The Thr to Met substitution of amino acid 118 in hepatitis B virus surface antigen escapes from immune-assay-based screening of blood donors

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Hepatitis B surface antigen (HBsAg) is the main diagnosis marker for hepatitis B virus (HBV) infection. In this study, a novel HBV mutant from an HBV-positive blood donor with false-negative results during HBsAg screening was identified. DNA sequencing discovered two mutations at nt 353 (A to T) and nt 349 (T to A), leading to Thr to Met and Ser to Thr substitutions at aa 118 and 117 of HBsAg, respectively. Further analysis showed that eight of ten HBsAg ELISA kits failed to detect this HBsAg mutant. A mutagenesis assay indicated that the Thr to Met substitution at aa 118 was the determinant for escape from HBsAg ELISA detection. A small-scale screening of blood donors identified two individuals infected by this unique HBV mutant, suggesting a certain level of prevalence among the general population.

In conclusion, our study identified the aa 118 mutation in HBV surface antigen and provided information for improvement of HBV diagnosis products.

INTRODUCTION

The hepatitis B virus (HBV) is a worldwide public health concern, which is responsible for at least one million deaths annually due to HBV-related cirrhosis, liver failure and hepatocellular carcinoma (Dienstag, 2008; WHO, 2015). A seroprevalence study indicated that China is a highly endemic area of HBV across the world (Ott et al., 2012). It has been reported that the prevalence of HBV infection is up to 7% in the adult population (age 19–49) in China (Ott et al., 2012). Moreover, HBV is also a threat for blood products due to blood-transfusion-mediated transmissions, especially when blood collection has been conducted during the undetectable window period of HBV infected donors (Candotti & Allain, 2009). HBV surface antigens are the main clinical markers indicating acute or chronic infection, as well as occult HBV infection if no HBV DNA screening is done (Shepard et al., 2006). Currently, ELISA-based hepatitis B surface antigen testing is the primary way to identify persons with chronic HBV infection (Shepard et al., 2006).

The HBV surface antigens are able to induce protection against HBV and therefore can be used as a vaccine (Szmusczynska et al., 1981). There are three surface antigens, large, middle and small, which share 226 common C-terminal residues (Tian et al., 2007). The small hepatitis B virus (HBV) surface antigen (HBsAg), which is 226 aa in length, is considered to be the major part of the viral envelope (Tian et al., 2007). The major hydrophilic region (MHR) between aa 99 and 169 in HBsAg harbours conformational epitopes and is the major target of neutralizing antibodies (Seddighi-Tonekaboni et al., 2000; Tian et al., 2007). Within this region, the area around aa 124 and 149, containing five cysteine residues, is essential to the immunogenicity of HBsAg (Mangold & Streeck, 1993). In many cases, amino acid variations were found in this region of HBsAg and they were associated with immune escape or diagnostic failure, due to the impaired ability to bind anti-HBV antibodies (Carman, 1997; Carman et al., 1990; Tian et al., 2007).

In this study, we identified a new mutation site of nt 353 (A to T), which led to a Thr to Met substitution in aa 118, of HBsAg in an HBV-positive blood donor (GenBank accession number FJ905226). Analysis of this variation indicated that the Thr to Met substitution changes the antigenicity of HBsAg and evades detection by most of the commercial ELISA kits used for HBV diagnosis in China. In a screen of a total of 39 blood samples from HBV-positive blood donors, two individuals infected by this novel HBV mutant were identified, indicating that this HBV
mutant had some level of prevalence and more attention should be paid for this novel mutant. In summary, our study provided useful information for the future development of HBV diagnosis products and vaccine development.

RESULTS

Identification of a novel HBsAg mutant and expression of this mutated HBsAg in HEK293T cells

During our routine HBV screening of samples from blood donors, two ELISA kits from different companies were used for HBsAg screening for every sample. When inconsistent results were shown, more tests were conducted to further confirm the inconsistent ELISA results. During our screening, ELISA results for one donor’s serum demonstrated inconsistent results in two ELISA kits targeting HBsAg. For safety concerns relating to blood transfusion and for determining the true HBV status of this donor, we conducted ELISA tests for other HBV antigens and antibodies, as well as PCR detection and DNA sequencing for HBV DNA. Eventually, this donor was confirmed to be HBV-positive but some HBsAg-based ELISA kits demonstrated a false-negative result for this donor’s sample. A follow-up visit to this donor indicated this blood donor was a 28-year-old male. He was a first time blood donor and blood screening indicated he was negative for anti-human immunodeficiency virus, anti-hepatitis C virus and anti-treponema pallidum antibodies.

