Author's response to reviews

Title: Molecular Characterization of Hepatitis B Virus in Liver Disease Patients and Asymptomatic Carriers of the Virus in Sudan

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Author's response to reviews: see over
RESPONSE TO REVIEWERS

Reviewer #1

Reviewer's report

Title: Molecular Characterization of Hepatitis B Virus in Liver Disease Patients and Asymptomatic Carriers of the Virus in Sudan

Version: 1 Date: 4 April 2013

Reviewer: Christophe COMBET

Reviewer's report:

Yousif et al. described in their manuscript a work to molecularly characterize circulating hepatitis B viruses in Sudan. This interesting and reliable work linked clinical data with viral sequences, which were extensively analysed. It will give a better picture of hepatitis B in Sudan, in the frame of the WHO-EMR request.

Some comments are given below in order to improve the manuscript.

Minor essential revisions:

1) The authors must be acknowledged for summarizing the data in Table 1. This helped reading of the paper. However, the table could be split in two tables, with one dealing with demographic/clinical data and the other with genotypic data.

This remark comes from the fact that genotype D/E and A column are not available for lines like Male:Female

The table has been revised and split into Tables 1 and 2

2) The paragraph “HBV serology and viral loads” in “Results” should be rewritten in order to better understand the given numbers. For example, the mean age could be put between round brackets. The 22 Females should be highlighted too.
We assumed that the number of females could be inferred. However, in order to aid comprehension we have now added the number. Although adding brackets is not conventional, we have added them.

3) In the paragraph “HBV genotyping and phylogenetic analysis” in “Results”, 81 HBV isolates were successfully genotyped by different techniques. Could the authors give precision about the choice of the different methods (why not only sequences were used?)? What about the 18 remaining isolates?

This has been done and highlighted on pages 9 & 10. See figure 3

4) If the analyses of genotype, recombination and mutation patterns are of clinical interest, more caution should be taken with the subtype analysis.

The authors are not sure if anything was required of them.

5) In the “Discussion”, the percentage should be 48% and not 60%, 60% is 48 genotype D isolates over 81 total isolates, not 99.

This has been changed in order to clarify the differences. Page 10 paragraph 2

6) Did the authors look for why the sM133T was observed?

The sM133T mutation could possibly compromise antibody neutralization and may represent potential vaccine escape mutant. This part has been added and highlighted in the manuscript. Page 16, paragraph 3

7) Did the authors look for resistance mutations in their sequence?

Although a number of mutations were found in the polymerase, these are neither primary resistance mutations, nor have been convincingly reported in an overt resistance during antiviral therapy. This information has been added to the manuscript.

Page 16, paragraph 3

Level of interest: An article whose findings are important to those with closely related research interests
Quality of written English: Acceptable

Statistical review: No, the manuscript does not need to be seen by a statistician.

Declaration of competing interests:

I declare that I have no competing interests
Reviewer #2

Comments to the Author

The Yousif and colleagues present the Molecular Characterization of Hepatitis B Virus in Liver Disease Patients and Asymptomatic Carriers of the Virus in Sudan, this does exist already in Sudan (Mahgoub S, Candotti D, El Ekiaby M, Allain JP. *Hepatitis B virus (HBV) infection and recombination between HBV genotypes D and E in asymptomatic blood donors from Khartoum, Sudan*. J Clin Microbiol. 2011 Jan;49(1):298-306). Our study is the first to molecularly characterize HBV strains from liver disease patients. The study of Mahgoub et al. 2011 is limited in that it investigated only healthy blood donors and not HBV-associated liver disease patients.

Overall, the manuscript is not well-organized and the data are not well presented and don't support most of the authors' objectives. We do not agree with these statements. Moreover, both other reviewers found the manuscript to be acceptable. In fact one reviewer states that: “The paper reads well and is well presented”. The following concerns about the manuscript have to be addressed by the authors before consideration for publication so that it can benefit other colleagues.

1. The manuscript requires careful editing as there are many grammatical errors, thus making the text difficult to read as there are several linguistic problems.

   The manuscript was thoroughly checked by the corresponding author, who is an English first language author and we are not sure what grammatical errors and linguistic problems the reviewer is referring to.

2. The title must be changed don't reflect the text (small size of group)

   In total 89 samples were molecularly characterized in the BCP/PC and/or complete S region. We believe that this is representative.

3. The authors reported that « chronic infection of more than 240 million people
worldwide however, most data report more than 350 million

This is the figure quoted in the latest WHO data (2012), please refer to reference 1. Moreover, this is the figure presented by Dr Hande Harmanci, Medical Officer, WHO at the 14th International Symposium on Viral Hepatitis and Liver Disease held in Shanghai in June 2012.

4. The authors said that genotype is important in treatment management this not true because the impact of genotype is not clear like HCV.]

