Author's response to reviews

Title: Enterovirus Genotypes Causing Hand Foot and Mouth Disease in Shanghai, China: A Molecular Epidemiological Analysis

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Author's response to reviews: see over
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Editor-in-Chief
BMC Infect Dis

Dear Editor,

Enclosed please find our revised manuscript, entitled “Enterovirus Genotypes Causing Hand Foot and Mouth Disease in Shanghai, China: A Molecular Epidemiological Analysis”. The authors thank the reviewers and the editors for their thoughtful and constructive comments on our manuscript. As follows are our point-to-point responses to the comments of the reviewers. We also highlighted the revision in red in the revised manuscript.

We appreciate very much the comments and suggestions from the reviewers and editors and believe that their input has improved the quality of our manuscript. We hope you will find all the revisions and responses acceptable. We welcome further suggestions to improve the clarity of our manuscript for publication in your journal.

Sincerely yours,

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Response to Reviewer 1

**Critique 1:** Line 52: replace ‘that’ with ‘as’.

**Response:** We have revised our manuscript as suggested on line 52.

**Critique 2:** Line 61-62: ‘The disease usually resolves…..’. References should be provided for this statement.

**Response:** Thank you for the question. We have already supported it by two references (N Engl J Med 1999; 341: 936-942; N Engl J Med 1999; 341: 929-935) and now we added two more references (Arch Dis Child 1980; 55: 583–588; Lancet 1974; 2: 112). Please see line 61-63 in the revised manuscript.

**Critique 3:** Line 72: replace ‘can’ with ‘has’.

**Response:** We have revised our manuscript on line 71 as suggested.

**Critique 4:** Line 79-80: replace ‘…make EV71 characterized as the most important cause of severe HFMD.’ with ‘..is considered an important cause of severe HFMD’.

**Response:** We have revised our manuscript on line 78-79 as suggested.

**Critique 5:** Line 81: typo, ‘compliations’ should be ‘complications’

**Response:** We apologize for our negligence. We have revised our manuscript on line 80 as suggested.

**Critique 6:** Line 84-86: ‘Sporadic reports demonstrated…’. References should be provided for this statement.

**Response:** Thank you for the question. We have already added some references

**Critique 7:** Line 129: replace ‘Afterward.’ with ‘A volume of...’

**Response:** We have revised our manuscript on line 136 as suggested.

**Critique 8:** Line 239-240: ‘CA16 has only one genotype B.....’. This statement is not true. Strains of CA16 have been divided into genogroups A and B (Arch Virol (2007) 000:1–8).

**Response:** Thank you for the question. We have revised our manuscript on line 245-247 as suggested.

**Critique 9:** Line 269: typo, ‘none-EV71’ should be ‘non-EV71’

**Response:** We have revised our manuscript on line 275 as suggested.

**Critique 10:** Line 270: replace ‘were’ with ‘are’

**Response:** We have revised our manuscript on line 276 as suggested.
**Response to Reviewer 2**

**Critique 1:**

Major compulsory revisions

The aim of the authors was to “identify any associations between enterovirus types and clinical manifestations”. I’m not sure that stool specimens are the most appropriate specimens for investigating enteroviruses associated with HFMD. Enteroviruses may be shed in the faeces 3-5 weeks after infection. Stool samples may thus contain a virus that has nothing to do with the symptoms. Therefore, the clinical samples used in this study are not adequate to fulfil the objectives claimed by the authors. Moreover, the clinical symptoms are not fully described in the methods or the results sections. In my opinion, the report should focus on the genotyping results. The phylogenetic analyses should be reinforced and improved as the analysis of the figures does not fit the data reported in the manuscript.

**Response:**

Thank you for the good question. We would try our best to response to the comment.

First, since this study was a retrospective study and the samples we collected were from the specimens sent routinely to virology laboratory, only adequate stool specimens could be taken. Second, in our another previous study, we try to detect and type the enterovirus by RT-PCR from throat swabs and CSF in patients with CNS infections, but for the reason of the lower viral load in these types of specimens, many specimens could not be directly genotyped by sequencing the PCR products of VP1 region. Owing to the much higher enterovirus load in the stool specimens compared with that in the throat swabs and CSF, it was easier to detect the enterovirus and to work out the genotype successfully by directly sequencing the RT-PCR products. Third, all the stool specimens we collected were from patients hospitalized for treatment of HFMD. That is to say, all the patients have been clinically diagnosed with HFMD. And all the specimens were collected during the early phase of the illness which could be able to represent the recent infection of the patients. Lastly, it is widely recognized that HFMD is a common disease of children
mostly associated with the human enterovirus species A. For each enterovirus positive stool specimen we detected in this study, only one single enterovirus genotype was identified. So we think that the enterovirus identified in the stool specimen could represent the pathogen causing HFMD and had a great relationship with the clinical symptoms.

