RESEARCH NOTE

REVISED Analysis of the complete genome of hepatitis B virus subgenotype C2 isolate NHB17965 from a HBV infected patient [version 3; referees: 2 approved, 1 approved with reservations]

Previously titled: Analysis of the complete genome of hepatitis B virus subgenotype C2 isolate NHB17965 from a patient with uncomplicated chronicity

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Latest published: 10 Sep 2018, **7**:1023 (doi: 10.12688/f1000research.15090.3)

Abstract
The burden of chronic hepatitis B virus (HBV) infections is increasingly detected nowadays. Herein, we report a complete genome of HBV subgenotype C2 (HBV/C2) from a HBV infected patient. Complete genome analysis revealed that the isolated strain was a non-recombinant wild type and had several regular substitutions in the reverse transcriptase domain and small surface proteins of HBV. This study may help clinicians and scientists gain in-depth knowledge on the current substitutions of HBV/C2 genome and to identify potential therapies against HBV infections.

Keywords
HBV/C2, Chronic, Non-recombinant, Bangladesh

Open Peer Review

Referee Status: ✔ ? ✔

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REVISED version 2 published 13 Aug 2018		? report	✔ report
version 1 published 09 Jul 2018	✔ report	✘ report	

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Comments (0)

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Author roles: Shaha M: Methodology, Writing – Original Draft Preparation; **Sarker PK**: Investigation, Validation, Writing – Review & Editing; **Hossain MS**: Data Curation, Methodology; **Das KC**: Conceptualization, Writing – Review & Editing; **Jahan M**: Data Curation, Methodology, Writing – Review & Editing; **Dey SK**: Investigation, Validation, Writing – Review & Editing; **Tabassum S**: Data Curation, Writing – Review & Editing; **Hashem A**: Supervision, Writing – Review & Editing; **Salimullah M**: Conceptualization, Supervision, Writing – Review & Editing

Competing interests: No competing interests were disclosed.

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REVISED **Amendments from Version 2**

The revised version of the manuscript contains a very little changes in some sentences that were suggested by the reviewers. The title has also been changed to "Analysis of the complete genome of hepatitis B virus subgenotype C2 isolate NHB17965 from a HBV infected patient".

See referee reports

Introduction

The burden of chronic liver disease caused by hepatitis B virus (HBV) is increasingly detected at present[1]. Globally, more than 2 billion people have been infected by HBV[2,3] and, according to the World Health Organization (WHO) approximately 257 million were living with HBV in 2017. In Bangladesh, the rate of HBV chronicity is 2–6%[4], which makes it relatively higher risk than some infectious diseases, for example, Hepatitis C virus[5] , Human immunodeficiency virus[6].

HBV genome comprises a partially double-stranded covalently closed circular DNA that encodes four highly overlapping major open reading frames[7]. Due to the absence of proof-reading activity, the mutation rate of HBV is high[8], which may induce the possible recombination events of the strains[9]. Most chronic HBV cases have a high possibility of causing liver cirrhosis[10] and hepatocellular carcinoma[11]. In Bangladesh, there is scarce of complete genome sequence of HBV chronic strain of subgenotype C2. Hence, we isolated the complete genome of a HBV/C2 strain collected from a patient having HBV infection carrying the virus for a long time.

