Author's response to reviews

Title: The variations of VP1 protein might be associated with nervous system symptoms caused by enterovirus 71 infection

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Author's response to reviews: see over
Dear Dr. Harris

Thanks very much for reviewing and considering our manuscript for publication in BMC Infectious Diseases. We have addressed the reviewers’ queries in our revised manuscript accordingly, and formatted the revised manuscript to the journals style. For your convenience, we would like to provide two versions of manuscript; one is for publication and the other one for showing the chances worded in Red color. Our point-by-point responses are shown as follows.

Response to Reviewer 1

1. Pg4. Since AF135899 has been excluded from all the comparisons, the total sequences would be 186.

Response: Sorry for the unclear statement. Strain AF135899 does not belong to none of B subgenotype from B1 to B5, but it does belong to subtype B. Therefore, this strain was included in the phylogenetic analysis. In our revised manuscript, we have stated clearly.

2. Supplementary table 1 should also include references of which the sequences were published. Most of the accession number pages do not reveal the clinical presentation.

Response: Thanks very much for your good suggestion. We have updated the sequence information and added the corresponding references in our revised manuscript.

3. Were all the VP1 sequences downloaded and looked at one by one? How thorough was the process and search? New sequences are available in Genbank every day, hence the date when sequences were accessed is important.

Response: We took the advantage of BLASTN tool to search VP1 gene of EV71, and cross-validated in the database of EXPASY. The sequences were doubly checked by going through 324 papers one by one. We have amended the information in the Section Nucleotide sequences screening (page 4, line 4-6). The sequences were collected up to Sep 30, 2013 (page 4, line 7).
4. How the authors decide which C4 sequences to choose? There are really loads of them. Same goes to other subgenotypes, since they would be more HFMD sequences compared to others. Authors need to demonstrate that sequences were not selected to fit into the results.

Response: Thanks very much for your good question. As the Reviewer pointed that a lot of sequences belong to the C4 subgenotype. We used the following criteria to select sequences from C4 genotype. All the sequences associated with nervous symptoms were selected by either EV 71 detected from cerebral spinal fluid (CSF) or isolated from patients with meningitis/encephalitis as described in detail. All the sequences without nervous symptoms were accepted by either annotated with HFMD or herpangina, or the referred to the EV71 patients with HFDM or herpangina.

5. Similarly, some genotypes may not be very well presented. The presence of mutations is very much subgenotype specific. More importantly, some EV71 subgenotypes are geographically distinct-C4 in China and Taiwan, and B4 and B5 in Malaysia. Can authors show or discuss how that can possibly skewed the correlation?

Response: Thank you very much for your constructive suggestion. It has been noted that some subgenotypes of EV71 took place with time and geographical specificity (e.g., Chan KF et al., Infect Genet Evol. 2010;10(3):404-12). If all the sequences were mixed and compared, false positive or negative correlation between the mutation and nervous system symptoms would be acquired. For example, the site of 164 in VP1 was identified with the residue of D in genotype C. If analyzing with combination of all subgenotypes, the position would be no significant correlation; in fact, variation of E164D/K in genotype B was correlated with neurovirulent phenotype. We have added the related information in discussion (page 7, line 16-19). We agree that some genotypes may not be very well presented, because partial sequences are not included. When more and more complete sequences are deposited in the database, this problem will be solved.

6. Discussion. Pg 7. 1st para. Most if not all the previous studies actually suggest that there is no correlation between EV71 subgenotype and neurovirulence. What’s probably different in this study is statistically methods were used to show the association.

Response: Statistical methods in this manuscript were same as other studies, but the attention was different. Such as in PLoS One 2011, 6(10):e26237, they paid attention to relationship between the genotypes/mutations to severe or mild symptoms in clinic, but not narrowed to nervous system symptoms. Another possible difference may be caused by limited number of cases in those reports.

Minor essential revisions

1. Pg 6. para 1. Change “To our surprising” to “to our surprise”
2. Discussion. Pg 8. Recently heparan sulfate has also been identified as the attachment receptor.
Response: We have corrected the errors and cited the reference as suggested.

Response to Reviewer 2

1. The background information of VP1 protein and other important proteins is not adequately provided. For instance, why VP1 is the selected protein here.

Response: Thanks for your good comment. We have added the following information in the Background section to show the importance of the VP1 as “VP1 protein is one of structural proteins of EV71 virus, which is involved in forming the pentameric icosahedral structure of the virus, important for virus to bind receptors and infect host cells” (page 3, line 21-23).

2. In phylogenetic analysis, normally, Maximum-likelihood (ML) method is better than Neighbor-joining (NJ) method. Can the author use ML method here or explain why they chose NJ method?

Response: The NJ method is well adopted and recognized as conventional tool for the classification of VP1 sequences of Enterovirus, e.g., BMC Evol Biol. 2010; 10: 294; Emerg Infect Dis. 2003; 9(4): 462–468; while Maximum-likelihood (ML) method with software of PAUP is too expensive for us to buy one.

3. The association between phenotype (e.g. nervous system symptom in the study) and the viral phylogeny can be determined by phylogeny-trait association statistics. It would be much better and convincing if the authors can test the phylogeny-trait association statistics instead of drawing the conclusion from the topology of NJ phylogenetic tree only.

Response: Thanks for your comments. As the phylogeny-trait association statistics has just developed recently, we have met some problems to run this program.