The responses to other comments were seen in the following responses to critique 3, critique 7, critique 10 and critique 14.

**Critique 2:**
- Page 5 – Sample collection:

The way by which the authors have collected clinical information for the patients included in the study should be indicated. Was the information collected prospectively or retrospectively?

**Response:**
Thank you for the question. Our study is a retrospective study. The clinical information for the patients included in the study was collected from the medical history after the detection. We have added more information on this on line 104-106.

**Critique 3:**
- Clinical complications should be more precisely described. For example, neurological complications following HFMD and herpangina have been clearly defined and classified in the manual entitled “guidance for clinical anagement of HFMD” available at http://www.wpro.who.int/publications/docs/GuidancefortheclinicalmanagementofHFMD.pdf.

**Response:**
Thank you for the good suggestion. The complications involve diseases in neurologic,
respiratory or circulatory system that caused by HFMD, such as encephalitis, acute flaccid paralysis, pulmonary edema, or cardiorespiratory failure, which were defined in the China Ministry of Health diagnostic criteria and “A Guide to Clinical Management and Public Health Response for Hand, Foot and Mouth Disease (HFMD)” from WHO. We have added more information on this on line 95-103, page 6.

**Critique 4:**
- Page 7 – Enterovirus genotyping: the approximate length of the amplified products for the partial VP1 sequence should be indicated.

It would be interesting for the scientific community if the sequences obtained in this study could be deposited in international databases.

**Response:**
Thank you for the question. The primers we used were obtained from the reference (J Clin Virol 2009; 44: 119-124). The PCR products used for the sequencing were about 683bp for the group A, 619bp for the group B and 497bp for the 5’UTR. We have added this information on line 145-146, page 7-8.

We have submitted the sequences obtained in our study to the NCBI database with accession numbers KC834832-KC834854 for CA6, KC834855-KC834877 for CA10, KC834878-KC834892 for CA16 and KC834893-KC835055 for EV71. We have added this information on line 143-144, page 7-8.

**Critique 5:**
- Page 7 – Phylogenetic analysis: the mathematic model used to estimate the genetic distances should be indicated.

**Response:**
The phylogenetic trees were constructed applying the neighbor-joining method with the 1000-bootstrap re-sampling implemented in the Molecular Evolutionary Genetics Analysis, version 4.1 program. We have added this on line 151-152, page 8.
Critique 6:
- Page 8, lines 158-160: The detection rates of each enterovirus types should be calculated with the number of positive clinical specimens, i.e 277: EV71 (66.7 %, 185/277) for example.

Response:
Thank you for the comment and good suggestion. When analyzing our data, we also considered which one is better for calculation of the detection rate of each enterovirus type: the number of all specimens or the number of positive clinical specimens. We think if with the number of all specimens as the denominator, we got the detection rate of each enterovirus type in all specimens. But if we use the number of positive samples, we just got the composition of each type in all enterovirus positive ones. So finally we chose to use the detection rate in all specimens.

Critique 7:
- Page 8, lines 163-164: The phylogenetic analysis of partial VP1 sequences of enterovirus 71 strains was performed with 165 sequences. The authors reported in the precedent section that 185 strains of EV71 were detected. Please explain why. I have the same remark for the other serotypes: CV-A16 (15 instead of 20), CV-A6 (23 instead of 24), CV-A10 (23 instead of 26).

Response:
Thank you for the comment. We have finally detected 185 strains of EV71 using RT-PCR with the primers from the VP1 junction region (163) in addition with 5’UTR (22) according to the methods described in J Clin Virol. 2009; 44(2):119-124. We feel very apologized for our negligence that the number of partial VP1 sequencing is 163. We have revised our manuscript on line 174. And only the 163 sequenced in the VP1 junction region could be used to construct the multiple-sequence alignment and the phylogenetic tree based on partial VP1 gene sequences. It was the same situation as CA16, CA6 and CA10.
Critique 8:
- Page 9, lines 175-178: the CV-A16 sequences are distributed in two clusters B1a and B1b by the authors. The authors should indicate on which criteria the clusters B1a and B1b have been defined, as bootstrap values do no support the differentiation of the two clusters.

Response:
Thank you for the comment. We distributed the CA16 sequences into Clusters B1a and B1b according to the references (J Med Microbiol 2011; 60: 349–358; J Clin Microbiol 2010; 48: 619-622.) which may not be supported by the bootstrap values.

Critique 9:
- Page 9, lines 179-183: same remark for the phylogenetic analysis of the CV-A6 sequences. Bootstrap values don’t allow distinguishing “two genetically distinct branches”.