![Fig. 1. Detection of HBsAg mutation that is able to escape from HBsAg ELISA tests and expression of the mutated HBsAg in HEK293T cells. (a) DNA sequencing of HBV HBsAg mutant. Arrows in the chromatogram indicate T to A and A to T mutations at nt 349 and 353, respectively. (b) Expression of HBsAg-M in HEK293T cells. HEK293T cells were transfected with pIRES-Neo3 vector or pIRES-Neo3 HBsAg-M for 48 h, and harvested for SDS-PAGE and Western blotting with anti-HBsAg antibody.](http://jgv.microbiologyresearch.org)

![Fig. 2. Test results from different HBsAg ELISA kits for the HBV mutant. (a) Ten commercial ELISA kits (assigned as nos 1 to 10) for HBsAg were used to test HBsAg-M and serum samples from the blood donor. The 0.5 ng ml⁻¹ Quality Control (QC) sample and a negative control (NC) from each ELISA kit were included as positive and negative controls, respectively. (b) Gradient dilution assay for the HBsAg-M serum sample. (c) Gradient dilution assay for the recombinant HBsAg-M (rHBsAg-M) expressed from HEK293T cells. The error bars represent the SD for each group. Significant differences between groups are shown by an asterisk, which indicates *P<0.05.*](http://jgv.microbiologyresearch.org)
According to his personal statement, he had no history of hepatitis or HBV-related symptoms. No HBV-infected individual was identified in his family who had close contact with him. He also denied being a drug user, having history of high-risk sex or receiving a blood transfusion in the past. Screening for other HBV antigens and antibodies demonstrated he was positive for anti-HBcAg antibody and HBeAg, but negative for anti-HBeAg. Moreover, the transaminase ALT level of this blood donor was normal (data not shown). Sequencing of HBV DNA from this donor indicated this HBV mutation belonged to HBV subgenotype C.

By analysis of the DNA sequence for the HBsAg region of this HBV mutant (GenBank accession number FJ905226), two novel mutation sites, nt 349 and 353, were identified (Fig. 1a). These two mutations led to Ser to Thr and Thr to Met substitutions in aa 117 and 118 of HBsAg, respectively. Notably, as Ser and Thr are hydrophilic amino acids

Fig. 3. HBsAg neutralization assay ELISA kits for the HBV mutant. (a) ELISAs for HBsAg-M serum sample, with or without adding the anti-HBV Ig. A Quality Control (QC) sample was included as a positive control. Only ELISA kits no. 6 and 10, which were able to detect HBsAg-M from serum, were used for ELISA. Asterisks (*) indicate an S/N value below the cut off ratio. (b) Calculation of the relative neutralization rate for ELISA kits no. 6 and 10. The error bars represent the SD for each group. Significant differences between groups are shown by an asterisk, which indicates $P<0.05$. 
but Met is a hydrophobic amino acid, the substitution of Thr to Met in aa 118 could significantly affect the protein structure and antigenicity of HBsAg, which may contribute the false-negative results during routine HBV screening. Therefore, to test this speculation, we expressed the HBsAg from a plasmid in HEK293T cells, introducing mutations to both aa 117 and 118. The HBsAg sequence containing both aa 117 and 118 mutations (hereafter referred to as HBsAg-M) was cloned into the pIRES-Neo3 vector for transient expression in HEK293T cells and successful expression of the recombinant protein was confirmed by immunoblotting using anti-HBsAg antibody (Fig. 1b).

**Mutated HBsAg from blood sample and HBsAg-M expressed from HEK293T cells could evade detection by many ELISA kits**

To test if HBsAg-M expressed in HEK293T cells could mimic the HBsAg from the blood donor, 10 commercial HBsAg ELISA kits collected from different manufacturers were used. Among all 10 ELISA kits, only kits no. 6 and 10 were able to detect HBsAg-M in the serum sample or recombinant HBsAg-M (rHBsAg-M) from the supernatant of HEK293T cells transfected with pIRES-Neo3-HBsAg-M (Fig. 2). For kits no. 6 and 10, although the S/N ratios for the donor’s serum were higher than for rHBsAg-M, these two kits did demonstrate a positive result for rHBsAg-M. However, all the other kits failed to detect HBsAg-M, although these kits maintained reactivity to the quantity control (QC) sample obtained from the Centers for Disease Control and Prevention (CDC) of China (Fig. 2a). To further confirm our results, gradient dilution assays using both ELISA kits no. 6 and 10 were conducted for HBsAg-M from serum and HEK293T-expressed rHBsAg-M. As demonstrated in Fig. 2(b, c), as the dilution fold increased, the S/N ratio decreased, which suggested a dose-dependent effect on the detectability by both kits.
for this mutated HBsAg. Moreover, neutralization assays were conducted using kits no. 6 and 10 as well. By incubating the serum sample along with anti-HBV Ig, the S/N ratio of the ELISA tests fell below the positive cut off value (2.1) for both kits (Fig. 3a), which suggested that this mutated HBsAg could be neutralized by anti-HBV Ig to evade ELISA detection. However, no significant difference in the neutralization ratio was observed between the serum sample and QC sample for both kits (Fig. 3b). Taken together, these results suggested that HBsAg bearing aa 117 Thr and 118 Met mutations could escape detection by most of the available HBsAg ELISA kits. Moreover, it also suggested that recombinant HBsAg-M expressed from HEK293T cells maintained similar antigen reactivity to the donor’s serum.

