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Short communication

Identification of a novel tri-genotypic recombinant Hepatitis B virus in Bangladesh

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ABSTRACT

We report a novel tri-genotypic recombinant Hepatitis B virus (HBV) strain circulating in Bangladesh. The strain is recombinant with the genotypes D, C and E, of which, genotype E was not reported before in Bangladesh. Additionally, the complete genome has a frameshift deletion of nine nucleotides from overlapping *Surface* and *Polymerase* genes, and a vaccine escape mutation, A128 V, in the surface protein. This is the first report with such unusual recombination event responsible for rapid liver cirrhosis in a 13 year old patient in Bangladesh. This report may alert the clinicians to take the measure to prevent an upcoming outbreak of recombinant HBV.

Genomic variation of Hepatitis B virus (HBV) occurs due to the tremendous mutations during virus replication (Hakami et al., 2013). Hepatitis B virus is a member of *Hepadnaviridae* family comprising a partially double-stranded DNA (Shaha et al., 2018), which uses self-encoded reverse transcriptase during replication (Ganem et al., 1994). The genome has four major overlapping open reading frames (ORFs) that mutate frequently to evade the actions of vaccine, antiviral drugs and immunoglobulin activity (Shaha et al., 2018). Furthermore, mutations in the HBV genome may render evolution of new genotypes. Currently, there are ten HBV genotypes (A to J), of which, genotypes C, D and A are prevalent in Bangladesh (Sunbul, 2014; Shaha et al., 2016). Different studies reported that genotype C and D are mostly involved in chronicity with more disease severity caused by genotype C (Sunbul, 2014). Genotype D was also reported to have increased mutation frequencies (Sunbul, 2014). However, genotype A was documented to have a strong association with acute infection and disease persistence after acute infections (Sunbul, 2014).Recently, the recombination between the HBV genotypes has been observed in different regions of the world, some of which were reported to cause HBV strain evolution (Simmonds e Midgley, 2005; Littlejohn et al., 2016). However, documentation about the effect of recombination on liver fibrosis is scarce. Herein, we report a novel tri-genotypic recombinant HBV strain isolated from a patient with liver fibrosis in Bangladesh.

The plasma sample was collected from a patient of 13 year old with liver fibrosis level F3 to F4 (cirrhosis) after getting a written consent from the patient and his guardian. The patient was diagnosed HBV positive by real-time PCR analysis with a viral load of 5.94×10^6 IU/ml in a hospital in Bangladesh. The patient was not introduced to syringe shearing, blood transfusion and surgery. There was no record of HBV infections of the patient's family members. Furthermore, the patient's plasma was tested seronegative for Human immunodeficiency virus and Hepatitis C virus by enzyme-linked immunosorbent assay (ELISA). HBV DNA was isolated from the plasma sample using QIAmp MiniElute Virus Spin kit (QIAGEN, Germany) and amplified by direct PCR using six primer pairs (Table 1) spanning the whole genome (Sugauchi et al., 2001). Firstly, 462 bp and 2744 bp of the genome were amplified by first round of PCR using 3F-3R and 4F-2R primer pairs respectively. Then the second round nested PCR was performed with 2744 bp fragment using the given primer pairs. All the PCRs of the used sample were performed three times to avoid experimental errors and observed identical DNA band in agarose gel electrophoresis (AGE). The amplified overlapping segments were purified using Purelink PCR Purification Kit (Thermofisher Scientific, USA) and sequenced using BigDye Terminator version 3.1 cycle sequencing kit (Applied Biosystems, USA).

The sequenced fragments were assembled and analyzed for phylogeny using SeqMan v.7 (Swindell e Plasterer, 1997) and NCBI-BLAST tool (Altschul et al., 1990) respectively. The genotyping and subtyping & mutation analysis of the sequence were performed using NCBI

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Table 1
Primer pairs used to amplify the complete genome of HBV strain NHB17963 (Sugauchi et al., 2001).

