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OPEN PEER REVIEW

TaqMan Multiplex Real-time PCR Assay for Crimean-Congo Hemorrhagic Fever Virus Diagnosis Using Armored RNA Technology

Saleh Jamehdor, Negin Hosseinirouzbahani, Seyed javad Hoseinishokoh, Khodayar Ghorban, Ali Teimoori, Mohammad Gholami, Parisa Agahi, Ghazale Azizi, Mojgan Mohammadimehr

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About this report

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1 Original submission

2 comments · 28 Jan 2023 – 30 Jan 2023

Referee 1 Reviewer

MAJOR REVISION 28 Jan 2023

The manuscript entitled “TaqMan Multiplex Real-time PCR assay for diagnosis CCHF virus by using Armored RNA technology” aimed to design and optimize a TaqMan Multiplex RT-Real time-PCR for rapid and sensitive diagnosis of CCHFV. The main drawback of the manuscript lies in the method section. It needs to be revised for clarity.

Comments to the authors:
-Overall, the manuscript would benefit from English usage editing.

Introduction:
-In the following sentence, “the Nairovirus of the Nairoviridae family and Orthonairovirus genus”, please remove the Nairovirus.
-Please don’t use “CCHFV virus”. Correct form: CCHFV or CCHF virus.
-In line 70: What do the authors mean by using “nature viruses”? It seems that natural/wild type is a better choice.

-Please mention the latest prevalence of CCHF in Iran using previous systematic reviews or review articles.

-It would be recommended to describe and explain the basic theory of armed RNA technology in the introduction section.

Method:

-Line 91 (2.2.1 section): “A plasmid containing a 1751bp fragment of MS2 phage that was previously fabricated in pET-32a was used in this study”; please add the gene name, i.e., maturase.

-Line 93: Please add a description of “SOEing PCR (Splicing Overlap Extension)”.

-Line 118: The term “bacteria plate” is not correct; please change it to “pellet”.

-The authors should add a new subheading to the method section entitled “Vectors and bacterial strains” and then introduce the characteristics of the bacterial strains and vectors used [antibiotic-resistant marker, type of promoter (T7 promoter), etc.].

-Line 115: The authors mentioned induction by IPTG for protein expression, but where is the result of the SDS-PAGE?

Line 138: The authors should remove sensitivity from the following sentence, “Moreover, the specificity and sensitivity of primers and probes”, because they evaluate only specificity in this section.
-It would be recommended to outline the workflow in the figure. Or outline the process of sequences in Table 1 as a figure (in a way that they overlap).

Discussion:
The discussion is concise and needs to be improved.

Referee 2 Reviewer

MAJOR REVISION 30 Jan 2023

Comments on Manuscript ID:134188

The authors have submitted a "Methodology" manuscript describing a designed TaqMan Multiplex Real-time PCR method using a constructed Armored RNA-based positive control for CCHF virus diagnosis. Given the reappearance of CCHF and annual reports of CCHFV in different countries, including Iran, designing a more efficient and reliable diagnostic method can facilitate the early diagnosis of the virus. I would recommend providing some additional information to improve the manuscript. Please consider the following comments to strengthen your submission.

Comment 1: Given the extensive range of tests available, please be very specific about how this study addresses a gap in the field and how it differs from other existing studies. Thus, the authors should address different Real-time PCR methods already used by research groups and diagnostic laboratories around the world and in Iran. Also, please mention whether there are currently any PCR methods targeting more than 1 gene of CCHF virus.

Comment 2: There is no information regarding PCR efficiency. In addition, please consider including an amplification plot and standard curve graphs for each assay and mention the cut-off Ct value of each assay.
Comment 3: The manuscript has many typos and grammatical errors. It needs to be reviewed by an English-speaking editor to correct the syntax errors in the paper. In the references section, the journal titles have not been abbreviated for some references. Some references also need to be corrected, e.g., ref.14, ref.17, ref.18, and ref.26. This section should be standardized based on the journal's EndNote style.

Abstract
Comment 4. (line 10): Write “Method”.
Comment 5. (Line 10): Write “area”.
Comment 6. (Line13): Remove “and”.
Comment 7. (Line 20). Please reformulate “Preliminary specificity of the sequences (primers and probes) based on NCBI database algorithms as well as experimentally by using samples of healthy subjects (DNA and RNA) and based on another infectious panel”.
Comment 8. (Line 28): “101 copies”.

Background
Comment 9. (page 1, Line 4): Genus and species should be written in italics, NOT the virus family. Please correct “Nairoviridae family”.
Comment 10. (Page 2, Line 33): Armored technology was developed around 1983, so it’s not a new method. Please omit “recently”.
Comment 11. (Page2, Line 33): “Armored” technology.
Comment 12. (Page3, Line 3): “Armored” technology.

Method
Comment 13. (Page3, line 14): Please mention the accession number of the sequence used for blast. Is it a reference sequence? Also, mention the number of aligned sequences. Which virus genotypes were included for blast and primer design?
Comment 14. (Page3, line 27): Write “second strand” instead of “one strand”.
Comment 15. (Page3, line 30): Update “The primer set and one-strand DNAs” to “The primer set and one-strand DNA sequences”.
Comment 16. (Page3, line 31): “tables 1 and 2”.
Comment 17. (Page3, line 31): Reformulate the sentence “To this description that…”. It is unclear.
Comment 18. (Page4, line 19): “Release”.
Comment 19. (Page 5, line 19): At what RNA concentration was specificity assessed? Please mention it in the manuscript. What volume of samples was used for extraction?
Comment 20. (Page 5, line 20): Please reformulate “by preliminary blasted all primers and probes in the NCBI database”.

