Author's response to reviews

Title: The immunity and clinical efficacy of an inactivated enterovirus 71 vaccine in healthy Chinese children: report from further observation

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Author's response to reviews: see over
Response to reviewers

Reviewer 1-Rogier Van Doorn

The authors describe a substudy of one of the three phase III vaccine trials of an inactivated EV-A71 C4 vaccine that were published from China in 2013-14 in the Lancet and the New England Journal of Medicine (refs 8-10). A subset of 1100 children was randomized to receive vaccine or placebo and were followed up until day 720 (unblinded after 1 year for publication of the larger trial) and their sera were analysed for neutralizing antibodies (all), IFN gamma and IL4 responses (10 sera from each of 4 agegroups at day 720) and cross-protection (20 sera from day 0 and 360).

The results are interesting, but there is some repetition of the data already presented in the trial paper (particularly the methods section). The authors show a lasting immune response in the 4 agegroups (with unexplained boosting after 1y in all agegroups, and little benefit in the oldest agegroup), some differences among IFN gamma values but no p-values or discussion and cross-protection among all subgenotypes but only on day 360 and in a small subset. I have added comments, questions and suggestions for improvement below.

Major compulsory

-p6 Can the methods section be reduced by referring to the original NEJM publication?

Thanks for the suggestion. We might prefer to keep the methods section as according to the comment of the editor, this section will be helping the readers to have a complete picture of the research.

-p10 There is no Results section, is this correct?

Thanks for the comment. In this manuscript, we combine the sections of results and discussion according to the BMC Medicine guide for author, but we forgot to put the subtitle of results in the manuscript. We’re terribly sorry for this mistake and any inconvenience this may cause to you.

-p12-13: The issue of lasting cross-protection is quite an important one for vaccine implementation strategies, yet the authors chose to only use a small selection and only look at day 0 and 360. Why not look at the other timepoints and look at the dynamics of the response, also beyond day 360. It would be interesting to see whether EV-A71 behaves as a single genotype with lasting cross-protection from day 0 to 720, or – like dengue or flu – has an initial broad response across all subgenotypes that wanes over time for the non C4’s. It would also be interesting to include some contemporary CA6 and CA16 strains.

Thank you for this point. In the revised version, we take your suggestion and supplement the results of cross-protection at day 56. As there is a remarkable tendency of cross-protection against different genotypes of EV71 based upon the primary results observed at these three different time points, further, the cross-protection capacity is identified to be not varied with the changing of time.
On the other hand, we did have performed the in vitro cross-protection assays on different enteroviruses, including CA16, CA7, CA9, CB2, CB6 and Echo, and the results showed that the sera from the immunized cohorts fail to cross neutralize other enterovirus (data are not yet published). And the protective potency results of phase III clinical trial confirm the same failure of this vaccine against CA16 and other enterovirus (Li, et al, NEJM, 2014, 370:829-37).

Minor essential
-Line numbers were missing, please add when resubmitting
Thank you very much. As the reviewer suggestion, the line numbers have been added for comfortably reading.

-Many numbers are presented with 2 decimals, while this level of exactness is often neither justified nor required
As suggested, the numbers are presented with 1 decimal in the revision.

-Clarify in abstract and introduction that the IFNgamma-IL4 and subgenotype work was only done on a small subset
The IFNgamma-IL4 and subgenotype work only done on a small subset has been clarified in abstract and introduction (page 3 and page 6-7).

-p5 In a published ... efficacy: please clarify there were three different phase 3 trials with three different C4 strains
In page 5 line 69-72, the sentences were re-written in revised manuscript.

-p7 …a phase III trial… : is this the same trial (ref 8) published in NEJM described above? The 1100 participants described here were also part of the enrolled population of this trial (ref 8)?
Yes, this study is the long-term observation subsequently followed the phase III trial (ref 8). So the participants in this study are the same as set in ref 8.

