Author's response to reviews

Title: Development and Evaluation of Reverse Transcription-Loop-Mediated Isothermal Amplification Assay for Rapid Detection of Enterovirus 71

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

We really appreciate you and reviewers for carefully reviewing our manuscript entitled “Development and Evaluation of Reverse Transcription-Loop-Mediated Isothermal Amplification Assay for Rapid Detection of Enterovirus 71” with a manuscript ID (MS: 3549511265251509) and giving us highly positive evaluation, helpful comments and constructive suggestions.

Based on the reviewers’ helpful comments and constructive suggestions, we have revised the manuscript according to the reviewers’ suggestions. The point-to-point responses for reviewers’ concerns were also enclosed in this letter. We believe that the quality of the revised manuscript was greatly improved under the help of the reviewers. Meanwhile, we hope that the revised manuscript will be satisfactory and acceptable for the publication in *BMC Infectious Disease*.

Sincerely yours

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Response to Reviewer (Panagiotis Karanis)

- Discretionary Revisions

Comment 1: THERE STILL MINOR MISTAKES TO CORRECT IN THE MS FE SPACE AFTER: Hand.. Six.. A total... Our.. in the Abstract p. 5. swabs,20
Response: Thank the reviewer for this comment. We have carefully checked the whole manuscript and corrected all mistakes corresponding to spaces, grammatical, spelling and typo errors in the revised manuscript.

- Minor Essential Revisions

Comment 2: Some comments in the tables are not completely accurate f.e. table 1: the description contains the primers' sequence not the design. table2: Findings on...
Response: Thank the reviewer for this comment. We have modified the table 1 title for the RT-LAMP primers and table 2.

Comment 3: In the results the authors report the optimal Temp. they do not report about the results with other temperatures tested.
Response: We have reported about the results tested at with other temperatures tested as shown in Fig. 2A. Except the optimal reaction temperature of 60 °C, clear bands of amplified products weren’t observed in gel electrophoresis at other tested temperatures (61, 62, 63, 64 and 65 °C).
Comment 4: p. 11 names of species please use block letter at the beginning. Commata, space, between

Response: We have used block letters for the names of species at the beginning in Page 11.

Comment 5: p. 12. They report on six temperatrues tested. This belong to the M & M.

Response: We have removed six temperatures description in Page p. 12.

Comment 6: p. 14. All (space!) p. 20. CVB, repre... (space)

Response: Thank the reviewer for this comment. We have revised all spaces in Page p. 14. and Page p. 20.
Response to Reviewer (Patchara Phuektes)

Comment 1: The manuscript described development and evaluation of Reverse Transcription Loop-mediated Isothermal Amplification (RT-LAMP) for detection of human enterovirus 71 (EV71). The RT-LAMP assay can be very useful for rapid and sensitive detection of EV71 from patients with HFMD. However, the manuscript still contains a number of badly worded/constructed sentences. The authors will have to check and refine the language carefully.

Response: We really appreciate the reviewer for this constructive suggestion. We have carefully modified this manuscript and corrected all grammatical, spelling and typo errors in the revised manuscript. Moreover, our revised manuscript was reviewed by native English speakers before re-submission.

Background

Comment 2: Ref 15 should be placed after the sentence “Recently, reverse transcription-PCR (RT-PCR) and real-time PCR assays have been used for EV71 detection”. Additionally, more references on real time PCR assays should be added.

Response: Thank the reviewer for this wonderful suggestion. In Page 2, we have placed
Ref 15 after the sentence “Recently, reverse transcription-PCR…” and added two references of “Simultaneous detection of human enterovirus 71 and coxsackievirus A16 in clinical specimens by multiplex real-time PCR with an internal amplification control” and “Rapid detection of enterovirus 71 by real-time TaqMan RT-PCR”.

-Materials and methods

Comment 3: –“pharynx swab” should be “pharyngeal swab”.

Response: –We have changed pharynx to pharyngeal in the revised manuscript.