Comment 21. (Page 5, line 21): Using the word “sensitivity” is incorrect. The negative control and other infectious agents were tested to determine specificity, not sensitivity.
Comment 22. (Page 5, line 23): Report the number of samples and replicates tested for each infectious virus. Also, please clarify how these viruses were chosen for inclusion in the mentioned panel. How were these viruses provided and verified?
Comment 23. (Page 5, line 31): Remove the period before “Serial dilutions (104, 103, 102, 101, and”. Also, please correct the arrangement and powers of the serial dilutions to “10-1,10-2,10-3,10-4, and 10-5”.
Comment 24. (Page 6, line 1): It appears that “containing” should be removed.
Comment 25. (Page 6, line 1): The statement “From each dilution, three multiplex reactions were repeated for each segment and two segments and RNase P control gene with designed primers and probes (Table 2) and repeatability was assessed in three different days by different users and three times for each reaction also three times in a day” is too long and unclear. Please rewrite it clearly and in several shorter sentences.
Comment 26. (Page 6): In the legend of table 2, should this be “The sequences of primers and probes used in the study”?
Result
Comment 27. (Page 6, line 9): The statement “For confirmation of the production of the VLP stained by negative staining with 1% phosphotungstic acid (PTA), and then the VLPs were shown by TEM was performed respectively. The result virus-like particles with ~30 nm diameter has shown” was mentioned in the method section and could be streamlined, e.g., as “The result of VLP confirmation using TEM virus-like particles with ~30 nm diameter has shown (Fig 1) and confirmed that the MS2 was successfully produced.”
Comment 28. (Page 6): In the legend of figure 1, update the “l” segment to “L”.
Comment 29. (Page 6, line 17): The statement “After treatment of viral-like particles with Ribonuclease enzyme, the Viral-like particles were analyzed by gel agarose electrophoresis (1%) and stained with gel-red” is repetitive and could be streamlined.

Comment 30. (Page 7, line 5): Is the statement “After induction Bactria with 1 mM IPTG, Bactria cell disrupted with sonication and briefly centrifuged at 9000 Rpm for 20 minutes. The supernatant collected in a 50ml canonical tube and treated by 100 U/ml RNaseA and 1 U/ml DNase I for 4 h in 37°C in order to digestion plasmid and RNA respectively. The VLP precipitated by PEG6000 and suspended in TSE buffer. Lan A, ladder 100pb (BIORON Gmbh), lane B and C: mobility of VLP in 1% gel agarose between 800 to 900 bp.” part of the figure 2 legend? If so, please reformulate it. There is no need to refer to the method in the legend. Please avoid repeating the method in the results section.
Comment 31. (Page 7, line 12): It would be best to update the statement “ The MS2+CCHF virus Viral-like particles were amplified by Biorabit one-step RT-PCR kit according to the following program, 20 minutes at 50°C for convert RNA to Cdna and followed 2 min at 95°C for inactivated RT and activated polymerase, then 40 cycles 95°C at the 20s, 40s at 60°C and 30 s was amplified” to “The MS2+CCHF virus Viral-like particles were amplified by Biorabit one-step RT-PCR kit according to the manufacturer’s instruction. Reverse transcription and amplification were performed under the following conditions :20 minutes at 50°C for RNA to cDNA conversion and followed 2 min at 95°C for RT inactivation and polymerase activation, then followed by 40 cycles of amplification at 95°C for 20s, 60°C for 40s and 72°C for 30s”.
Comment 32. (Page 7, line 12): Is no final extension cycle at 72°C included in the thermocycler protocol?
Comment 33. (Page 7): Update the legend of figure 3 to “Agarose gel electrophoresis of RT-PCR amplification products of the MS2+CCHF virus viral-like particle, 1% agarose”.
Comment 34. (Page 7, line 23): Is the statement “The result showed that 135bp and 110bp of CCHF. . ..” part of the figure 3 legend? If so, it should be clarified and moved to the results of “PCR Amplification of MS2+CCHF virus Viral-like particles”.

Comment 35. (Page 7, head 3.3): “PCR Amplification of MS2+CCHF virus Viral-like particles” should be moved to the “method” section, and only the results should be reported in the “result” section.

Comment 36. (Page 7, line 28): The method and protocol should be mentioned in the Method section. Please move this information to the related part of the method section.
Comment 37. (Page 8, line 2): Write “containing 5µl” instead of “containing the 5µl”.
Comment 38. (Page 8, line 2 and 3): Write “nmol” instead of “Nanomole”.
Comment 39. (Page 8, line 4): Update “L, S” to “L and S”.
Comment 40. (Page 8, line 8): Update “Analytical specificity the assay” to “Analytical specificity of the assay”.
Comment 41. (Page 8, line 9): Should this be “influenza A and B”?
Comment 42. (Page 8, line 10): This would be better phrased as “so were suggested specificity to be 100% at a cut-off value …”.
Comment 43. (Page 8, line 12): Reformulate the statement “Reproducibility and LOD of designed primers and probes Multiplex RT-TaqMan Real-time PCR (probes and primers listed in the materials and methods section) were evaluated”, e.g., as “Reproducibility and LOD of Multiplex RT-TaqMan Real-time PCR were evaluated”.
Comment 44. (Page 8, line 14): To assess reproducibility, three independent analyses should be run in triplicate at each concentration. Reproducibility should then be evaluated using the intra-assay and inter-assay mean CT values and reported as the coefficient of variation.

Comment 45. (Page 8, line 16): What is meant by “After preparing the serial dilution from the VLPs RNA samples and performing real time RT-PCR. The results showed that the L and S segments primer are specific for the CCHF virus”? Were serial dilutions of VLP tested to evaluate specificity or sensitivity?
Comment 46. (Page 8, line 22): It would be better to replace the references with more up-to-date references.