The 118 Thr to Met substitution is responsible for changing the reactivity of HBsAg in ELISA tests

As this unique HBsAg contained two mutation sites, determination of the exact mutation site causing the false-negative result was necessary. Therefore, we generated different HBsAg-M constructs by introducing point mutations at nt 349 and 353. In addition to the HBsAg-M

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Fig. 5. Identification of HBV mutants with 118 Met in HBsAg from samples of blood donors. Serum samples (134) from HBV-positive blood donors were subjected to DNA extraction and sequencing. Sequencing data of 39 samples (donors no. 1 to 39) were compared. Donor no. 5 demonstrated 118 Met in HBsAg.
described above, two more constructs were generated: HBsAg117S118T (the same amino acid residues as the reference sequence) and HBsAg117T118T (the same amino acid as the reference sequence at residue 118). Expression of these two constructs in HEK293T cells was also confirmed by Western blotting (Fig. 4a).

After generation of these constructs, we examined the reactivity of these recombinant HBsAgs with same ELISA kits used above. As shown in Fig. 4(b), the HBsAg117S118T (the same amino acid residues as the reference in both aa 117 and 118) demonstrated similar results to the QC control and could be detected by all ELISA kits. A mutation at residue 117 from Ser to Thr in HBsAg117T118T did not change the test results, as it could also be detected by all ELISA kits. The results indicated that the 118 Met mutation was responsible for the false-negative results observed with all the ELISA kits except kits no. 6 and 10 (Fig. 4b). Moreover, all ELISA kits showed the same positive results for both HBsAg117T118T and HBsAg117S118T as the QC sample, which suggested that mutation of 117 Thr did not affect the antigenicity of HBsAg. Taken together, these data indicated the 118 Thr to Met mutation significantly changed the antigenicity of HBsAg and was responsible for the evasion of detection by most of the ELISA kits used in this study.

Detection of 118 Met HBV mutant from blood donors

Since the false-negative result observed during the routine HBV screening of blood donors could be a significant safety concern, it was important to determine the prevalence of the HBV mutant bearing 118 Met (HBV118M) in the normal population. A total of 134 HBsAg positive serum samples from our routine blood screening for blood donors were collected. ELISA screening was conducted using ELISA kits no. 6 and 10 to avoid false-negative results. DNA extraction and sequencing were also conducted for all the positive samples to examine the DNA sequence of HBsAg. As the serum HBV DNA level was variable among infected individuals, DNA sequencing data were only readable for 39 samples, including the one we used in this study.

By analysis of all the HBsAg sequences we collected in this study, we observed a sample from donor no. 5 bearing a single 118 Met mutation, while its residue 117 was still Ser (Fig. 5). Moreover, a single 117 Thr mutation was also detected in donors no. 4 and 10 (Fig. 5). Therefore, the HBV118M mutation has a certain prevalence among HBV-positive individuals.

**DISCUSSION**

Although HBV is a DNA virus, it needs reverse transcriptase to replicate. HBV polymerases are composed of four domains, including a reverse transcriptase (RT) domain, which shows significant homology to retroviral RTs (Barholomeusz et al., 2004; Radziwill et al., 1990). As a result, there is a high mutation rate of HBV reverse transcriptase due to the lacking of proofreading activity, which is the main cause of HBV mutation (Deng & Tang, 2011). HBV is a very compact virus with highly efficient usage of its genome. The small S gene of HBV is fully embedded in the polymerase gene. HBV mutations that occur in the S gene not only result in false-negative results for some diagnostic tests due to the mutations of HBsAg but also probably link with drug resistance (Deng & Tang, 2011; Mizuochi et al., 2006). Antivirus therapy was extensively accepted in clinics in the past two decades, and more and more mutants with drug resistance have been reported. On the other hand, large-scale vaccination and application of hyper-immune antibodies to HBV have also led to HBV escape mutants (Zuckerman, 2000).

