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OPEN PEER REVIEW

Detection of Hepatitis B Virus Genome with Mutations Outside the Major Hydrophilic Region in the Surface Antigen Isolated from Patients with Coexisting HBsAg and Anti-HBs

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About this report

This open peer review (OPR) report shows the public comments made on this article during peer review, by the reviewers, the associate editor and the editor-in-chief, for every revision round, together with the authors' replies. Comments appear only with the consent of the people who wrote them. Reviewers who chose to sign their report are named; the others are shown as "Referee". Comments are edited for language only, and confidential remarks to the editors are not included.

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1 Original submission

1 comment · 5 Nov 2022

Editor-in-Chief

MAJOR REVISION 5 Nov 2022

Dear Authors,

Please follow the peer-review comments and submit a revision.

Regards,
EIC

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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 Associate Editor,

Hepatitis Monthly

Thank you for taking the time to review the manuscript. Your very constructive and valuable comments and questions will definitely improve the manuscript. I have included some of them in the manuscript and have tried to answer some of your questions in this letter. However, this project was not epidemiologically conceived from the beginning. Because of this, I am unable to respond to some of the comments you and other reviewers have made. Based on the reviewers' comments and questions, we changed the title of the manuscript to cover and clarify the aim of the study.

Comments and questions

Reviewer: Include a STROBE flowchart for patient selection.

Response: a STROBE flowchart was included based on available data.

Reviewer: Could the authors differentiate between chronic HBV cases who had active and inactive disease?

Response: As this was not part of our project, we did not perform tests such as viral load or biochemical tests to indicate patient status.

Reviewer: Line 35> small surface antigen (HBsAg) is not correct term.

Response: Corrected

Reviewer: Were the sequences deposited in Genk Bank? I suggest including the codes.

Response: As you suggested, I have submitted the sequence to GenBank (Submission ID 2647746), but I am still waiting for a response from GenBank to get the accession number.

Reviewer: Have you repeated the antibody (anti-HBs) titer with another kit?

Response: No. We did not.

Reviewer: What's the inclusion and exclusion criteria? (such as coinfection with another viral hepatitis).

Response: The methods section included inclusion and exclusion criteria in the revised version of the manuscript. In addition, some new sentences have been added to the methods section.

Reviewer: What's the necessity and differences and between two set of standard PCR? (Section 2.3 and section 2.4).

Response: In Section 2.3, a PCR was performed for the first time to confirm whether HBsAg-positive patients were also DNA-positive with a diagnostic kit. In Section 2.4, 83 patients with chronic hepatitis B virus infection were tested for detecting the complete sequence of the HBsAg gene and then for specific regions of MHR using specific primers.

Reviewer: Please include gel agarose image in the manuscript.

Response: A revised version of the manuscript included photographs of agarose gels.

Reviewer: HBsAg amplification indicating the band size of 681bp. 541 bp fragment of HBsAg is truncated molecule for full length analysis of S Ag.

Response: In this study, we obtained three different fragments of HBsAg. A 353 bp fragment using commercial kits to confirm the presence of HBV DNA in clinical specimens. A 927 bp fragment of the complete HBsAg sequence and a 541 bp fragment of the MHR containing "a" determinant.

Reviewer: HBV genotypes should be determined by sequence and molecular evolutionary analysis. Reference HBV sequences should be aligned by CLUSTALX, and genetic distances estimated with the several parameter methods in the Hepatitis Virus Database (http://s2as02.genes.nig.ac.jp/).

Response: As mentioned earlier, this project was not conceived from an epidemiological point of view. In addition, previous studies found that more than 99% of HBV in our area were type D. Therefore, we did not consider it necessary to genotype the isolates.

Reviewer: Based on obtained distances, phylogenetic trees should be constructed by the neighbor-joining method with the mid-point rooting option. To confirm the reliability of the phylogenetic trees, bootstrap resampling tests should be performed 1000 times for analysis by appropriate software.

Response: In this project, we wanted to investigate whether there is any change in the nucleotide sequence of HBV DNA isolated from patients with HBsAg and anti-HBs coexistence. We have yet to study the epidemiological aspects of the isolates and their relationships to other isolates in our region or worldwide.

Reviewer: The viral load of all HBV samples should be reported.

Response: I do not think it is necessary for the study. We did not perform real-time PCR to quantify the virus.

Reviewer: The language needs a comprehensive consideration. Please set the whole text of revised version to be checked by a professional language editor too regarding its grammar, spelling and etc. Some typographic error (line 113> Hydrooathy).