Methods

Isolation and sequencing

An HBV-positive plasma sample was collected from a 45-year-old male patient in a tertiary hospital in Dhaka, Bangladesh after obtaining the patient's written informed consent. The infected patient might have chronic liver disease, as determined by ultrasonography. The patient was diagnosed with the possibility of chronic HBV infection, suggested by the presence of ascitis and enlarged spleen, after the positive reaction of anti-HBc total and with a high viral load in the plasma. However, the patient was not showing signs of jaundice, though was affected by fever, nausea, vomiting and fatigue. The study was approved by the Research Ethics Committee of National Institute of Biotechnology, Bangladesh (NIBREC2015-01). The patient was not taking any antiviral therapy and was diagnosed 1 month prior to obtainment of the plasma sample. HBV DNA was extracted from the sample using the QIAamp MinElute Virus Spin kit (Qiagen, Germany). The complete HBV genome was amplified by six sets of primer pairs used previously in another study[12] using a conventional PCR method. The primer sequences and their annealing temperatures were as follows: set 1, forward- AAGCTCTGCTAGATCCCAGAGT, reverse- AGTTGGCGAGAAAGTGAAAGCCTG, 56°C; set 2, forward- CCTATTGATTGGAAAGTATGTCA, reverse- AACAGACCAATTTATGCCTA, 48°C; set 3, forward- GAGACCACCGTGAACGCCCA, reverse- CCTGAGTGCTGTATGGTGAGG, 56°C; set 4, forward- TTCACCTCTGCCTAATCATC, reverse- ATAGGGGCATTTGGTGGTCT, 52°C; set 5, forward- TCAGGCAACTATTGTGGTTTCA, reverse- GGGTTGAAGTCCCAATCTGGATT, 51°C; set 6, forward- GGGTCACCATATTCTTGGGAA, reverse- CGAGTCTAGACTCTGTGGTA, 51°C. For a mixture of 25 µl reaction volume, 12.5 µl of 2X MasterMix (Thermo Fisher Scientific, USA), 1 µl each of forward and reverse primers (IDT, USA), 9.5 µl of nuclease-free water (Thermo Fisher Scientific, USA) and 2 µl of template DNA were used. The condition of the PCR reaction was 1 cycle at 95°C for 10 min, 35 cycles at 95°C for 1 min, with the aforementioned annealing temperatures for 1 min and 72°C for 1 min, and a final cycle for 10 min at 72°C. Sanger sequencing was performed using the BigDye Terminator version 3.1 cycling sequencing kit (Applied Biosystems, USA) by ABI 3130 Genetic Analyser (SeqGen, CA, USA) and by thermal cycler (Sigma-Aldrich, Germany) using the described annealing temperatures as per manufacturer's instructions after the purification of PCR products using PureLink PCR Purification Kit (Thermo Fisher Scientific, USA), performed in accordance with the manufacturer's protocol. Next, the sequenced contigs were assembled using the Seqman tool of DNASTAR Lasergene version 7.2[13].

Analysis

The subgenotyping and mutation analysis of the sequenced genome were performed using the HBV Geno2Pheno tool version 2[14] using the default parameters, comparing against the HBV genotypes consensus sequences. Recombination analysis of the sequence was performed using the NCBI genotyping tool. The complete genome was deposited in the GenBank under the accession number MH220971.

Results and discussion

Analysis of the complete genome denotes that the isolate studied here, termed NHB17965, comprises HBV genotype C and subgenotype C2 (HBV/C2) with a GC content of 48.77%. Recombination analysis using the NCBI Genotyping tool showed that NHB17965 is a non-recombinant wild-type HBV isolate (Figure 1).

The patient was diagnosed with chronic HBV infection. Although, the patient was tested positive 1 month prior to obtainment of the plasma sample, he might be infected much earlier as he had a minor surgery few years back and his brother was positive for HBV years ago. Isolate NHB17965 was observed to have amino acid substitutions H9Y, N13H, I91L, P109S, T128N, I269L and V278I in the polymerase domain and S53L, P120T, I126T and S210N in the small hepatitis B surface protein as analysed by HBV Geno2Pheno tool[14], compared against the HBV genotypes consensus sequences. These substitutions may be the results of regular genomic changes to HBV because of a lack of proof-reading