-p8 Additionally … EXCEL software: Unclear. Were 20 patients selected from whom samples collected on day 0 and 360 were tested, or were 20 sera -collected on day 0 and 360 from 10 patients - tested? Were 20 patients selected from both vaccinees and placebo-control group, or 20 in total (if so, add how many were vaccinees and how many received placebo)?
Thank you for your question. The EXCEL analysis is performed according to the following procedure: firstly, the participants whose sera are collected at days 0, 56, 180, 360, 540, and 720, respectively are screened from all the vaccine and placebo-controlled groups and subsequently divided into 2 groups (vaccine and placebo). Then the selected participants in each group are assigned randomly with an unique new number via RAND() function in EXCEL. Finally, the sera collected from the above-selected first 20 participants based upon the new assigned numbers at days
0, 56 and 360 are used for the cross-protection assay.

-p8 A total ... Asia-Pacific countries: clearly indicate which strains/isolates were used

Thank you. The strains/isolates were described in the supplemental data of the manuscript. Please see the following table for details.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Name</th>
<th>Genebank No</th>
<th>Isolated area</th>
<th>Isolated time</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>9522</td>
<td>AY258300</td>
<td>Malaysia</td>
<td>2003</td>
</tr>
<tr>
<td>C2</td>
<td>8M/6/99</td>
<td>AY126012</td>
<td>Malaysia</td>
<td>1999</td>
</tr>
<tr>
<td>C3</td>
<td>001-KOR-00</td>
<td>AY125966</td>
<td>Korea</td>
<td>2000</td>
</tr>
<tr>
<td>C4</td>
<td>FY-23</td>
<td>EU812515</td>
<td>China</td>
<td>2004</td>
</tr>
<tr>
<td>C5</td>
<td>VN5559</td>
<td>AM490158</td>
<td>Vietnam</td>
<td>2005</td>
</tr>
<tr>
<td>B3</td>
<td>13903</td>
<td>AY207648</td>
<td>Malaysia</td>
<td>1997</td>
</tr>
<tr>
<td>B4</td>
<td>A10/4</td>
<td>AF376067</td>
<td>Malaysia</td>
<td>2000</td>
</tr>
<tr>
<td>B5</td>
<td>15431</td>
<td>NA*</td>
<td>Malaysia</td>
<td>2006</td>
</tr>
<tr>
<td>A</td>
<td>BeCr</td>
<td>U22521</td>
<td>USA</td>
<td>1970</td>
</tr>
</tbody>
</table>

*NA* mean not available.

-p8 ... as described in previously published reports: Reference these reports, particularly development and validation of ELISPOT assays for EV71, which stimulating peptides were used etc

The reference and stimulating peptides used in our ELISPOT assays are provided based upon your comments (page 9 line 155).

-p9 stimulating peptides: Define

The sequences of the stimulating peptides are defined in the revised version (page 9 line 154-159).

-p9 considered to be susceptible: Define

The level of neutralizing antibodies against C4 genotype of EV71 lower than 1:8 before immunization is defined as the susceptible participants (page 8 line 137-139).

-p10 recent epidemiological studies: this reference is from 2002, use a more recent reference or delete the word recent

The word “recent” has been deleted.

-p11 in clinical trials: Not sure what this addition means or why reference 17 is inserted here
Thank you for your suggestion. The reference 17 has been deleted.

-p11 although … 100%: Delete, as this is repeated with more accuracy below
Yes, it has been deleted.

-p11 In some cases ... earlier results: What does this mean, infection with a related
virus? (Asymptomatic) re-infection with EV71? Can this be discussed in more detail, ie. was this effect caused by a strong increase in a small number of children or was it
a common observation?
This sentence means that the Nab levels at days 540 and 720 post-immunization
raised, comparing with those at days 360. And the sentence has been modified in
revised manuscript (page 11-12 line 212-217).

-p11 the effect of a natural infection ... by the vaccine: What data do the authors have
to support this statement?
Thank you very much. The references, which support this statement, were added in the
revision (page 12 line 227).

-p12 synthesized VP1 peptide: Reference
The details of VP1 stimulating peptide were supplemented in the revision (page 9 line
155).