Discussion: This should be entirely rewritten using STROBE guidance. The authors should summarize the main finding and make a greater effort to reference the existing literature reporting CCHF virus PCR detection methods. The strengths and limitations of the study should be outlined in the “discussion” part. The first paragraph provides the context of the study, e.g., geographic distribution, incubation, and clinical findings. It would be better placed in the introduction. However, some parts are mentioned in the introduction section and could be streamlined.
Comment 48: The results of this study need to be validated on clinical samples to ensure the efficacy of this test. The lack of access to clinical samples and to the appropriate biosafety level for CCHFV could also be added as a limitation, or validation could be suggested as a future study in the discussion section.

Comment 49. (Page 9, line 19): Mention which technique is meant in the statement “With this technique, we could detect viral genomes by the lowest LOD”. Also, is the cited reference correct?
Comment 50: The “Conclusion” section should provide a summary of the key discussion points in the manuscript.

2

Revision 1

AUTHORS' RESPONSE TO REVIEWERS

Authors' reply to reviewers

The next pages contain the authors' point-by-point reply to the comments of this revision round, as submitted with the revised manuscript. It is published as the authors wrote it.

Dear Reviewer 1,

We would like to appreciate your precious time in reviewing our paper and providing valuable comments. We have carefully considered the comments and tried our best to address every one of them. We hope the manuscript after careful revisions meet your approval. Hereby, We provide the point-by-point responses. All modifications in the manuscript have been highlighted.

Question 1. Overall the manuscript would benefit from English usage editing.

Answer: Thanks a lot for your comment. All of the manuscript corrected with a native.

Question 2. In the following sentence “the Nairovirus of the Nairoviridae family and Orthonairovirus genus” please remove the Nairovirus.

Answer: We removed it base of your comment.

Question 3. Please don’t use “CCHFV virus”. Correct form: CCHFV or CCHF virus.

Answer: We corrected it in all of the manuscript (CCHFV).

Question 4. In line 70: What do the authors mean by using “nature viruses”? It seems that the natural/Wild type is a better choice.

Answer: We corrected it based of your comment to "natural viruses".

Question 5. Please mention the latest prevalence of CCHF in Iran using previous systematic or review articles.

Answer: We added an update article with " Crimean-Congo Hemorrhagic Fever Virus in Central, Eastern, and South-eastern Asia" title (2023).

Question 6. It would be recommended to describe and explain the basic theory of armed RNA technology in the introduction section.

Answer: We described it in the manuscript (introduction section).

Question 7. Line 91 (2.2.1 section): “A plasmid containing a 1751bp fragment of MS2 phage that was previously fabricated in pET-32a was used in this study” please add the gene name, i.e. maturase

Answer: We added the name of the gene (maturase).

Question 8. Line 93: Please add a description about “SOEing PCR (Splicing Overlap Extension)”

Answer: We added this section and an reference with "Splicing by overlap extension PCR to obtain hybrid DNA products" title.

Question 9. Line 118: bacteria plate is not correct please edit it to “pellet”

Answer: We corrected it.

Question 10. The authors should add a new subheading to the method section entitled “Vectors and bacterial strains” and then introduce the characteristics of bacterial strains and vectors used [antibiotic-resistant marker, type of promoter (T7 promoter), etc].

Answer: We added this section “Vectors and bacterial strains”.

Question 11. Line 115: The authors mentioned induction by IPTG for protein expression, but where is the result of the SDS-PAGE?

Answer: Thanks a lot for your appreciate comment. We fabricated it with SDS-PAGE in another work and published it "Preparation and Evaluation of Ribonuclease-Resistant Viral HIV RNA Standards Based on Armored RNA Technology", so in this study we approved

VLPs (end products) with Electronic microscope, cloned segments with Sanger sequencing and the CCHFV *L* and *S* segments RNA with Real-time RT-PCR.

Question 12. Line 138: The authors should remove sensitivity from the following sentence "Moreover, the specificity and sensitivity of primers and probes" because they just evaluate the specificity in this section.

Answer: Thanks for your comment. We removed it.

Question 13. It would be recommended to outline the workflow in the figure. Or outline the process of sequences in Table 1 as a figure (in a way that they overlap).

Answer: We added it in Table 1 as a figure.

Question 14. The discussion is concise and needs to be improved.

Answer: We changed this section totally based on your comment.

Dear Reviewer 2,

We would like to appreciate your precious time in reviewing our paper and providing valuable comments. We have carefully considered the comments and tried our best to address every one of them. We hope the manuscript after careful revisions meet your approval. Hereby, We provide the point-by-point responses. All modifications in the manuscript have been highlighted.

Question 1. As the extensive range of tests available, please be very specific about why this study contributes to a gap in the field, why it is different compared to other existing studies. Thus, authors should address different Real-time PCR methods already used by Research groups and diagnosis laboratories around the world and Iran. Also, please mention if currently there is no PCR methods targeting more than 1 gene of CCHF virus?

Answer: We mentioned all of them to discussion and conclusion section and changed these sections totally.

Question 2. There is no information regarding PCR efficiency. In addition, please consider including an amplification plot and standard curve graphs for each assay and mention the cut-off Ct value of each assay.

Answer: Thanks a lot for your comment, it's a really good comment but this study is quantitative, so it doesn't have any cut-off Ct value and standard curve also PCR efficiency evaluation.

Question 3. The manuscript has many types and grammatical errors. It needs to be reviewed by an English-speaking editor to correct the syntax errors in the paper. In the references section, the journal titles have not been abbreviated for some references. Some references also need to be corrected e.g.: ref.14, ref.17, ref.18, and ref.26. This section should be standardized based on the end note style of the journal.

Answer: The manuscript has been improved with a native and correct the references. We removed ref. 14 and 17. Reference "Zeller H. Laboratory diagnosis of Crimean-Congo hemorrhagic fever. Crimean-Congo Hemorrhagic Fever: Springer; 2007. p. 233-43" is a

book chapter and it is correct, moreover reference " Jerome KR. Lennette's laboratory diagnosis of viral infections: CRC Press; 2016" is correct too.

