Faculty of Biotechnology and Biomolecular Sciences

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Dear Dr. Ma,

Ms. Ref. No.: 6628
Title: Phage display creates innovative applications to combat hepatitis B virus

Many thanks for informing us to revise our manuscript based on the comments from three reviewers and an editorial member. We were very happy to receive such a positive review and appreciated the comments given and have made some changes that we hope address all the points raised. The changes in the revised manuscript are highlighted by using blue coloured text. Responses to the comments are as follows. We hope that you will find the revised manuscript satisfactory.

Reviewer #02538313:

General comments:

The authors have provided a comprehensive and excellent review of the literature with regard to various applications of phage display technology and have specifically included literature review on HBV. There are good numbers of references from the beginning days of using phage technology up to the past several years. And of course, the authors have very well used their long term experience using this technique. The reference 169 is especially very interesting. And the tables are very well prepared. In order to prevent ambiguity, it is especially valuable that the authors have summarized each section before the beginning of a new section on application of phage display. There are also sections on drawbacks of the use of phage display for certain applications.

Response: Many thanks for you kind words. We appreciate the effort and time you spent to review our manuscript. We find your comments are very constructive and useful to improve the quality of this manuscript.

Specific comments/questions:

Comment 1: There were minor spelling, grammatical corrections that are highlighted in gray. Please note and see if the changed words still convey your understanding of the literature.

Response: The spelling and grammatical errors highlighted in gray have been rectified. The changes made are as follows:

i) Page 4, line 29: We thank the reviewer for deleting the "comma" after the word "activities".
ii) Page 5, line 22: A reference, Voet *et al* (2006) has been added in the text and also the list of reference.
iii) Page 6, lines 3-4: "At this writing" has been replaced with "At the time of writing this manuscript".
iv) Page 13, lines 21: "the individual becomes a chronic carrier" has been replaced with "these individuals become chronic carriers"
v) Page 14, line 8: "doubting the" has now been replaced with "doubt in".
vi) Page 15, line 28: The comma after the word "technology" has been deleted.
vii) Page 17, line 29: "that resistant" has been replaced with "that are resistant".
viii) Page 17, line 30: "thread" has been replaced with "threat".
ix) Page 18, lines 8 and 20: "druggable target" is replaced with "targeted protein".
x) Page 19, line 1: "interfere" has been changed to "interfere with".
xi) Page 19, line 8: Comma has been deleted after the word "infectivity".
xii) Page 19, lines 18-19: "relative simpler and straightforward" has been changed to "relatively simpler and more straightforward".
xiii) Page 21, line 12: "been" has been replaced with "to be".
xiv) Page 21, lines 19-21: The sentence has been replaced with "Up to 85% of the isolated phages are associated with S-HBsAg in relative to only 45% of the soluble VHHs expressed intracellularly."
xv) Page 22, line 21: The typographical error "polypeptides" has been rectified.
xvi) Page 23, line 25: "comma" has been replaced with "semicolon".
xvii) Page 23, line 28: "ampliphathic" has been replaced with "amphipathic".
xviii) Page 24, line 26: "interact" has been changed to "interacts".
xix) Page 25, lines 21 and 22: "tedious, laborious" has been replaced with "tedious and laborious techniques".
xx) Page 27, line 19: A reference, Berman *et al* (2000) has been added.
xxi) Page 28, line 25: "e" has been deleted from "Several e epitope mappings".
xxii) Page 30, line 12: "bearing the with a motif" has now been replaced with "bearing the motif".
xxiii) Page 30, line 18: "contains" has been replaced with "contained". "match" has been changed to "matched".
xxiv) Page 31, line 11: "of " has been deleted.
xxv) Page 31, line 12: comma has been replaced with semicolon.
xxvi) Page 31, line 12: "Besides" has been removed.

Comment 2: The authors have noted the importance of HIV-HBV coinfection in the abstract and introduction. One may wonder if there is any unpublished introductory work by the authors or other authors in this area that worth mentioning in the conclusion as well.

Response: Sorry, we do not have any unpublished work on the application of phage display in HIV-HBV coinfection issue. Neither do we know our collaborators working on bacteriophage are addressing this issue. We think T4 phage can be used to display HBV and HIV immunodominant regions on a single phage particle by exploiting two different capsid proteins. This information is stated in the manuscript (page 10, lines 9-13).

Comment 3: Under introduction: "The virion is enveloped by a lipid bilayer derived from the liver cell membranes". Although this sentence is generally right, I have usually seen it in references as" The virion is enveloped by a lipid bilayer derived from host cell membranes". (Volker Bruss. Envelopment of the hepatitis B virus nucleocapsid. Virus Research, 2004, 106:199-209).

Response: Thanks for the comment. We have now replaced "liver cell" with "host cell" in the revised manuscript (page 4, line 23). The reference has been cited in the text and added in the list of references.

Comment 4: Phage display technique has been experimentally used for design of HBV vaccine in late 1990s and early 2000s; the authors have cited these articles and mentioned a few in late 2000s:
a) The main question that arises is that why a vaccine for HBV using phage display has not been worked on more vigorously? In what ways the use of Phage display techniques for vaccine design can be improved?