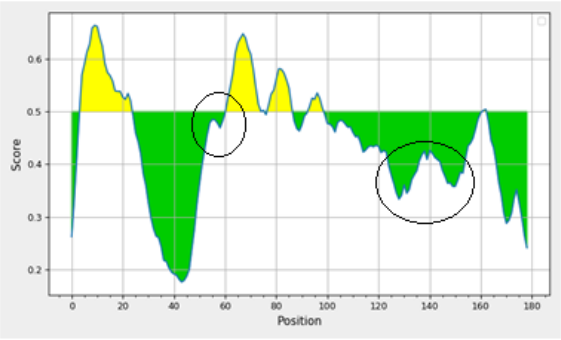
Response: The manuscript was revised for grammar and spelling using premium Grammarly software and edited by a native English speaker.

Reviewer: All sequences should be aligned with Iranian HBV HBsAg sequences. Also, all sequences should be aligned with HBsAg with accession number GQ183486-s (nearest HBsAg similar to Iranian isolates).

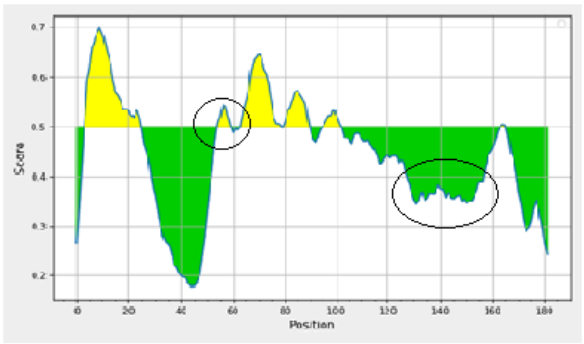
Response: All sequences have been aligned to the one suggested by the reviewer (GQ183486-1). However, between 97% and 99% similarity was found between the nucleotide sequences of the isolates and that proposed by the reviewer.

Reviewer: Please clarify the reasons for analysis of Hydropathy index for HBsAg? A hydrophilicity plot is a quantitative analysis of the degree of hydrophobicity or hydrophilicity of amino acids of a protein. It is used to characterize or identify possible structure or domains of a protein. Identification of topogenic sequences requires a way to scan sequence databases for segments that are sufficiently hydrophobic to be either a signal sequence or a transmembrane anchor sequence. Topogenic sequences can often be identified with the aid of computer programs that generate a Hydropathy profile for the protein of interest.

Response: We wanted to determine whether changing the nucleotide sequences (deletion, insertion or substitution) of HBsAg would change the amino acid of the protein and consequently the antigenic determinant of the protein. Because replacing one amino acid with another can affect the shape of a peptide's epitopes, which can affect the immunological response. Based on the reviewers' suggestion, we examine the HBsAg hydropathic profile and epitope predictions using the Immune Epitope Database Analysis Resource (http://tools.iedb.org/main/). Below are the results. I hope this will satisfy the reviewer. As has been shown, there are some changes in the epitope sequences and some small changes in the phenotypic change of the antigens. However, there is no evidence that this change could affect the antigen-antibody reaction



No.	Start	End	Peptide	Length
1	5	24	NSQSPTSNHSPTSCPPTCPG	20
2	62	74	LIPGSSTTSTGPC	13
3	76	76	T	1
4	78	87	TTPAQGTSMF	10
5	94	99	KPSDGN	6
6	162	163	LS	2



No.	Start	End	Peptide	Length
1	4	25	GQNSQSPTSNHSPTSCPPTCPG	22
2	55	60	QGMETL	6
3	65	79	LIPGSSTTSTGPCRT	15
4	81	90	MTTAQGTSMY	10
5	96	102	TKPSDGN	7
6	164	166	ILS	3

Left: HBV genome reference GQ183486

Right: Our isolate: patient No.4

Reviewer: What measures have been taken to prevent carry over contamination? Regarding high risk of false positive in nested PCR sets. Please note that all sequence contains I198T and R207S mutations.

Response: The measures have been included in the methods section in the revised version of the manuscript.

Reviewer: Authors should be more discuss about the detected mutations and HBsAg-HBsAb coexistence.

Response: A new paragraph has been added to the discussion section regarding some possible reasons for the coexistence of HBsAg and anti-HBs antibodies.

Reviewer: What's the immune epitopes changes (B cell, Th cells and CTL) affected by mutations in the inside and outside of MHR.

Response: No significant changes were found in the immune epitopes of Th and CTL based on the Immune Epitope Database Analysis Resource as mentioned above. However, changes in one nucleotide affected the B-cell-dependent epitopes as mentioned above. We have not examined these effects.

2 Revision 1

1 comment · 19 Feb 2023

Editor-in-Chief

MAJOR REVISION 19 Feb 2023

Dear Authors,

Please follow the peer-review comments and submit a revision.