Question 4. (line 10): Write “Method”.

Answer: We corrected it base of your comment.

Question 5. (Line 10): Write “area” .

Answer: We corrected it base of your comment.

Question 6. (Line13): Remove “and”.

Answer: We removed it base of your comment.

Question 7. (Line 20). Please reformulate” Preliminary specificity of the sequences (primers and probes) based on NCBI database algorithms as well as experimentally by using samples of healthy subjects (DNA and RNA) and based on another infectious panel”

Answer: Thank you for your valuable comment. We reformulated it.

Question 8. (Line 28): “101 copies”.

Answer: We corrected it base of your comment.

Question 9. (page 1, Line 4): Genus and species should be written italic NOT virus family. Please correct “Nairoviridae family”.

Answer: We corrected it base of your comment.

Question 10. (Page 2, Line 33): Armored technology was developed about 1983. So it's not a new method. Please omit "recently".

Answer: We corrected it base of your comment.

Question 11. (Page2, Line 33): "Armored" technology.

Answer: We corrected it base of your comment.

Question 12. (Page3, Line 3): "Armored" technology.

Answer: We corrected it base of your comment.

Question 13. (Page3, line 14): Please mention the accession number of sequence used for blast. Is it a reference sequence? also, mention the number of aligned sequences. Mention what genotypes of virus were included for blast and primer design?

Answer: We mentioned all of these base of your comment.

Question 14. (Page3, line 27): Write "second strand" instead of "one strand".

Answer: We corrected it base of your comment.

Question 15. (Page3, line 30): Update "The primer set and one-strand DNAs" to "The primer set and one-strand DNA sequences".

Answer: We corrected it base of your comment.

Question 16. (Page3, line 31): "tables 1 and 2".

Answer: We corrected it base of your comment.

Question 17. (Page3, line 31): Reformulate the sentence “To this description that…”. It is unclear.

Answer: We reformulated it.

Question 18. (Page4, line 19): “Release”

Answer: We corrected it base of your comment.

Question 19. (Page 5, line 19): With what concentration of RNA was the specificity assessed? Please mention it in the manuscript. What volume of samples were used for extraction?

Answer: These sections added to the manuscript based of your comment " Finally 200µl MS2 RNA genome of phages were extracted by RNA extraction kit based on instruction manual (GeneAll Biotechnology, Corea)" and " The monoplex and multiplex reactions were performed by using the Biorabit TaqMan master mix(4X CAPITAL™ 1-Step qRT-PCR Probe Master Mix) with the final reaction volume of 20µL containing 5µl buffer, 200 nmole of each forward and reverse primers, 100 nmole of each probe (Table 2), 5 µl of each Armored RNA contain a segment of *L* and *S* (totally 10 µl)".

Question 20. (Page 5, line 20): Please reformulate “by preliminary blasted all primers and probes in the NCBI database”

Answer: We reformulated it.

Question 21. (Page 5, line 21): Using the “sensitivity” word is incorrect. Negative control and other infectious agents tested to determine specificity not sensitivity.

Answer: Thanks for your appreciate comment. We corrected it.

Question 22. Comment 22. (Page 5, line 23): Report the number of samples and replicates tested for each infectious virus. Also, please clarify how were these viruses chosen to include in mentioned panel? How were these viruses provided and verified?

Answer: We clarified them in 3.4 section "Analytical specificity of the assay (with 10µl concentration) showed negative results for 20 healthy subjects and a panel of infectious agents (8 samples for each virus) with three repetition (COVID-19, Influenza A, and B, B19, HBV, HCV, HIV, HTLV-1, CMV, HSV1, VZV) that clarified in AJA university of medical science diagnostic laboratory, so clinical and analytical specificity were suggested to be 100% for CCHFV"**.**

Question 23. (Page 5, line 31): Remove the point before "Serial dilutions (104, 103, 102, 101, and". Also, please correct the arrangement and power of serial dilutions as "10-1,10-2,10-3,10-4, and 10-5"

Answer: We corrected it base of your comment.

Question 24. (Page 6, line 1): It appears "containing" should be removed.

Answer: We corrected it base of your comment.

Question 25. (Page 6, line 1): The statement "From each dilution, three multiplex reactions were repeated for each segment and two segments and RNase P control gene with designed primers and probes (Table 2) and repeatability was assessed in three different days by different users and three times for each reaction also three times in a day" is too long and not clear. Please rewrite it clearly and in several shorter sentences.

Answer: We rewrite it base of your comment.

Question 26. (Page 6): In the legend of table 2, should this be, "The sequences of primers and probes used in the study

Answer: We corrected it base of your comment.

Question 27. (Page 6, line 9): The statement of "For confirmation of the production of the VLP stained by negative staining with 1% phosphotungstic acid (PTA), and then the VLPs were shown by TEM was performed respectively. The result virus-like particles with ∼30 nm diameter has shown" was mentioned in method section and could be streamlined e.g. as "The result of VLP confirmation using TEM virus-like particles with ∼30 nm diameter has shown (Fig 1) and confirmed that the MS2 was successfully produced."

Answer: We rewrite it base of your comment.

Question 28. (Page 6): In the legend of figure 1, update “l “segment to “L”

Answer: We corrected it base of your comment.

Question 29. (Page 6, line 17): The statement of “After treatment of viral-like particles with Ribonuclease enzyme, the Viral-like particles were analyzed by gel agarose electrophoresis (1%) and stained with gel-red” is repetitive and could be streamlined.

Answer: We corrected it base of your comment.