Comment 4: A section on “virus” may be added to provide information on virus strains and/or genotype used for specificity and sensitivity tests. How many EV71 strains were tested in this study?

Response: Thank the reviewer for this comment. We have added information on virus strains in the revised manuscript. In this study, four virus strains of EV71, CVA4, CVA16 and CVB3 were used for specificity and sensitivity tests.

Comment 5: Please provide manufacturers for “betaine” and “Bst DNA polymerase”

Response: The manufacturers for “betaine” and “Bst DNA polymerase” have been provided in the revised manuscript.

Comment 6: Please provide information on “preservation solution”

Response: We have added the information of preservation solution (Hanks solution containing 10 µg/ml gentamicin and 0.25 µg/ml amphotericin B) in the revised manuscript.
-Results

Comment 6:  Fig 2A: DNA marker should be included.

Response: Thank the reviewer for this comment. We have added the DNA marker in molecules in size have been marked with 100 bp ladder in Fig. 2A.

Comment 7:  Fig 3: please check figure legend; lane 3 is positive??

Response: We have corrected the error. The results indicated that lane 3 should be positive.

Comment 8:  Fig 4: lane 1-4 should be specified as RT-LAMP products of EV71

Response: Lanes 1-4 have been specified as RT-LAMP products of EV71. Among them, Lanes 1 and 3 are RT-LAMP product digested with HinfI and TaqI, while Lanes 2 and 4 are RT-LAMP amplified product.

Comment 9:  Fig 5: the sensitivity seems to be at 10 PFU/ml rather than 1 PFU/ml, the picture of GoldView staining of each dilution reaction should be included as figure 5B. Please check figure legend; 10-3 PFU/ml.

Response: Thank the reviewer for this comment. In our study, RT-LAMP assay showed that the sensitivity was 1 PFU/ml when combined with Goldview staining of serial dilution reaction. In addition, we have added the picture of GoldView staining of each dilution reaction and changed 10-3 PFU/ml to $10^{-3}$ PFU/ml.
**Comment 10:** Fig 6: each line may be labeled as 105, 104 PFU/ml……10 PFU/ml.

**Response:** Thank the reviewer for this good suggestion. We have labeled $10^4$, $10^3$, $10^2$ and 10 PFU/ml in each line.

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**Discussion**

**Comment 11:** Line 1: “enterovirus” should be changed to “human enterovirus”. This sentence should be modified.

**Response:** We have changed enterovirus to human enterovirus.

**Comment 12:** Line 17: the sentence “In this study, a one step………” need to be rephrased.

**Response:** Thank the reviewer for this comment. We have revised the sentence for “In this study, a one-step and simple assay for EV71 virus without requirement of thermal cycling can be completed within 1 h in a single tube containing the mixture of buffer, primers, reverse transcriptase and DNA polymerase. In order to ensure its accuracy and reliable amplification, it is very important to optimize the reaction temperature.”

**Comment 13:** Line 24-27: “In the present study, the amplified product....non-specific products could not be detected in LAMP assay due to without digestion” this sentence is rather confusing and need to be clarified.

**Response:** Thank the reviewer for pointing this unclear description. We have modified the sentence for “In the present study, the specific products could be digested by HinfI and TaqI restriction enzymes to result in a series of bands in agarose gel electrophoresis;
in contrast, non-specific products could not be detected due to without digestion”.

**Comment 14:** In the result section “Diagnosis of 123 clinical specimens” : the sentences “Two positive samples detected by RT-LAMP assay were then inoculated into RD cells and the positive culture was identified by indirect immunofluorescence test:however, only one sample was verified as positive EV71 infection by PCR fluorescence probing assay. This result is different from the explanation in the discussion section on line 33-36. Please clarify and explain the result.

**Response:** Thank the reviewer for paying attention to this issue. We have modified the sentence for “Two positive samples detected by RT-LAMP assay but PCR fluorescence probing assay negative were then inoculated into RD cells and the positive culture was identified by indirect immunofluorescence test. However, only one sample was verified as the positive EV71 infection”