Even though the impact of HBV genotype on disease progression is not as clear cut as that for HCV, HBV genotypes are important in both disease prognosis and treatment management. Thoroughly reviewed in references 18,19

5. chronic hepatitis (CH) patients had abnormal ALT is not enough because also depends on viral load > 3.3 log10 IU/mL) for at least 6 months and histological signs of moderate/severe necroinflammation.

The CH patients were HBsAg positive for longer than 6 months, HBeAg negative. There is no need for liver biopsy for CH classification.

6. « Acute hepatitis (AH) cases were diagnosed based on clinical presentation (symptoms and clinical presence of jaundice) plus high ALT » not valid because acute hepatitis B depends on HBc-Ab IgM.

All 7 samples classified as acute hepatitis were positive for HBcAb IgM. We inadvertently did not include this information in the original manuscript. This has now been added page 5. Thank you for noticing this omission.

7. « Real-time PCR quantification of HBV DNA » what is about the standard ?
The details of the real-time PCR protocol have been added to provide this information.

8. «When the complete S ORF could not be amplified, a RFLP assay was used» why the S ORF was not amplified if you are success to amplified the PreS?

The relatively longer amplicon of the S region 2.1 kb in length, compared to the shorter regions used for the short S and RFLP amplifications, meant that not all samples could be amplified in the longer region successfully. We have added this information in the text, page 9, last paragraph

9. «12 had neither HBeAg nor anti-HBe » how to explain this serology?

There are two possible explanations. First that they seroconverted long ago and the antibody levels have slowly dwindled. The second possibility is that they were originally infected with an HBe antigen negative variant so have never been exposed to HBeAg.

When 8 of the HBV isolates were sequenced in the BCP/PC, two had 17621764 mutations and one had a 1814 start codon mutation. Although the other 5 had wild-type BCP/PC, they had relatively low viral loads. We could not sequence the remaining four, so the serological profile is probably because of very low viral loads as well.

Furthermore, the currently available HBeAg/anti-HBe test systems are not optimal in sensitivity compared to highly sensitive HBsAg tests.

10. «HBV genotyping and phylogenetic analysis » this part must be rewritten

This has been done

11. «The BCP/PC region of 70 isolates was sequenced and 62 had genotype assignments “what happen for 8 samples?”

We agree that this needed clarification and we have thus revised the sentence to: “Of the 70 isolates amplified and sequenced in the BCP/PC region, 62 amplified in the S region and thus had genotype assignments.” Thank you!
12. In discussion section I don't understand this 60% of patients had genotype D? and in abstract 59%???? and I don't found this value in table and I don't understand the difference with previous Sudanese study. **This has been corrected and clarified.**

13. In overral, giving your small number of group your discussion and conclusion are not supported by your results.

We are aware of the limitation of small numbers in our study and had stated it in the discussion. Please see page 16, paragraph 2 where we had stated that: ‘However, the numbers were small to reach any firm conclusions.’
Reviewer #3

Reviewer's report

Title: Molecular Characterization of Hepatitis B Virus in Liver Disease Patients and Asymptomatic Carriers of the Virus in Sudan

Version: 1 Date: 13 May 2013

Reviewer: Vincent Thibault

Reviewer's report:

This manuscript provides an original and interesting molecular characterization of HBV isolates from Sudan. There is no concern about the originality of the work as very few data are available on HBV molecular epidemiology in Africa, in general. The paper reads well and is well presented except for some minor points highlighted below. The main weakness lies certainly in the overall description of the methodology and most points are detailed in the following comments. Authors should also be careful when interpreting clinical data from this small cohort as each group includes rather low numbers and statistical interpretation of such data is questionable. It is probably advisable to remain purely descriptive in this context. I am convinced that an effort in the presentation of the data would really improve the quality of the manuscript.

Specific comments

1- Methods (P5-6): in order to strengthen the scientific impact of their work, the authors should provide more details on their methodologies. Please, specify what exact region was amplified in the "short region of the S" (even if it is from a previous paper). The modifications applied to the method described initially by Günther et al. should also be briefly described.
The methods have been expanded and additional text in the materials and methods is highlighted.

2- In line of previous comment, a general outline of your approach should be proposed either as a figure or in the text (table?). How many genomes could be fully sequenced and if not, how many were only sequenced in the S gene or only analyzed by RFLP? You report on only 4 full genome analyses: is it because you only managed to perform full amplification of these samples or is it because you decided for some reasons (to be specified) to further document these very samples? (Page 8).

An explanation for this has been added under HBV genotyping and phylogenetic analysis and figure 3 has been added. Because of financial and time constraints the complete genome of a number of representative samples was sequenced. This has been stated in the text on page 10, paragraph 3.

3- For each applied strategy, the genotype (and subtype) distribution should be reported. This would insure that your technical approach did not bias the genotype distribution. Please provide as much information on the 18 samples that could finally not be genotyped.