Question 30. (Page 7, line 5): The statement “After induction Bactria with 1 mM IPTG, Bactria cell disrupted with sonication and briefly centrifuged at 9000 Rpm for 20 minutes. The supernatant collected in a 50ml canonical tube and treated by 100 U/ml RNaseA and 1 U/ml DNase I for 4 h in 37°C in order to digestion plasmid and RNA respectively. The VLP precipitated by PEG6000 and suspended in TSE buffer. Lan A, ladder 100pb (BIORON Gmbh), lane B and C: mobility of VLP in 1% gel agarose between 800 to 900 bp.” is part of figure 2 legend? If so, please reformulate it. No need to refer to method in the legend. Please consider to avoid repeat the method in results section.

Answer: We corrected it base of your comment.

Question 31. It would be best to update the statement “ The MS2+CCHF virus Viral-like particles were amplified by Biorabit one-step RT-PCR kit according to the following program, 20 minutes at 50°C for convert RNA to Cdna and followed 2 min at 95°C for inactivated RT and activated polymerase, then 40 cycles 95°C at the 20s, 40s at 60°C and 30 s was amplified” to “The MS2+CCHF virus Viral-like particles were amplified by Biorabit one-step RT-PCR kit according to the manufacturer’s instruction. Reverse transcription and amplification were performed under the following conditions :20 minutes at 50°C for RNA to cDNA conversion and followed 2 min at 95°C for RT inactivation and polymerase activation, then followed by 40 cycles of amplification at 95°C for 20s, 60°C for 40s and 72°C for 30s”.

Answer: We updated it base of your comment.

Question 32. (Page 7, line 12): Is no final extension cycle of 72°C included in the thermocycler protocol?

Answer: Thanks for your comment. As a matter of fact, Real-Time PCR doesn't need 72°C in final extension cycle.

Question 33. (Page 7): Update legend of figure 3 to "Agarose gel electrophoresis of RT-PCR amplification products of the MS2+CCHF virus viral-like particle, 1% agarose"

Answer: We updated it base of your comment.

Question 34. (Page 7, line 23): Is the statement of "The result showed that 135bp and 110bp of CCHF….." part of figure 3 legend? If so, it should be cleared and moved to results of "PCR Amplification of MS2+CCHF virus Viral-like particles".

Answer: We corrected it base of your comment.

Question 35. (Page 7, head 3.3):" PCR Amplification of MS2+CCHF virus Viral-like particles" should be moved to "method" section and only the results should be reported in the "result" section.

Answer: We moved it to "method" section.

Question 36. (Page 7, line 28): Method and protocol should be mentioned in Method section. Please consider it to related part in method section.

Answer: We considered it and evaluated all of the manuscript base of your comment.

Question 37. (Page 8, line 2): Write "containing 5µl" instead of "containing the 5µl"

Answer: We rewrite it base of your comment.

Question 38. (Page 8, line 2 and 3): Write "nmol" instead of "Nanomole"

Answer: We corrected it base of your comment.

Question 39. (Page 8, line 4): Update “L, S” to “L and S”.

Answer: We corrected it base of your comment.

Question 40. (Page 8, line 8): Update “Analytical specificity the assay” to “Analytical specificity of the assay”

Answer: We updated it base of your comment.

Question 41. (Page 8, line 9): Should this be “influenza A and B”?

Answer: These are influenza A and B and we corrected them.

Question 42. (Page 8, line 10): This would be better phrased as “so were suggested specificity to be 100% at a cut-off value …”.

Answer: Actually, it is a quantitative study, so based on the control samples, no negative sample was found and only the positive sample was positive, moreover we approved it bioinformatically and experimentally but this study needs more work to approve with patient samples and we added it in discussion section.

Question 43. (Page 8, line 12): Reformulate the statement of “Reproducibility and LOD of designed primers and probes Multiplex RT-TaqMan Real-time PCR (probes and primers listed in the materials and methods section) were evaluated”. E.g. as “Reproducibility and LOD of Multiplex RT-TaqMan Real-time PCR were evaluated”.

Answer: We reformulated it base of your comment.

Question 44. (Page 8, line 14): To assess the reproducibility, three independent analyses should be run in triplicates on each concentration. Then, evaluated using mean CT of intra-assay and inter-assay and reported as the coefficient of variation.

Answer: We corrected it base of your comment.

Question 45. (Page 8, line 16): What is meant by “After preparing the serial dilution from the VLPs RNA samples and performing real time RT-PCR. The results showed that the L and S segments primer are specific for the CCHF virus”? serial dilutions of VLP tested to evaluate specificity? or sensitivity?

Answer: We are so sorry for this fault. We corrected it base of your comment.

Question 46. (Page 8, line 22): It would be better replaced references with more updated references.

Answer: We replaced it base of your comment.

Discussion: This should be entirely rewritten taking benefit of STROBE guidance. The authors should summarize main finding, make a deeper effort to reference the existing literature reporting CCHF virus PCR detection methods. Strengths and limitations of the study should be outlined in the “discussion” part.

Answer: We totally replace it base of STROBE guidance.

Question 47. The first paragraph provides context of study e.g. geographic distribution, incubation, and clinical findings. It should be better placed in the introduction. Although, some parts are mentioned in introduction section and could be streamlined.

Answer: We transferred them to introduction section.

Question 48. The results of this study need to be validated on clinical samples to ensure the efficacy of this test. Due to not having access to clinical samples and not having the appropriate biosafety level of CCHFV, this could also be added as a limitation or suggested as future study in the discussion section.

Answer: We added this section in the end of discussion section.

Question 49. (Page 9, line 19): Mention what technique is meant in the statement “With this technique, we could detect viral genomes by the lowest LOD”. Also, does the referred reference is correct?

Answer: We are sorry for this error. We corrected it base of your comment.

Question 50. “Conclusion” section should provide a summary of the key discussion points in the manuscript.

Answer: We changed it totally base of your comment.

2 Revision 1

0 comments

No public comments in this round.