Response:
Thank you for the comment. We apologize for our negligence. We have revised the “two genetically distinct branches” as “two branches” on line 191-192, page 10.

Critique 10:
Supplemental material Table 1: It is not clear why the babinski’s and the brudzinski’s signs have been combined in the same line in the table, as they do not correspond to the same clinical information.

Considering the aim announced by the authors to “identify any associations between enterovirus types and clinical manifestations”, it is not clear why the table is included as supplemental material.

Response:
Thank you for the comment. The babinski’s and the brudzinski’s signs are not the same clinical sign. The two signs are considered to be related to the central nervous
system symptoms, so we brought the two signs together when collecting the clinical information. However only very few patients showed one or two of these signs, we just combined them in the same line in the table like “the babinski’s signs or/and the brudzinski’s sign”. We have revised it in the table 1.
We showed the table as a formal one, not as supplemental material.

Critique 11
- Page 11, lines 212-221: this paragraph could be shortened. It does not really correspond to a discussion of the obtained results.

Response:
Thank you for the comment. We have already shortened the paragraph as suggested.

Critique 12:
- The authors should point out the weakness of not using more appropriate specimens (vesicles, throat or buccal swabs) for the diagnostic of enterovirus infections associated with HFMD and discuss how it could have influenced the results.

Response:
Thank you for the good question. As we discussed in response to the reviewer’s major compulsory revisions, we have some reasons for not collecting other specimens. However, we felt regretted for this. We believe our study would be more credible and abundant if we could also detect and compare the data from different kinds of specimens. In the subsequent study, we will try to develop a prospective study for further investigation of the pathogens causing HFMD. We will collect stools together with other types of specimens such as throat swab, vesicles and/or CSF. We have added more information on this on line 280-292, page 14.
**Critique 13:**
- What was the seasonality of enterovirus infections?

**Response:**
Thank you for the good comment. We have analyzed the seasonality but there was no obvious seasonal distribution difference of each enterovirus genotype. Please see the fig. s1 below.

**Critique 14:**
- The improvement of the phylogenetic analyses could allow a better analysis of genetic relationships between strains isolated in Shanghai with other Chinese strains and with strains isolated in other countries.

**Response:**
Thank you for the comment. We consciously collected the sequences form NCBI as much as we can. We analyze the strains isolated in our study by comparing with other strains isolated in Shanghai or other provinces, and with strains isolated in other countries to well understand the evolution and genetic relationships.

**Critique 15:**
For each figure corresponding to phylogenetic analyses, the length of the VP1 sequences should be indicated, as well as the number of sequences of reference strains used in the analyses.

**Response:**
Thank you for the comment. We have marked the length of the VP1 sequences and the number of sequences of reference strains in each legend of the figures.

**Critique 16:**
For clarity, only the bootstrap values of over 70 % can be indicated on the
dendograms.

Response:
We have revised the figures as suggested.

Critique 17:
The country in which the reference strains used were collected should be indicated.

Response:
We have added the country of the reference strains in each figure.

Critique 18:
- Page 6, line 110: “Human enterovirus was identified with highly conserved 5’UTR primers”. The 5’UTR region is used for the diagnostic of enterovirus infection. The term “identify” is not appropriate here.

Response:
We have selected “preliminary detected” instead of “identify” in our manuscript as suggested on line 117, page 6.

Critique 19:
- page 5, line 81: “severe compliations”. Please correct.

Response:
We apologize for our negligence. We have revised our manuscript on line 80, page 4.

Critique 20:
- Reference section: lines 320 and 323: “Oberate” should be replaced by Oberste.

Response:
We have revised our manuscript on line 343, 346 and 396.
Critique 21:

Response:
We have revised our manuscript on line 351, page 18.

Critique 22:
- Line 442: “Clin Micrboiol Infect” : Clin Microbiol Infect

Response:
We have revised our manuscript on line 455, page 21.

Editor's Comments:
* Please deposit your sequence data in a public repository and include details of this within your methods section.
* Please deposit your alignment and phylogenetic tree data in a public repository and include details of this within your methods section.
* Please clarify within your methods section if you obtained informed consent from patients for participation in the study. If the ethics committee waived this and if samples were taken as part of standard patient care please include these details.

Response:
Thank you very much for all the suggestions. We have submitted the sequences obtained in our study to the NCBI database with accession numbers KC834832-KC835055. This information had been added on line 153-156, page 8.

We checked many references and asked some experts but we could not get any information about how to deposit phylogenetic tree in a public repository. I’m sorry about this. If you can provide me the repository internet site, please let us know.

Because the specimens were collected in the normal course of patient care, no informed consent was required for this study. This was approved by the Ethics Committee of the Children’s Hospital of Fudan University. We have added this
Fig S1. Season distribution of enterovirus genotypes of HFMD patients in 2010-2011