Primer name	Primer sequence (5'-3')	Polarity	Nucleotide position
HBV1F	AAGCTCTGCTAGATCCCAGAGT	Sense	18-39
HBV1R	AGTTGGCGAGAAAGTGAAAGCCTG	Antisense	1107-1084
HBV2F	CCTATTGATTGGAAAGTATGTCA	Sense	970-992
HBV2R	AACAGACCAATTTATGCCTA	Antisense	1803-1784
HBV3F	GAGACCACCGTGAACGCCCA	Sense	1611-1630
HBV3R	CCTGAGTGCTGTATGGTGAGG	Antisense	2072-2048
HBV4F	TTCACCTCTGCCTAATCATC	Sense	1824-1843
HBV4R	ATAGGGGCATTTGGTGGTCT	Antisense	2314-2278
HBV5F	TCAGGCAACTATTGTGGTTTCA	Sense	2190-2211
HBV5R	GGGTTGAAGTCCCAATCTGGATT	Antisense	2987-2965
HBV6F	GGGTCACCATATTCTTGGGAA	Sense	2814-2834
HBV6R	CGAGTCTAGACTCTGTGGTA	Antisense	256-237

Genotyping tool (Rozanov et al., 2004) and Geno2Pheno tool (http://hbv.geno2pheno.org/index.php) respectively. The recombination analysis of the isolated strain was done using NCBI Genotyping tool (Rozanov et al., 2004) and RDP4 software (Martin et al., 2015). The complete genome sequence of the tri-genotypic recombinant strain is deposited to the NCBI GenBank under the accession number MH220970.

The isolated strain NHB17963 comprises of 3206 nucleotides with 48.7% GC content and was mostly aligned with the HBV subgenotype D2. However, whole-genome analysis revealed that the complete genome is a recombination of three genotypes, D (81.29%), C (18.71%) and E (9.36%) (data not shown). The genome region ~(1800–2100) spanning the pre-C and C genes denoted genotype E (which was not reported in Bangladesh before), nucleotides ~(2400–3000) spanning the large S and P genes showed genotype C and the rest of the nucleotides denoted genotype D (Fig. 1)

Interestingly, the genome sequence contains a frameshift deletion of nine nucleotides (from 42 to 50 nucleotides) in overlapping large S, middle S and P genes, which may involve in promoting the severity of the disease and have an additional effect on developing fibrosis in the early age of the patient. Furthermore, the strain was observed to have a mutation, A128 V in the surface protein, which was previously documented as a secondary mutation responsible for vaccine escape (Shaha et al., 2016). Moreover, the strain was observed to have a stop codon at 28th position of the core protein.

The more the recombination, the more the probability to increase the pathogenicity of the virus, as documented previously (Simon-Loriere e Holmes, 2011). Recombination is the late stage of a virus evolution. HBV may gain new properties to develop a disease rapidly through recombination. Hence, the goal of this study was to report a novel recombinant HBV strain associated with advanced liver fibrosis isolated from a boy of 13 year of age in Bangladesh.

The isolated strain NHB17963 was highly recombinant comprising tri-genotypic recombination of genotypes D, C and E with sharing nucleotide percentage of 81.29%, 18.71% and 9.36% respectively. The genotype D and C was documented predominant in acute and chronic cases respectively of HBV strains in Bangladesh (Shaha et al., 2016; Munshi et al., 2017). However, there was no previous report of HBV genotype E in Bangladesh. Hence, the existence of genotype E in Bangladesh may be transported from its prevalent areas such as Saudi Arabia or Central Africa (Sunbul, 2014) and considered as a possible evolution forming the recombination event in the isolated strain in Bangladesh. One plausible reason of the recombination event may be that the HBV genotypes are getting weaker due to mass vaccination program and hence, they are trying to be stronger by exchanging the inter-genotypic genes. According to the recent studies, genotype C and D are strongly associated with the disease severity causing hepatocellular carcinoma (HCC) and increased mutation frequencies respectively (Sunbul, 2014). Studies also documented HBV genotype E as the most difficult one to treat (Sunbul, 2014). In this regard, such recombination of the genotypes D, C and E may impose threat to the global health and