3

Revision 2

AUTHORS' RESPONSE TO REVIEWERS

Authors' reply to reviewers

The next pages contain the authors' point-by-point reply to the comments of this revision round, as submitted with the revised manuscript. It is published as the authors wrote it.

Dear Reviewer 1,

We would like to appreciate your precious time in reviewing our paper and providing valuable comments. We have carefully considered the comments and tried our best to address every one of them. We hope the manuscript after careful revisions meet your approval. Hereby, We provide the point-by-point responses. All modifications in the manuscript have been highlighted.

Question 1. Overall the manuscript would benefit from English usage editing.

Answer: Thanks a lot for your comment. All of the manuscript corrected with a native.

Question 2. In the following sentence “the Nairovirus of the Nairoviridae family and Orthonairovirus genus” please remove the Nairovirus.

Answer: We removed it based of your comment (line 35).

Question 3. Please don’t use “CCHFV virus”. Correct form: CCHFV or CCHF virus.

Answer: We corrected it in all of the manuscript (CCHFV).

Question 4. In line 70: What do the authors mean by using “nature viruses”? It seems that the natural/Wild type is a better choice.

Answer: We corrected it based of your comment to "natural viruses" (line 73).

Question 5. Please mention the latest prevalence of CCHF in Iran using previous systematic or review articles.

Answer: We added an update article with "Crimean-Congo Hemorrhagic Fever Virus in Central, Eastern, and South-eastern Asia" title (2023) (lines 75-77).

Question 6. It would be recommended to describe and explain the basic theory of armed RNA technology in the introduction section.

Answer: We described it in the manuscript (introduction section) (lines 69-73).

Question 7. Line 91 (2.2.1 section): “A plasmid containing a 1751bp fragment of MS2 phage that was previously fabricated in pET-32a was used in this study” please add the gene name, i.e. maturase

Answer: We added the name of the gene (maturase) (line 103).

Question 8. Line 93: Please add a description about “SOEing PCR (Splicing Overlap Extension)”

Answer: We added this section and an reference with "Splicing by overlap extension PCR to obtain hybrid DNA products" title (lines 104-107).

Question 9. Line 118: bacteria plate is not correct please edit it to “pellet”

Answer: We corrected it (line 133).

Question 10. The authors should add a new subheading to the method section entitled “Vectors and bacterial strains” and then introduce the characteristics of bacterial strains and vectors used [antibiotic-resistant marker, type of promoter (T7 promoter), etc].

Answer: We added this section "Vectores and bacterial strains" (lines 94-100).

Question 11. Line 115: The authors mentioned induction by IPTG for protein expression, but where is the result of the SDS-PAGE?

Answer: Thanks a lot for your appreciate comment. We fabricated it with SDS-PAGE in another work and published it "Preparation and Evaluation of Ribonuclease-Resistant Viral HIV RNA Standards Based on Armored RNA Technology", so in this study we approved

VLPs (end products) with Electronic microscope, cloned segments with Sanger sequencing and the CCHFV L and S segments RNA with Real-time RT-PCR.

Question 12. Line 138: The authors should remove sensitivity from the following sentence “Moreover, the specificity and sensitivity of primers and probes” because they just evaluate the specificity in this section.

Answer: Thanks for your comment. We removed it and rewrite all of the discussion section (lines 221-250).

Question 13. It would be recommended to outline the workflow in the figure. Or outline the process of sequences in Table 1 as a figure (in a way that they overlap).

Answer: We added it in Table 1 as a figure.

Question 14. The discussion is concise and needs to be improved.

Answer: We changed this section totally based on your comment.

Dear Reviewer 2,

We would like to appreciate your precious time in reviewing our paper and providing valuable comments. We have carefully considered the comments and tried our best to address every one of them. We hope the manuscript after careful revisions meet your approval. Hereby, We provide the point-by-point responses. All modifications in the manuscript have been highlighted.

Question 1. As the extensive range of tests available, please be very specific about why this study contributes to a gap in the field, why it is different compared to other existing studies. Thus, authors should address different Real-time PCR methods already used by Research groups and diagnosis laboratories around the world and Iran. Also, please mention if currently there is no PCR methods targeting more than 1 gene of CCHF virus?

Answer: We mentioned all of them to discussion and conclusion sections and changed these sections totally (lines 221-258).

Question 2. There is no information regarding PCR efficiency. In addition, please consider including an amplification plot and standard curve graphs for each assay and mention the cut-off Ct value of each assay.

Answer: Thanks a lot for your comment, it’s a really good comment but this study is quantitative, so it doesn’t have any cut-off Ct value and standard curve also PCR efficiency evaluation.

Question 3. The manuscript has many types and grammatical errors. It needs to be reviewed by an English-speaking editor to correct the syntax errors in the paper. In the references section, the journal titles have not been abbreviated for some references. Some references also need to be corrected e.g.: ref.14, ref.17, ref.18, and ref.26. This section should be standardized based on the end note style of the journal.

Answer: The manuscript has been improved with a native and we corrected the references. We removed ref. 14 and 17. Reference "Zeller H. Laboratory diagnosis of Crimean-Congo hemorrhagic fever. Crimean-Congo Hemorrhagic Fever: Springer; 2007. p. 233-43" is a book chapter and it is correct, moreover reference " Jerome KR. Lennette's laboratory diagnosis of viral infections: CRC Press; 2016" is correct too.

Question 4. (line 10): Write “Method”.

Answer: We corrected it based of your comment (line 9).

Question 5. (Line 10): Write “area” .

Answer: We corrected it based of your comment (line 10).

Question 6. (Line13): Remove “and”.

Answer: We removed it based of your comment (line 13).

Question 7. (Line 20). Please reformulate” Preliminary specificity of the sequences (primers and probes) based on NCBI database algorithms as well as experimentally by using samples of healthy subjects (DNA and RNA) and based on another infectious panel”

Answer: Thank you for your valuable comment. We reformulated it (lines 20-22).