-p12 The result ... enterovirus-infected cases: Was boosting of EV-A71 NAbs
observed after infection with other EVs, both in vaccinees and placebo-controls?
Thanks. The cases in each group have been supplemented in revised paper (page 13
line 252-255). A total of 15 EV71-infected cases (total in placebo group), along with
11 CA16-infected cases (3 in vaccine and 8 in placebo) and 29 other
enterovirus-infected cases (12 in vaccine and 17 in placebo), were identified with an
RT-PCR assay in a total of 1,100 children in this cohort for a two-year period after
the vaccination.

-p12 These data … older children: What data do the authors have to support this
statement?
In order to avoid the confusion, we re-write our statement. See the revised version
(page 13 line 250)

-p12 Notably … p13 clinical trials: Does this add anything to the results already
published in the NEJM paper (ref 8)?
In this manuscript, the neutralizing antibodies and cross-protection are assayed by
using the sera of the sub-cohorts (1,100 participants) in a period of two years, and the
protective efficacy is analyzed in parallel in these 1100 participants for the HFMD
cases caused by the EV71, CA16 and other enteroviruses. In contrast, in the NEJM,
only the neutralizing antibodies are assayed by using the sera of the sub-cohorts
(1,100 participants) in a short period of 11 month, but the protective efficacy are
analyzed in a total of 12,000 participants for the HFMD cases caused by the EV71, CA16 and other enteroviruses.

-p12 Any p-values for the IFNgamma/IL4 work? Why are the levels so much lower in the oldest agegroup. How can the authors explain that there is seemingly no difference in the third agegroup? Which differences were significant? Was the sample large enough to be representative?

The statistical analyses were performed in figure 3. And the p-values were marked in the figure. And we have tried our best to explain the reason lead to the strange result in the oldest agegroup in revision (page 13 line 242-245). And further studying should be performed in the future.

-p13 Whereas ... issue: Reference the recently published papers showing full cross-protection against all EV-A71 subgenotypes after natural infection and among vaccinees (PLoS NTD 7(2):e2067, PLoS One 8(11):e79599, PLoS One 9(6):e100545) and discuss what is new here compared to these. Reference the recent paper from Taiwan showing a C4 to B5 subgenotype switch (PLoS One 10(3):e0116322)

Thank you for the suggestion. We’d like to clarify our understanding as follows: Like the reports in the published paper (PLoS One 8(11):e79599, PLoS One 9(6):e100545 and PLoS NTD 7(2):e2067), the antibodies induced by our vaccine could cross-protect against the infections caused by enteroviruses of genotypes B4, B5, C2, C4 and C5. However, the neutralizing capacity against A genotype was identified to be different from those reported by Dr. Huang (PLoS NTD 7(2):e2067 and Dr. Luo PLoS One 10(3):e0116322), which might be attributed by the different phylogenetic distance of genotype A from other strains by Dr. Luo (PLoS One 10(3):e0116322) and Dr. Yip (Virology Journal 2013,10:222). On the contrary, according to the findings of Dr. Chan (Infection, Genetics and Evolution 10(3):404-412) and Dr. McMinn (JVI 75(16):7732-7738), the phylogenetic distance among all the genotypes A, B and C are quite similar, which might be providing some evidence for understanding the identification of genotype A neutralizing capacity in our study.

Table 1: Also show data for the placebo group

We agree on the reviewer’s concern. The data of placebo group have been added in revision.

Figure 1: The dropout rate is quite high, higher than for the entire study it seems, comments on this?

The reason is mainly due to the relocation of some families, which has been happening between rural and urban areas in recent years in China. It was supplement in the revision.

Figure 2 and furthers: Numbering of legends and figures doesn’t match, same with cross-referencing in the body of text

We are very sorry for the mistakes. They have been corrected in revision.
Figure 2 (Figure 3 legends): The boosting of the Nabs level after day 360 in the three youngest agegroups is quite striking, as is the fact that in the oldest agegroups the levels among vaccinees and placebo-controls are virtually indistinguishable after 56 days.

Thank you for your suggestion. It is modified in revision.

Figure 3 and 4: p values?
The statistical analyses were performed in figure 3 and 4. And the p-values were marked in the figures.