Response: In general, vaccine productions for human are monopolized by giant pharmaceutical companies because the cost of clinical trials and product development could amount to about US$300 million or higher. Currently, the recombinant vaccines based upon HBsAg produced in yeast are very effective in preventing HBV infection and in reducing the incidence of liver cancer. These vaccines have been licensed to giant pharmaceutical companies which have spent tremendous amount of money and time to develop and market the products. We don't think the companies would spend the same amount of money and time to develop an equally effective vaccine based upon phage display. Phage display will only be given priority by the companies if this technology can reduce the cost of production drastically. For a start, to test the phage display techniques for vaccine production, we think new biotechnology companies can develop vaccines for animals which do not require a very stringent clinical trial compared to human vaccines. Some of this information has been summarized in the conclusion in question format (page 33, lines 15 to 19) to stimulate scientists and entrepreneurs to take the challenge.

b) What are the drawbacks of using this methodology in vaccine design? The authors have pointed to induction of immune response against phage antigens too and pointed out on ways to induce specific immunity against HBsAg. How applicable these methods are in practice? Although, it has to be acknowledged that items discussed as positive points for justification of the use of phages for vaccine design on pages 12 and 13 are important to be considered. In addition, the paragraph on the use of VLPs under conclusion is very well written.

Response: The limitation of phage display is that the displayed peptide is not post-translationally modified as in eukaryotic systems. HBsAg are glycoproteins; the S-HBsAg is either glycosylated or un-glycosylated at Asn-146 of the S region and the M-HBsAg has an additional glycosylation site at Asn-4 of the PreS2 region. A myristyl group is linked to the glycine residue at the N-terminus of L-HBsAg. These post-translational processes plus the formation of cysteine bonds play significant roles in stimulating both humoral and cellular immune responses. This information is stated on page 10 (lines 19-27) of the revised manuscript.

Phage particles are normally very immunogenic and may mask the immunogenicity of HBsAg displayed on the particles. In order to reduce the immunogenicity of the phage particles, the immunogenic regions of the phage particles have to be studied in depth before mutagenesis can be employed to reduce the immunogenicity of phage particles. This may enhance and focus the antibody response against the HBsAg displayed on the phage particles. In practical, van Houten *et al* (2006) demonstrated that deletion of the immunodominant region of the pIII protein of a fusion phage enhanced the antibody response against the chemically-conjugated synthetic peptide.

Comment 5: Although it is admirable that the authors have spent time to describe each section of the article from the early work performed. As a reader, I found some history and introductory sentences such as those provided on page 13 and 14 to be repetitious. Therefore, I wonder if the editor finds it necessary to be included in the article. For example: "As a recap for the development of HBV diagnostic assays from the very beginning of the discovery of the Australia antigen, a typical Ouchterlony agar gel …….. The powerful nucleotide sequencing method developed by Sanger and colleagues…… The introduction of PCR by Karry B Mullis and colleagues [72], which enables a DNA molecule to be amplified rapidly and specifically in vitro, has revolutionized diagnostic assays……."

Response: Thanks for the comment. The repetitious information has now been removed in the revised manuscript (page 13, line 30 to page 14, line 9). Other repetitions which have also been removed include:

i) Page 30, line 23: "Currently there are about 370 million chronic carriers worldwide despite the existence of effective vaccines".
ii) page 33, line 24: "We witness a rapid adaptation of innovative technologies for detecting HBV, from the Ouchterlony agar diffusion test to fully automated ELISA, real-time PCR and DNA sequencing.".

Comment 6: Considering the facts about the "human microbiome", that is a cutting edge issue these days, if one uses phage display techniques in drug targeting and similar in vivo work, doesn't it change the pattern of microbiome of a healthy human and leads to other long term or even short term diseases? Thank you for the opportunity to review this very well written article.

Response: Before a phage can be used as a drug or immunogen delivery vehicle for human, the specificity of the phage towards bacteria in human must be studied in depth. This could prevent or reduce the change of microbiome patterns in a healthy individual. This information has now been added in the revised manuscript (page 31, lines 16-19).

Reviewer # 02861260:

Comments: In this article, the authors have reviewed the innovative applications of phage display in epitope mapping and the development of vaccines, therapeutic agents, diagnostic reagents, as well as gene and drug delivery systems to combat HBV. This is a very interesting review article. The findings of this study will definitely contribute to scientific literature and improve our understanding the applications of phage display on HBV research.

Response: Many thanks for you kind words. We appreciate the effort and time you spent to review our manuscript.

Reviewer # 01800545:

Comments: The authors reviewed the association between phage display and HBV. The issues about phage display were well documented. But there were many comments about HBV, which had been recognized as the common knowledge, therefore, I felt that this review was redundant.

Response: The common knowledge of HBV is meant to provide some background information for the applications of phage display technology. In addition, this provides some background information for readers, not only to researchers but also to entrepreneurs, who have no common knowledge on HBV. However, the general information about HBV particularly the history of diagnostic development has been deleted in the revised manuscript (page 13, line 30 to page 14, line 9). To the best of our knowledge, so far there is no review paper summarizing the applications of phage display on HBV research. Therefore, the main aim of this paper is to provide a comprehensive and critical review on the innovative applications of phage display to combat HBV. In this review, we have provided some new opinions on the innovative applications of phage display technology in vaccine development, epitope mapping, development of diagnostic reagents, identification of inhibitors and drug delivery. We have revised the manuscript as suggested by all the reviewers and the science editor in order to meet the aims and scope of the journal.

Minor comment: In Figure 2, 4, and 7, there was no new information.

Response: Thanks for your comment. We have now deleted Figures 2 and 7 as suggested by the reviewer. However, we feel that Figure 4 provides a clear picture to readers and compliments the explanation in the text. Therefore, we would like to maintain Figure 4.

Science Editor's comments:

Comment: For the figures, decomposable figures are required. It means that the fonts and lines can be edited or moved. It can be made by ppt. Please list and define all abbreviation appearing in the tables or figures. Please check across the text. Thank you!

Response: Thanks for the comment. Decomposable figures have now been provided. Abbreviations appeared in the tables and figures are defined in their legends.

Comment: The format should be like this, please revise. Thank you!

Response: The references in Tables 1 and 2 have been reformatted according to the journal's requirement.

Many thanks.

Yours sincerely,

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