Question 8. (Line 28): “101 copies”.

Answer: We corrected it based of your comment (line 27).

Question 9. (page 1, Line 4): Genus and species should be written italic NOT virus family. Please correct “Nairoviridae family”.

Answer: We corrected it based of your comment (line 35).

Question 10. (Page 2, Line 33): Armored technology was developed about 1983. So it’s not a new method. Please omit “recently”.

Answer: We removed it based of your comment (line 69).

Question 11. (Page2, Line 33): “Armored” technology.

Answer: We corrected it based of your comment (lines 175 and 247).

Question 12. (Page3, Line 3): “Armored” technology.

Answer: We corrected it based of your comment (lines 175 and 247).

Question 13. (Page3, line 14): Please mention the accession number of sequence used for blast. Is it a reference sequence? also, mention the number of aligned sequences. Mention what genotypes of virus were included for blast and primer design?

Answer: We mentioned all of these based of your comment (lines 9 and 85-87).

Question 14. (Page3, line 27): Write “second strand” instead of “one strand”.

Answer: We corrected it based of your comment (line 108).

Question 15. (Page3, line 30): Update “The primer set and one-strand DNAs” to “The primer set and one-strand DNA sequences”.

Answer: We corrected it based of your comment (line 111).

Question 16. (Page3, line 31): “tables 1 and 2”.

Answer: We corrected it based of your comment (line112).

Question 17. (Page3, line 31): Reformulate the sentence “To this description that…”. It is unclear.

Answer: We reformulated it (lines 112-114).

Question 18. (Page4, line 19): “Release”

Answer: We corrected it based of your comment (line 131).

Question 19. (Page 5, line 19): With what concentration of RNA was the specificity assessed? Please mention it in the manuscript. What volume of samples were used for extraction?

Answer: These sections added to the manuscript based of your comment " Finally 200µl MS2 RNA genome of phages were extracted by RNA extraction kit based on instruction manual (GeneAll Biotechnology, Corea)" [lines 134-136] and " The monoplex and multiplex reactions were performed by using the Biorabit TaqMan master mix(4X CAPITAL™ 1-Step qRT-PCR Probe Master Mix) with the final reaction volume of 20µL containing 5µl buffer, 200 nmole of each forward and reverse primers, 100 nmole of each probe (Table 2), 5 µl of each Armored RNA contain a segment of L and S (totally 10 µl)" [lines 172-176].

Question 20. (Page 5, line 20): Please reformulate “by preliminary blasted all primers and probes in the NCBI database”

Answer: We reformulated it (lines 153-154).

Question 21. (Page 5, line 21): Using the “sensitivity” word is incorrect. Negative control and other infectious agents tested to determine specificity not sensitivity.

Answer: Thanks for your appreciate comment. We removed it in all of the manuscript.

Question 22. Comment 22. (Page 5, line 23): Report the number of samples and replicates tested for each infectious virus. Also, please clarify how were these viruses chosen to include in mentioned panel? How were these viruses provided and verified?

Answer: We clarified them in 3.4 section "Analytical specificity of the assay (with 10µl concentration) showed negative results for 20 healthy subjects and a panel of infectious agents (8 samples for each virus) with three repetition (COVID-19, Influenza A, and B, B19, HBV, HCV, HIV, HTLV-1, CMV, HSV1, VZV) that clarified in AJA university of medical

science diagnostic laboratory, so clinical and analytical specificity were suggested to be 100% for CCHFV" (lines 206-210).

Question 23. (Page 5, line 31): Remove the point before “Serial dilutions (104, 103, 102, 101, and”. Also, please correct the arrangement and power of serial dilutions as “10-1,10-2,10-3,10-4, and 10-5”

Answer: We corrected it based of your comment (lines 164-165).

Question 24. (Page 6, line 1): It appears “containing” should be removed.

Answer: We corrected it based of your comment (line 164).

Question 25. (Page 6, line 1): The statement “From each dilution, three multiplex reactions were repeated for each segment and two segments and RNase P control gene with designed primers and probes (Table 2) and repeatability was assessed in three different days by different users and three times for each reaction also three times in a day” is too long and not clear. Please rewrite it clearly and in several shorter sentences.

Answer: We rewrite it based of your comment (lines 165-167).

Question 26. (Page 6): In the legend of table 2, should this be, “The sequences of primers and probes used in the study

Answer: We corrected it based of your comment (line 168).

Question 27. (Page 6, line 9): The statement of “For confirmation of the production of the VLP stained by negative staining with 1% phosphotungstic acid (PTA), and then the VLPs were shown by TEM was performed respectively. The result virus-like particles with ∼30 nm diameter has shown” was mentioned in method section and could be streamlined e.g. as “The result of VLP confirmation using TEM virus-like particles with ∼30 nm diameter has shown (Fig 1) and confirmed that the MS2 was successfully produced.”

Answer: We rewrite it based of your comment (lines 182-183).

Question 28. (Page 6): In the legend of figure 1, update “l “segment to “L”

Answer: We updated it based of your comment (line 185).

Question 29. (Page 6, line 17): The statement of “After treatment of viral-like particles with Ribonuclease enzyme, the Viral-like particles were analyzed by gel agarose electrophoresis (1%) and stained with gel-red” is repetitive and could be streamlined.

Answer: We corrected it based of your comment (lines 188-189).

Question 30. (Page 7, line 5): The statement “After induction Bactria with 1 mM IPTG, Bactria cell disrupted with sonication and briefly centrifuged at 9000 Rpm for 20 minutes. The supernatant collected in a 50ml canonical tube and treated by 100 U/ml RNaseA and 1 U/ml DNase I for 4 h in 37°C in order to digestion plasmid and RNA respectively. The VLP precipitated by PEG6000 and suspended in TSE buffer. Lan A, ladder 100pb (BIORON Gmbh), lane B and C: mobility of VLP in 1% gel agarose between 800 to 900 bp.” is part of figure 2 legend? If so, please reformulate it. No need to refer to method in the legend. Please consider to avoid repeat the method in results section.