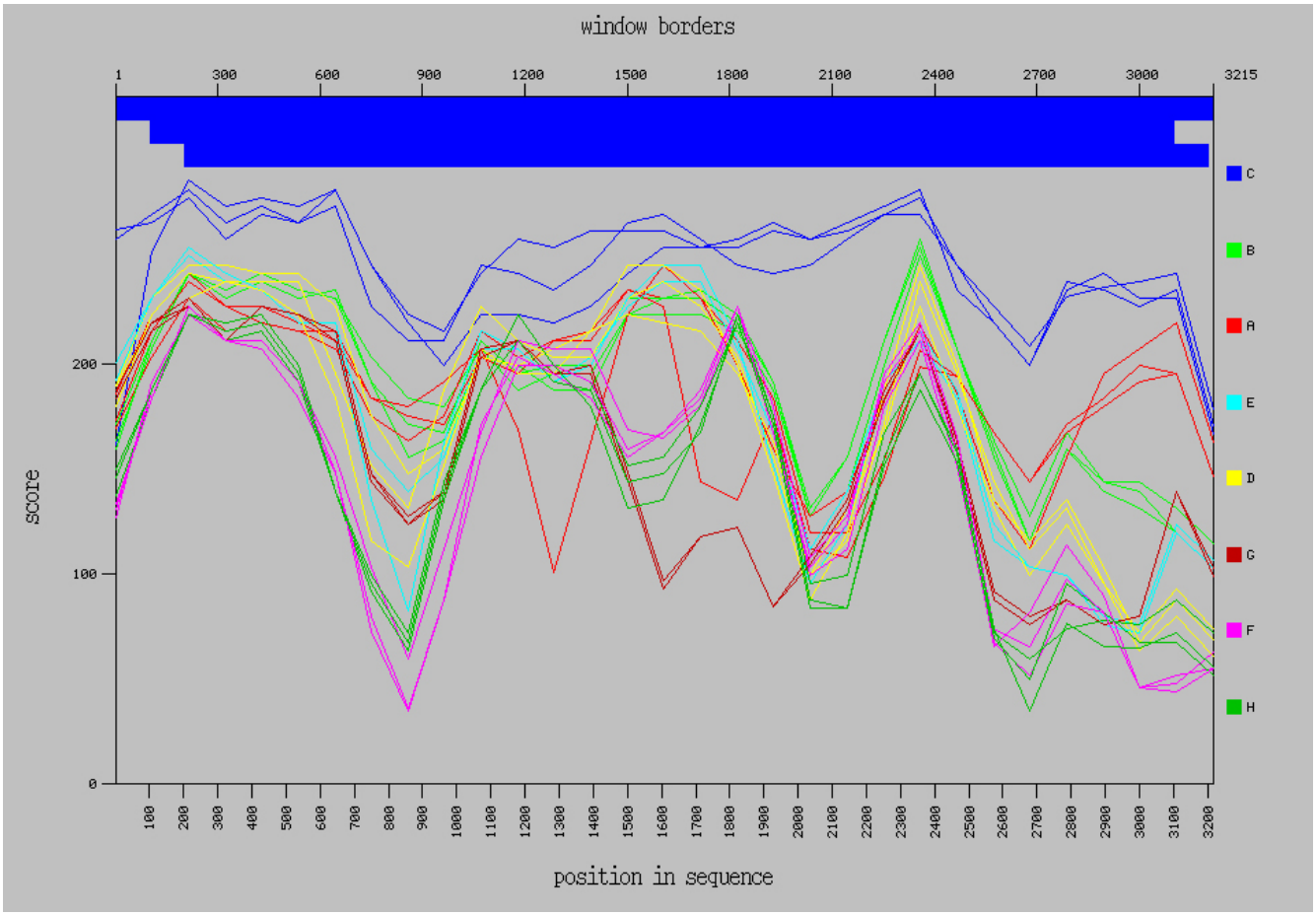

Figure 1. Recombination analysis of the isolate NHB17965. The Simplot diagram was generated using the NCBI Genotyping tool.

activity of the viral reverse transcriptase, and may not signify any danger.

The findings of this study may help clinicians and scientists to gain substantial knowledge about the current genomic substitutions of HBV/C2 and to decide treatments against chronic HBV infections.

Data availability

Genome of the HBV strain isolated in this study, MH220971.

Grant information

The study was supported by the National Institute of Biotechnology, Ministry of Science and Technology, Bangladesh.

