please add this in the discussion.

**Reply:**

We have added it in revised manuscript (Lines 245-249, the first paragraph, Page 12 and ref. no. 30).