Regards,
EIC

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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 Associate Editor,

Hepatitis Monthly

Thank you for taking the time to review the manuscript. Your very constructive and valuable comments and questions will definitely improve the manuscript. I have included them all in the manuscript and in the supplemental data file and have tried to answer some of your questions in this letter. I hope the answers are clear enough to satisfy the reviewers. All new changes have been highlighted in green in the revised manuscript.

Thank you.

Comments and questions

1. Reviewer: Although the substance of the paper is worthy to be considered for publication, I can't proceed further for the review process due to the undisclosed information about the author sequence. You may resubmit after this issue is cleared accordingly. Meanwhile, please kindly check the annotated manuscript. You should be insert the Accession numbers of sequences or attached the Notepad of the file of all sequences as supplementary data.

Response: All seven DNA nucleotide sequences have been included in the supplementary data.

2. Reviewer: In the section 2.3> line 110> to the best of my information, CinnaGen company does not have a standard PCR-based kit (requirement to Gel electrophoresis) for HBV diagnosis. All kits are the based on Real-time PCR method. Please include the lot No. of kit.

Response: The catalog number is given in the methods section to answer this question and confirm the tests. Also attached is a copy of the kit instructions for further confirmation. Part of this project was completed a few years ago, and its continuation has been completed in recent years. For this reason, the first part of the project was carried out using the available kits (CinnaGen Company). Although currently, the real-time PCR method has replaced the previous kits.

3. **Reviewer:** As a mentioned in Revision 1, you have several HBsAg sequences. You should be report the HBV subtypes and draw the phylogenetic tree.

Response: Since only seven samples were sequenced and the prevalence of HBV genotype D1 is predominant in Iran, these sequences were compared with reference numbers related to HBV genotype D in GenBank. Following the sequencing results and input, we examined these seven specimens with HBV genotype D and drew a phylogenetic tree, which we included in the supplementary data section. I hope this answer convinces the reviewer.

4. **Reviewer:** As a mentioned in Revision 1, all sequence contains I198T and R207S mutations. Please clarify this finding. Contamination is very likely to occur.

Response: Since a negative control was included in each PCR run, the presence of contamination is unlikely.

5. **Reviewer:**Please include gel agarose image for 353 bp band in the manuscript.

Response: A sample of the agarose gel electrophoresis for the **353** bp band is included in Figure 2C.

3 Revision 2

2 comments · 20 Mar 2023 – 20 Mar 2023

Associate Editor

MINOR REVISION 20 Mar 2023

The authors have addressed the peer-review comments; however, there are a few minor corrections:

1. The authors should refer to all the supplementary materials in the manuscript text.

2. The results of phylogenetic analysis and genotyping/serotyping should be added to the results section.

3. The supplementary materials should be well organized and numbered to let authors track them through the main text.

Thanks

Editor-in-Chief

MINOR REVISION 20 Mar 2023

Dear Authors,

Please follow the peer-review comments and submit a revision.

Regards,
EIC

4

Revision 3

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 Associate Editor,

I want to express my gratitude for taking the time to review our manuscript for Hepatitis Monthly. Your feedback has been incredibly valuable, and I am grateful for the constructive comments and questions you provided. I have incorporated all of your suggestions into the manuscript and the supplemental data file and attempted to address your questions in this letter. I hope my responses are clear enough to satisfy the reviewers.

I want to assure you that we have taken your comments seriously and have made changes accordingly. All of the new changes we have made to the manuscript have been highlighted in green in the revised version. We appreciate your time and effort in reviewing our work, and we hope you find the revised manuscript much improved.

Thank you once again for your assistance in improving our manuscript. We look forward to hearing from you soon.

Sincerely,

Corresponding author

Reviewers' Comments:

> Associate Editor's Comments:

Associate Editor 1:
Authors have addressed the peer-review comments, however, there are few minor corrections:

1. ***Authors should refer to all the supplementary materials in the manuscript text.***

Authors' response: Done.

2. ***The results of phylogenetic analysis and genotyping/serotyping should be added to the results section.***

Authors' response: Due to the limited sample size and the suboptimal visual quality of the phylogenetic tree, we have provided the results of the phylogenetic analysis in the supplementary materials (**Fig 1 S**). Nonetheless, I have briefly elucidated in the main text the rationale for presenting the phylogenetic analysis outcomes as supplementary files instead of incorporating them in the main text.

3. ***The supplementary materials should be well-organized and numbered to let authors track them through the main text.***

Authors' response: Done

4 Revision 3

1 comment · 27 Mar 2023

Editor-in-Chief

ACCEPT 27 Mar 2023

Dear Authors,

I am delighted to inform you that your manuscript is accepted for publication in Hepatitis Monthly journal.

Regards,
EIC