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Current Referee Status: ✔ ? ✔

Version 3

Referee Report 19 September 2018

doi:10.5256/f1000research.17752.r38142

? **Paul Klapper**
University of Manchester, Manchester, UK

As outlined in previous reviews of this m/s there is no laboratory evidence that this patient had chronic hepatitis or chronic hepatitis B infection. He was diagnosed with HBV infection only 1 month prior to the investigation described in the manuscript.

At the end of the introduction the authors state the virus was obtained from *"A patient having HBV infection for a long time".* There is no evidence presented for this, he was only diagnosed 1 month prior to investigation.

In results and discussion, paragraph 2, we are told *"the patient was diagnosed with chronic HBV infection"* there then follows an attempt to justify this statement.:- he had minor surgery a few years back, his brother was diagnosed with HBV a few years ago. This is circumstantial rather than clear proof that this patient had chronic hepatitis.

It appears that without retesting the patient more than 6 months after initial diagnosis, there is no way that this patient can be said to have chronic hepatitis or chronic HBV infection.

Introduction 2nd paragraph "*In Bangladesh, there is scarce of complete genome sequence of HBV chronic strain of sub-genotype C2"* might be better expressed as '*In Bangladesh there is a lack of complete genome sequences of HBV sub-genotyoe C*' - as outlined above, there appears to be no concrete evidence that the strain of HBV analysed in this paper caused chronic hepatitis.

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Version 2

Referee Report 04 September 2018

doi:10.5256/f1000research.17379.r37523

Ashesh Kumar Chowdhury
Department of Immunology, BIRDEM General Hospital, Dhaka, Bangladesh

The reporting of a sequence analysis of complete genome of subgenotype C2 of hepatitis B virus is new in Bangladesh perspective. The article as 'research note' is quite acceptable.

But, regarding the 'chronic status' of patient, it may be changed at all portion of the article except in 'results and discussion' part where it may be kept as a possibility of being chronic (because 'history of minor surgery few years back and brother was positive for HBV years ago'). The chronic status of the patient was not established by serological evidence (e.g. HBsAg positivity 6 months back).The change of 'chronic' status starts from title- where 'from a patient with uncomplicated chronicity' may be changed to 'HBV infected patient'

In the abstract, 'a patient with chronic HBV infection' may be changed to 'HBV infected patient' and from 'to identify potential therapies against chronic HBV infections' 'chronic' word may be omitted.

In the introduction part- 'Most of the HBV cases are chronic, …. hepatocellular carcinoma' is misleading. It needs rewriting as 'Most chronic HBV cases have a high possibility causing liver cirrhosis and hepatocellular carcinoma'. In last line, 'from a patient with chronic HBV infection carrying the virus for a long time' may be replaced by 'from a patient having HBV infection'.

In the methods part- 'The infected patient……..determined by USG. The Patient was diagnosed with chronic HBV infection, suggested by presence of the presence of ascitis…..high viral load in the plasma.' These two lines need rewriting, mentioning the possibility of being chronic. But, again lack of serological evidence needs to be mentioned.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Not applicable

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 06 Sep 2018

Modhusudon Shaha, National Institute of Biotechnology, Bangladesh

We would like to thank the reviewer for his constructive comments on the manuscript. Herein, the responses to the reviewer's comments are given.

From the title- 'from a patient with uncomplicated chronicity' is changed to 'HBV infected patient'.

The abstract is rewritten considering the valuable suggestions of the reviewer. The introduction part is revised according to the reviewer's comments. The commented sentences are revised in the methods section.

Competing Interests: No competing interests were disclosed.

Referee Report 21 August 2018

doi:10.5256/f1000research.17379.r37107

Paul Klapper
University of Manchester, Manchester, UK

The authors have made a reasonable attempt to address the points raised in my previous review of this manuscript however a fundamental concern remains. There is no laboratory evidence that this patient had chronic HBV infection, there is only evidence that the patient had chronic hepatitis and that hepatitis B infection was detected at the point the patient was investigated.
It is not possible to say that the patient had chronic hepatitis B virus infection.