Figure 5: The levels among placebo-controls appear to be quite low, considering the levels shown in Figure 2 (Figure 3 legends)

Thanks for this question. In figure 2, the neutralizing antibodies are measured from sera collected from 1,100 sub-cohorts in pre-protocol analysis, whereas in figure 5, the cross-neutralizing antibodies are measured from 160 collected sera as described in EXCEL analysis section (page 8 line 140-142)

Reviewer 2-Yen-Hung Chow
Major Compulsory Revisions:
The manuscript entailed The immune response and efficacy of an inactivated enterovirus 71 vaccine in healthy Chinese children: report from further observation by Longding Liu, et al., has several editions need to be fixed before submission.
1. Figures and legends can not be fitted in the content of article; suc as Figure 2's legend sould be Figure 4, Figure 3’s legend sould be Figure 2, Figure4's legend sould be Figure 3,
We are very sorry for these mistakes. They are corrected in the revision.

2. In Figure 3 (after edition) the P value for IFN-r and IL-4 should be marked to index that the statasitical difference between vaccine vs. placebo groups. Because of the standard error bar on the column are wide, I wonder their significance difference. If not, then the description in the content of result (page 12) should be edited and conclusion need to be corrected.
Thank you for pointing out the imperfections. The p values have been marked in figure 3 and 4.

Again in figure 4 (after edition), the statastical analysis should be marked.
Thank you very much. The results of statistical analysis have been marked in the figure.

Reviewer 3-Xianghua Luo
This review only focuses on the design, statistical methods used, and interpretation of statistical results in the paper.
Major Compulsory Revisions:
1. According to the manuscript, a stratified randomization (by age group) was performed. It is not clear if simple randomization or blocked randomization scheme or any other specific randomization scheme was used within each age group. 

*Thank you for the question. According to the protocol of phase III clinical trial (supplemental material of NEJM, Li et al, 2014, 829-837), a total of 12000 participants are recruited and stratified into 4 groups: 3500 in 6- to 11-month-old group, 3500 in 12- to 23-month-old group, 3000 in 24- to 35-month-old group and 2000 in 36- to 71-month-old group. The participants in each stratified group are randomly sub-divided into vaccine and placebo groups at a ratio of 1:1. The 10% of the recruited participants in each group are randomly selected for composing the 1100 subcohorts divided again into the vaccine and placebo groups at the same ratio of 1:1.*

2. Was there a formal power analysis and sample size justification performed for this trial? Details should be provided in the Statistical Analysis section.

*Yes. The sample size justification is the same with ref. 8, because that the manuscript is the long-term observation of the phase III clinical trial (ref. 8). And the other details of the statistical analysis have been supplement in the revision.*

3. Most content in the Discussion section should be moved to the Results section. The Results section is currently missing.

*In this manuscript, we combine the sections of results and discussion according to the BMC Medicine guide for author, but we forgot to put the subtitle of results in the manuscript. We’re terribly sorry for this mistake and any inconvenience this may cause to you.*

4. Figure titles and descriptions don’t match the pink figure labels, e.g. Figure 2’s description in page 21 is apparently for the page labeled as “Figure 4” (in pink).

*We are apologized for these mistakes and correct them in the revision.*

5. The statistical methods for the Kaplan-Meier curves were not described in the Statistical Analysis section (page 9). What method was used for comparing two curves? Group comparison p-values should be provided in the Figure and in the text.

*The Kaplan-Meier method was used for the comparison of vaccine efficacy, and the Mantel-Haenszel chi-square method was assessed as the difference in the proportions of children in the two groups (page 10 line 172-175). And the p-values were marked in the figures.*

Minor Essential Revisions:

1. Fig. 2’s name appeared in the text after Fig. 3 and Fig. 4. If Fig. 3 and 4 need to be discussed earlier, they should be called Fig. 2 and Fig. 3 instead.

*Many thanks. The figures have been re-ordered in revised manuscript.*

2. Follow-up rate (%) should be provided following N = # for each cell in the right
panel of Figure 1.

The follow-up rate (%) has been provided in Figure 1 form the revision.