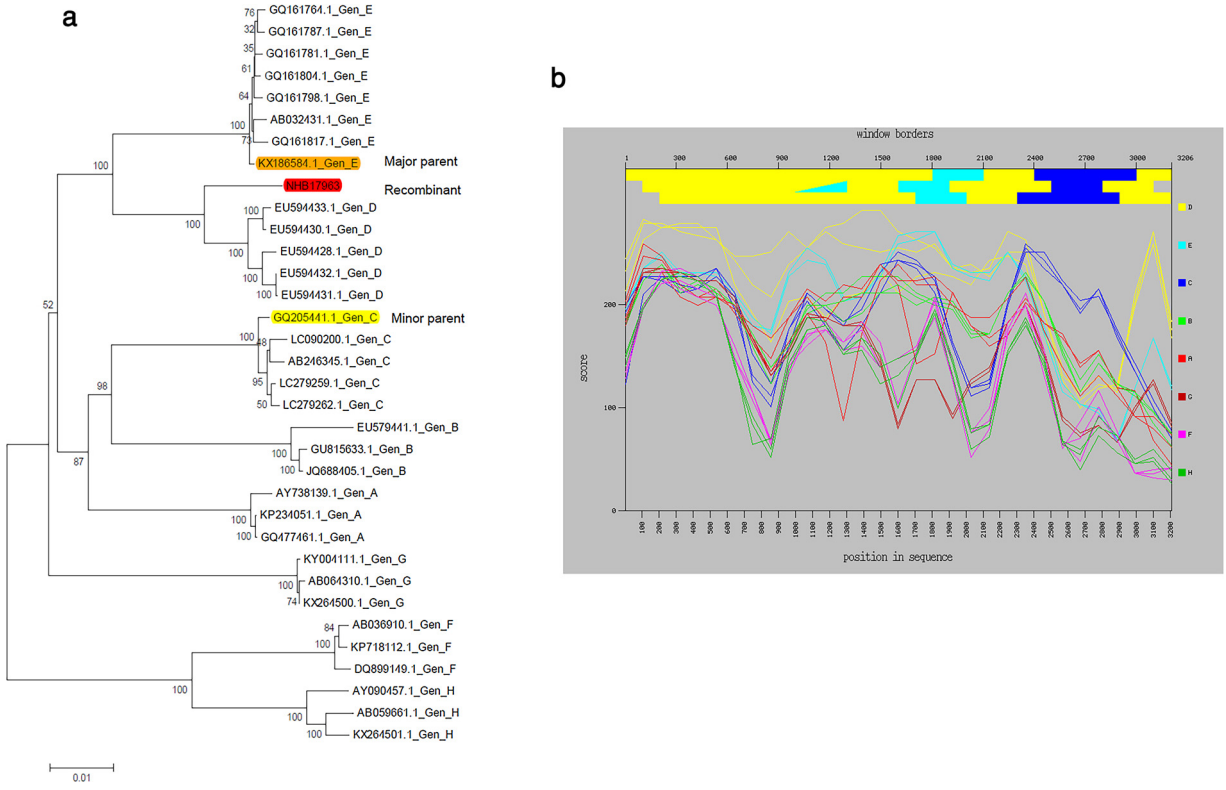

Fig. 1. Analysis of the recombination of the isolated HBV strain. (a) Phylogenetic analysis of the recombinant strain NHB17963 prepared by RDP4 software; (b) Graphical representation of the recombination events, developed by NCBI Genotyping tool.

may increase the severity of liver disease, which is supported again by that the patient has liver fibrosis level F3 to F4. Furthermore, the average age of the HBV infected patients to develop cirrhosis was 55–64 as documented in a study (Mendy et al., 2010). However, in this study, the patient with the unusual recombinant strain was of only 13 year old to develop cirrhosis, which again reflects the severe infectious capability of the isolated strain.

On the other hand, there was a nine nucleotides deletion at 42–50 position in the overlapping large S, middle S and P genes, which may play a major role in increasing pathogenicity of the strain. The frameshift deletion may also render to promote the progression of liver cirrhosis as well as to create a platform to stabilize the strain after recombination, though to conform the hypothesis more studies are needed. Furthermore, a secondary mutation, A128 V was found in the surface protein which was documented to be responsible for vaccine failure before (Shaha et al., 2016) and may cause the vaccine unresponsive if the child was administered with a HBV recombinant vaccine as it was included in the regular extended program on immunization (EPI) schedule for children (http://www.dghs.gov.bd/index.php/en/mis-docs/epi) in Bangladesh since a long ago.

This is the first report of a tri-genotypic recombination of HBV causing liver cirrhosis in the very young age of the patient. This unusual recombination may promote an outbreak of HBV in the future. Thus, high-risk population, such as healthcare professionals, should need to be alert of new HBV infections, and the clinicians should think considering new strategies to prevent infections caused by the recombinant strains. Further studies are recommended to investigate the HBV recombination events causing the liver disease more severe and other clinical associations in more detail and take initiatives to prevent such infections among children and elders as well.

Conflict of interest

Authors have no conflict of interest.

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