Abstract: *"Hence, we isolated the complete genome of a HBV/C2 strain collected from a patient with chronic HBV infection carrying the virus for a long time"*.
Yet the text of the paper does not support the statement of virus carriage for a long time, in methods we find:
"The infected patient had chronic liver disease, as determined by ultrasonography. The patient was diagnosed with chronic HBV infection, suggested by the presence of ascitis and enlarged spleen, after the positive reaction of anti-HBc total and with a high viral load in the plasma"

and in Results/Discussion we find:
"The patient was diagnosed with chronic HBV infection. Although, the patient was tested positive 1 month prior to obtainment of the plasma sample, he might be infected much earlier as he had a minor surgery few years back and his brother was positive for HBV years ago"
These findings confirm that the patient had chronic hepatitis they do not confirm that the patient had chronic hepatitis B infection. As previously outlined, the standard definition of chronic hepatitis B infection is the detection of hepatitis B surface antigen in serum for more than 6 months. As there is no laboratory evidence of carriage of hepatitis B virus surface antigen for more than 6 months it is only possible to say this patient had chronic hepatitis and suggest that it is likely to represent chronic hepatitis B infection.
Thus the title text "*a patient with uncomplicated chronicity*" is misleading.
The abstract text "*from a patient with chronic HBV infection*" is misleading.
The introduction text "*HBV chronic strain of subgenotype C2*" is misleading as there is no laboratory proof

that the patient had chronic hepatitis B infection

The methods text "*The patient was diagnosed with chronic HBV infection*" is misleading as there is no laboratory proof that the patient had chronic hepatitis B infection

Results and discussion text "*The patient was diagnosed with chronic HBV infection*" is also misleading the result present show the patient had chronic hepatitis And an HBV infection and that is all without laboratory proof that the patient had chronic hepatitis B infection.
Also "*current genomic substitutions of HBV/C2 and to decide treatments against chronic HBV infections"* is again misleading as there is no laboratory proof that the patient had chronic hepatitis B infection

There is a further statement in the introduction "*Most of the HBV cases are chronic"* that is not factually correct – most cases of vertical transmission of HBV result in chronic infection, most cases of post-natal acquired HBV infection result in clearance of HBV surface antigen. All cases of HBV infection result in virus latency in the form of HBV cccDNA in liver.

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 06 Sep 2018

Modhusudon Shaha, National Institute of Biotechnology, Bangladesh

We would like to thank the reviewer again for his constructive comments on the manuscript.

The manuscript is revised considering the comments and suggestions of the reviewer.

Competing Interests: No competing interests were disclosed.

Version 1

Referee Report 07 August 2018

doi:10.5256/f1000research.16434.r36692

Paul Klapper
University of Manchester, Manchester, UK

This paper reports a sequence analysis of a strain of hepatitis B virus. However, there are some aspects of the paper that merit attention. In the Abstract and again in the Introduction the authors state: *The number of chronic cases of hepatitis B virus (HBV) is increasing rapidly in the world".* I found this an interesting statement and wondered what was the evidence base for this. In the Introduction the authors cite MacLachlan and Cowie (2015). It appears to me that the authors are mis-quoting this reference. The reference actually says "T*he burden of chronic HBV infection is increasingly being recognized*", this is

substantially different from suggesting that the number of cases is increasing. There is a general lack of comprehensive epidemiological information on chronic hepatitis B infection as many developing countries (the epicentre of chronic HBV infections) lack surveillance to provide data. We do not know how WHO programmes to prevent vertical transmission of HBV are impacting on chronic hepatitis B and I believe we lack information to support the assertion of a global increase in numbers.

Also in the abstract - the third line ""*with current common amino acid substitutions"* is not a meaninglful statement. The sentence needs rewriting to make clear what the authors actually mean.

Introduction 1st paragraph
last 2 lines: "*which makes it relatively higher risk than other infectious diseases*" ; it is unclear what is meant here, a rate of 2-6% is clearly low compared with risk of, for example, influenza or rotavirus infection.