Answer: We corrected it based of your comment (lines 193-194).

Question 31. It would be best to update the statement “ The MS2+CCHF virus Viral-like particles were amplified by Biorabit one-step RT-PCR kit according to the following program, 20 minutes at 50°C for convert RNA to Cdna and followed 2 min at 95°C for inactivated RT and activated polymerase, then 40 cycles 95°C at the 20s, 40s at 60°C and 30 s was amplified” to “The MS2+CCHF virus Viral-like particles were amplified by Biorabit one-step RT-PCR kit according to the manufacturer’s instruction. Reverse transcription and amplification were performed under the following conditions :20 minutes at 50°C for RNA to cDNA conversion and followed 2 min at 95°C for RT inactivation and polymerase activation, then followed by 40 cycles of amplification at 95°C for 20s, 60°C for 40s and 72°C for 30s”.

Answer: We updated it based of your comment (lines 171-178).

Question 32. (Page 7, line 12): Is no final extension cycle of 72°C included in the thermocycler protocol?

Answer: Thanks for your comment. As a matter of fact, Real-Time PCR doesn't need 72°C in final extension cycle.

Question 33. (Page 7): Update legend of figure 3 to "Agarose gel electrophoresis of RT-PCR amplification products of the MS2+CCHF virus viral-like particle, 1% agarose"

Answer: We updated it based of your comment (lines 201-202).

Question 34. (Page 7, line 23): Is the statement of "The result showed that 135bp and 110bp of CCHF….." part of figure 3 legend? If so, it should be cleared and moved to results of "PCR Amplification of MS2+CCHF virus Viral-like particles".

Answer: We corrected it based of your comment (lines 201-202).

Question 35. (Page 7, head 3.3):" PCR Amplification of MS2+CCHF virus Viral-like particles" should be moved to "method" section and only the results should be reported in the "result" section.

Answer: We moved it to "method" section (lines 196-199).

Question 36. (Page 7, line 28): Method and protocol should be mentioned in Method section. Please consider it to related part in method section.

Answer: We considered it and evaluated all of the manuscript based of your comment.

Question 37. (Page 8, line 2): Write "containing 5µl" instead of "containing the 5µl"

Answer: We rewrite it based of your comment (line 174).

Question 38. (Page 8, line 2 and 3): Write "nmol" instead of "Nanomole"

Answer: We corrected it based of your comment (line 175).

Question 39. (Page 8, line 4): Update “L, S” to “L and S”.

Answer: We corrected it based of your comment (line 217).

Question 40. (Page 8, line 8): Update “Analytical specificity the assay” to “Analytical specificity of the assay”

Answer: We updated it based of your comment (line 206).

Question 41. (Page 8, line 9): Should this be “influenza A and B”?

Answer: These are influenza A and B and we corrected those (lines 156-157).

Question 42. (Page 8, line 10): This would be better phrased as “so were suggested specificity to be 100% at a cut-off value …”.

Answer: Actually, it is a quantitative study, so based on the control samples, no negative sample was found and only the positive sample was positive, moreover we approved it bioinformatically and experimentally but this study needs more work to approve with patient samples and we added it in discussion section (lines 249-250).

Question 43. (Page 8, line 12): Reformulate the statement of “Reproducibility and LOD of designed primers and probes Multiplex RT-TaqMan Real-time PCR (probes and primers listed in the materials and methods section) were evaluated”. E.g. as “Reproducibility and LOD of Multiplex RT-TaqMan Real-time PCR were evaluated”.

Answer: We reformulated it based of your comment (lines 213-214).

Question 44. (Page 8, line 14): To assess the reproducibility, three independent analyses should be run in triplicates on each concentration. Then, evaluated using mean CT of intra-assay and inter-assay and reported as the coefficient of variation.

Answer: We corrected it based of your comment (lines 213-214).

Question 45. (Page 8, line 16): What is meant by “After preparing the serial dilution from the VLPs RNA samples and performing real time RT-PCR. The results showed that the L and S segments primer are specific for the CCHF virus”? serial dilutions of VLP tested to evaluate specificity? or sensitivity?

Answer: We are so sorry for this fault. We corrected it based of your comment (line 219).

Question 46. (Page 8, line 22): It would be better replaced references with more updated references.

Answer: We replaced it base of your comment.

Discussion: This should be entirely rewritten taking benefit of STROBE guidance. The authors should summarize main finding, make a deeper effort to reference the existing literature reporting CCHF virus PCR detection methods. Strengths and limitations of the study should be outlined in the “discussion” part.

Answer: We totally replace it base of STROBE guidance (lines 221-250).

Question 47. The first paragraph provides context of study e.g. geographic distribution, incubation, and clinical findings. It should be better placed in the introduction. Although, some parts are mentioned in introduction section and could be streamlined.

Answer: We transferred them to introduction section (lines 73-75).

Question 48. The results of this study need to be validated on clinical samples to ensure the efficacy of this test. Due to not having access to clinical samples and not having the appropriate biosafety level of CCHFV, this could also be added as a limitation or suggested as future study in the discussion section.

Answer: We added this section in the end of discussion section (lines 249-250).

Question 49. (Page 9, line 19): Mention what technique is meant in the statement “With this technique, we could detect viral genomes by the lowest LOD”. Also, does the referred reference is correct?

Answer: We are sorry for this error. We corrected it based of your comment (lines 242-244).

Question 50. “Conclusion” section should provide a summary of the key discussion points in the manuscript.

Answer: We changed it totally base of your comment (lines 253-258).

3 Revision 2

0 comments

No public comments in this round.