Of the 99 isolates, (18/99) could not be genotyped using any of the three methods. No sequences could be obtained for 10 of these isolates and for the remaining 8 only the BCP/PC region was sequenced, which is not sufficient to differentiate between genotypes D and E. Figure 3 has been added in order to explain this.

4- All primers used should be gathered in a table indicating exactly the one used as forward or reverse primers. "In addition to the sequencing primers used previously…” is not really informative to the reader and it is therefore difficult to
understand the overall strategy. The same applies to the BCP/PC region amplification strategy that is very superficially described.

The primers used have been added to the manuscript and their sequences are available from the cited references. The sequences of new primers used have been added.

5- Results: overall, it is very difficult to figure out how many samples could effectively be sequenced in each region of the genome and from which region the genotype was deduced. An effort should be made to present a clear overview of the sequencing and phylogenetic analyses. Were the S and BCP/PC phylogenetic analyses fully concordant? If not, this information should clearly be stated. If your strategy had been clearly exposed, it would have been easier to understand your analysis and the proposed conclusions. For instance, (discussion p11) you state that some samples were classified as A1 based on BCP/PC analysis; why were those samples not analysed using the S region? Many questions arise from the imprecise description of your strategy.

Figure 3 has been added in order to explain this. Except for three isolates, S and BCP/PC phylogenetic analyses gave fully concordant genotyping results. This information has been highlighted in the manuscript and figure 3.

6- Table 1: it appears that all included samples could not be genotyped. The numbers of not genotyped samples (failure) should be reported and percentages should take into account these failures. So table 1 is misleading as the percentage is calculated according to genotyped samples from each group rather than percentage of the entire included samples of each group. 

This has been done

7- Since previous works have reported genotype D/E recombinants, why did you
not include genotype E in your Simplot analysis? Are there more reasons to
include other genotype D subtypes than genotype E?

**The recombination observed in D4 and D6 recombination, that is the reason to include all the subgenotypes of D, rather than genotype E. The pre-S1 deletion was 33 nt long, which is characteristic of genotype D. Genotype E has a 3 nt deletion.**

8- The abstract should report how many samples were finally genotyped, as only 81 of the initial 99 (82%) could be genotyped and only 53 of the 81 (65% or 54% of the initial population) could phylogenetically be characterized. The same remark applies to the discussion (page 11). While 99 samples were considered, genotyping could be performed for 81 of them; all numbers should be rigorously reported in order to convey a scrupulous message.

**This has been done**

9- Page 9: The rtT237P motif has been first reported in most genotype "D7" (D8?) strains (Meldal, J. Gen. Virol. 2009). This information should be discussed in light of their findings, as well as the preS2 motif I42T.

**This had already been discussed please see page 11, paragraph 1**

10- Discussion (page 11). The last part of the first paragraph is not really informative and compares patients from different studies; its pertinence is questionable. The viral load in HCC patients is not significantly higher than in the other groups; it is therefore not an outstanding finding. Could the higher viral load observed in the HCC group, be due to the higher percentage of genotype E in this group? Since high ALT levels were one of the criteria to identify AH, it is expected to be higher in this group than in the other. This finding is not very impressive. Moreover, the AH group includes only 7 patients; it is difficult to conclude anything on so small numbers.
By comparing the age of the HCC patients with those infected with genotype A we are trying to highlight HBV genotype may be important. We agree and the sentences referring to ALT and AH have been deleted.

11- Figure 1: the little box representing a phylogenetic tree of complete genomes is not well introduced in the legend. All information regarding this specific tree should be mentioned.

**This has been added**

12- Figure 3: the genotype distribution (according to the analyzed region S or BCP/PC) for each mutated region should be shown. This information would help to understand the discussion (page 13) where a discrepancy between S and BCP/PC for 3 isolates is raised.

**We have modified the figure legend to clarify this point. This figure is now figure 4**

13- Discussion, page 13: I believe it is rather cloning followed by sequencing rather than the opposite as written.

**This has been corrected**

14- Discussion, page 13: you cannot state "there was a significant correlation between the pre-S2…" as you cannot apply any statistical test to justify it, mainly because you have too small numbers. So, your sentence should remain descriptive; yet, I agree that this deletion was only observed in HBV-E/HCC patients.

**We agree and have deleted this.**

Minor point:

1- Table 1: the male:female ratio is improperly expressed on 2 columns (37:05:00…).
This has been corrected

2- Discussion, page 12: "In agreement with Sudanese blood donors…"; this sentence requires attention.

This has been corrected

3- Is figure 3 clearly needed?

This figure illustrates the combination of mutations that occur in the BCP/PC region and therefore has not been deleted.

Level of interest: An article of importance in its field

Quality of written English: Acceptable

Statistical review: No, the manuscript does not need to be seen by a statistician.

Declaration of competing interests:

I declare that I have no competing interests