Introduction, 2nd Paragraph "*the mutation rate of hepatitis B is high" and " hence, recombinant strains are evolving with a common pattern".* Mutation is a random event how can a random event lead to a commonly evolving pattern. The authors need to recast the sentence to explain what they really mean.

Introduction, end of 2nd paragraph. the genome was isolated from "*a patient without liver complication"* yet in methods we are told the patient has chronic liver disease as adjudged by ultrasonography.

Methods: line 4. Was formal staging not used to describe liver disease e.g. relating shear wave elastography to fibrosis score? Were liver function tests performed?

Methods, line 5 "*The patient was diagnosed with chronic hepatitis B recently*". This seems strange, the standard definition of chronic hepatitis B infection is the detection of hepatitis B surface antigen in serum for more than 6 months. This patient - with blood taken only one month post diagnosis would not seem to meet the definition. No hepatitis B markers results are given for the patient and so understanding of the phase of chronic illness (see EASL guidance;Journal of Hepatology 2017 vol. 67: 370–398) is not possible

Analysis - reference needed for the HBV Geno2Pheno software used.

What evidence is there that the HBV/C2 isolate sequenced is a common phenotype in chronic hepatitis B virus infection in Bangladesh? Without such evidence it is difficult to see how the conclusions "*The findings of this study will help....*" are justified. This could simply be an single instance of this virus produced through random mutation.It is therefore also difficult to understand how this could be considered a 'reference strain' for chronic hepatitis B as it may represent a single instance and further as the patient does not appear to meet a case definition of having chronic hepatitis B infection, can it be considered as a reference strain for chronic hepatitis B infection.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Not applicable

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Author Response 08 Aug 2018

Modhusudon Shaha, National Institute of Biotechnology, Bangladesh

We would like to thank the reviewer for his constructive comments on the manuscript. Herein, the responses to the comments are given.

'The number of chronic cases of hepatitis B virus (HBV) is increasing rapidly in the world'- The sentence is corrected in the revised manuscript.
"with current common amino acid substitutions"- this portion of the sentence is removed from the revised manuscript.
"which makes it relatively higher risk than other infectious diseases"- the sentence is re-written with substantial clarity.
"the mutation rate of hepatitis B is high" and " hence, recombinant strains are evolving with a common pattern"- the sentence is re-written in the revised manuscript.
"a patient without liver complication"- the sentence is corrected in the revised manuscript. Particular liver function tests were not performed. However, the liver was observed normal using ultrasonography.
"The patient was diagnosed with chronic hepatitis B recently"- The sentence is edited in the revised manuscript. The detail of diagnosis of the chronicity is described in the revised manuscript. Reference is given that study used the HBV Geno2Pheno tool.
"The findings of this study will help...."- the sentence is re-written in the revised manuscript.

Competing Interests: No competing interests were disclosed.

Referee Report 23 July 2018

doi:10.5256/f1000research.16434.r35849

Mohammad Ariful Islam
Jagannath University, Dhaka, Bangladesh

The overall level of the paper is good: even if it is quite simple, it is well written and some important considerations are highlighted.

The manuscript talks about the complete genome analysis of hepatitis B virus and represent the complete genome sequence of HBV subgenotype C2 in Bangladesh.
The study is a short research note. Although the findings of the study is limited and like a genome announcement, it signifies to be documented. The study is scientifically acceptable.
I have some minor queries

- what does the isolate NHB17965 indicates?
- any reference sequences used in this study? If used, what are these?"

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Not applicable

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 01 Aug 2018

Modhusudon Shaha, National Institute of Biotechnology, Bangladesh

We would like to thank the reviewer for his constructive comments on the manuscript.

NHB17965 indicates the sample identification number given by the laboratory.

There are no reference sequences used in this study for the analysis. The isolated sequence was analyzed using NCBI Genotyping tool and Geno2Pheno tools as given in the manuscript.

Competing Interests: No competing interests were disclosed.