4. The first paragraph in Results needs some rewriting.

---“A phylogenetic dendrogram was constructed by using 891 nucleotides of VP1 sequences of 187 EV71 strains (Fig. 1 and supplementary Fig1A/B for genotype B, C). These strains were divided into three groups: A, B (B1–B5) and C (C1–C4) genotypes. Only strain AF135899 belonged to B genotype.” The description of phylogenetic tree is unclear. Is the strain AF135899 another subtype of B (instead of B1-B5)?

Response: Yes. The strain AF135899 belonged to B subtype, but none of B1-B5. The sentence of “Only strain AF135899 belonged to B genotype.” was replaced with “The strain AF135899 was included in B genotype, but did not belong to B1-B5.”

---“Except subtypes B1 and B2, there were no concentrated clusters in NS-EV71 strains in other subtypes of genotype B and C.” Here needs more description and Figure S1 should be cited here.
Response: We have added a sentence “More importantly, some EV71 subgenotypes are geographical and time specific, such as, C4 in China and Taiwan, and B4-5 in Malaysia” in the text, and Figure S1 has been cited in the revised manuscript.

5. The third paragraph in Results needs some rewriting.

---Consensus changes found in different genotypes are very common and generally they are the major characteristics of the genotypes. It is totally unnecessary to mention here because genotype is not associated with nervous system symptoms in the previous analysis (the second paragraph in Results).

Response: We think it’s better to keep the original description. Normally, people only analyze nucleotide sequences but not the protein sequence whereas that may not cause residue changes. In fact, residue substitution is much more important as it may reflect functional chance. Therefore, it is necessary to analyze the variations of amino acid.

---“There were seven sites where variations displayed three times or more in NS-EV71 and nNS-EV71 cases (Table 2).” “Three (N31D, V170A and V262I) of eight sites with three or more variations were linked with nervous system symptoms (Table 2).” What do these sentences mean?

Response: To make it more clear, we have revised our description as “all subtypes had the consensus amino acids 43E, 58T, 184T and 240S; and seven other residues displayed high frequency of substitution in both NS-EV71 and nNS-EV71 cases (Table 3)” (page 5, line 22-23, and page 6, line 1-2); and “Three residue substitutions (N31D, V170A and V262I) were associated with nervous system symptoms in C genotype (Table 3)” (page 6, line 7-8).

---“The OR and 95% CI of N31D were 3.044 and 1.002–9.252; In the case of V262I, they were 0.367 and 0.136–0.989, respectively. More interestingly, we noted that residue 262I almost displayed in all C4 subgenotype. Normally, V262I substitution mainly exhibited in subgenotype C1 to C3. To our surprising, the risk of V262I in subgenotype C1–3 was negatively associated with neurovirulence(#2=6.262, p=0.012; OR, 0.239; 95% CI, 0.075–0.756).” Here needs some rewriting to improve the grammatical errors and help understanding.

Response: We have rewritten the sentences as “The OR and 95% CI of N31D were 3.044 and 1.002–9.252, indicating that N31D variant was significantly associated with EV71 neurovirulence. More interestingly, we noted that C4 subtype had a consensus residue 262I. To our surprise, the risk of V262I substitution in subgenotype C1–3 was negatively associated with neurovirulence (χ²=6.262, p=0.012; OR, 0.239; 95% CI, 0.075–0.756)”. 
As the occurrence rate of V170A was too low, what does it mean? Should it be “A170V” instead, according to Table 2?

Thanks for pointing out the typo. We have corrected it as A170V.

6. The discussion section is weak.

The structure of Discussion is not well organized. The first paragraph of Discussion can be moved to Background section and the authors can summarize the key findings instead. It would be better to discuss the findings between genotype/subtypes and nervous system symptoms first and then move on to the specific amino acid changes.

Response: We have reorganized the Discussion section. The first paragraph has been moved to the Background as suggested.

It has been reported that D164E variation was associated with severe cases of EV71 infection [14]. We postulated that the neurovirulence of EV71 may somehow be connected with nervous system tropism. Therefore, the residue variations of VP1 protein would play an important role in neurological complications. The connection between D164E and “variations of VP1 protein would play an important role in neurological complications” is weakly discussed here.

Response: We have discussed in deep in the revised manuscript. We have added one more paragraph to discuss D164E and neurological complications (page 7, the second paragraph).

The specific amino acid changes found here may have important role in binding the receptors. But the mechanism or potential relationship between the genetic variation and neurovirulence is poorly addressed.

Response: Up to now, the mechanism is poorly understood. Data from this study provides a clue for further investigating the underlying mechanism. The residue variations in VP1 protein may result in the affinity change between EV71 virions and host receptors on the neuron cells.

The limitation of the study is not stated.

Response: We have stated the limitation of the study.
Some minor comments (Minor Essential Revisions):

1. There are some typos or errors in the manuscript. For instance, 1) Second sentence in the first paragraph of Results: “A phylogenetic dendrogram”. “A phylogenetic tree” is much better; 2) Second paragraph of Results: “subgenotype” should be “subtype”; 3) First paragraph of Discussion: “to nervous system tropism [13,14]” should be “and nervous system tropism”.

2. Sequence of table 2 and 3 should be changed because table 3 is cited before table 2 in the main text. Actually table 3 is totally unnecessary.

Response: We have corrected typos and errors. We would like to keep Table 2 that would show the difference among the genotypes clearly.

3. Figures 1 and S1 can be improved.

Response: We have increased the resolution for Figures 1 and S1.

We wish we have successfully addressed all the queries raised by reviewers. Thanks again for your consideration.

Sincerely Yours,

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