In this study, we identified a novel Thr to Met mutation in aa 118 of the HBsAg, which has never to our knowledge been reported before. It is still unclear what caused this mutation, because the donor denied an HBV vaccination history or any contact with other HBV carriers. At the same time, we noticed the aa 117 Thr mutation as well. However, restoration of aa 117 to Ser did not improve the diagnosis result for all the kits used in this study (data not shown). However, reconstitution of Thr at aa 118 caused a significant improvement in all the diagnosis results. These data suggest that the Thr to Met mutation at aa 118 is the contributing factor for the false-negative results during diagnosis.

The major neutralizing targets in HBsAg are conformational epitopes locating two MHRs between aa 99 and 169 (Seddigh-Tonekaboni et al., 2000; Tian et al., 2007). The reconstitution of amino acid sequence will affect the three-dimensional conformation or the hydrophilicity of this region, which changes the antigenicity of HBsAg. Although the majority of the ELISA kits used in this study failed to detect the HBsAg118M mutant, two kits were still capable of recognizing it. This may be due to the different antibodies used to coat the ELISA plates. However, even those two kits demonstrated reduced sensitivity for the 118M mutant. After reconstitution of aa 118 from Met to Thr, the S/C value demonstrated a significant improvement, indicating these two kits only partially recognized the 118M mutant.

During our small-scale screening for normal blood donors with an HBsAg positive result, only 39 serum samples were DNA positive, which was consistent with our previous experience that less than 30 % of the HBsAg positive samples were HBV DNA positive and could be sequenced (unpublished data). An earlier report demonstrated that there was a lack of correlation between HBsAg and HBV DNA levels in blood donors who tested positive for HBsAg and anti-HBcAg, and HBV DNA levels in HBsAg-positive, anti-HBc-reactive blood donations can be extremely low (Kuhns et al., 2004). In our centre, the PCR-detection-based nucleic acid amplification testing (NAT) could detect 100 copies of viral genome, which was confirmed by using an HBV standard sample (unpublished data). One possible explanation is that HBV-positive blood donors generally come from a clinically healthy population.
without HBV-related symptoms, which may bear lower HBV DNA levels than individuals with demonstrated HBV-related symptoms.

It is undeniable that the 118M mutation has a significant impact on the sensitivity of these commercial HBsAg ELISA kits. However, since the HBsAg gene overlaps with HBV Pol gene, the mutation of aa 118 in HBsAg also led to a Gln to His mutation in aa 471 of Pol. It is still unclear if this mutation in HBV Pol could affect the replication of HBV. However, analysis of a blood sample from this donor showed that the HBV load in the blood did not change much, which implied this mutant could replicate effectively and maintain a sustained infection status. It is still unclear what led to this unique mutation. In China, many HBV mutation studies have been conducted by the CDC or hospitals, and their subjects are limited to HBV patients demonstrating clinical symptoms. Little attention is paid to HBV-positive blood donors that are clinically healthy. Whether this mutation was caused by immunization or administration of anti-HBV immunoglobulin still needs further investigation.

The false-negative results of HBV-infected blood donors due to mutation had been documented (Echevarria & Avello´n, 2008; Weber, 2006). In accordance, manufacturers of HBV diagnosis products have made efforts to improve their ELISA kits to avoid false-negative results. However, as the virus is capable of mutating frequently due to the unique replication process, HBV always generates new mutations to evade the host immune system, as well as current surveillance methods. On the other hand, the large-scale immunization with HBV vaccine or administration of anti-HBV sera has caused pressure for HBV to mutate. China has been considered a highly endemic area for HBV. Although epidemiology investigation suggests that 7 % of the total population have been chronically infected by HBV, our knowledge about the infection caused by HBV mutants in the population is limited. Our research subjects were blood donors from a clinically healthy population, and were all interviewed for potential HBV infection history or risk before blood collection. In this specific population, the HBV infection ratio was much lower than the 7 % in normal population; these 134 HBsAg positive samples were identified from more than 20 000 blood donors (HBV-positive ratio less than 1 %). Therefore, it is very hard for us to conduct larger scale screening for this specific HBV mutant. Since demand for blood transfusion during surgery is extremely high due to the large population in China, the existence of HBV mutants evading current detection methods will be a high risk for blood transfusion recipients. A brief screening of the samples available in our centre demonstrated this new HBsAg 118M has limited prevalence. To assess the exact prevalence rate of HBV118M, large-scale screening will be needed. Although the prevalence of this mutant in large populations is unclear, improvement of current detection methods is needed.

**METHODS**

**General information and ethics statement.** All participating subjects were formally informed for the purpose of this study and a letter of consent was signed by every subject involved. To minimize the effect of the general information on the final result, a systematic analysis was conducted of the gender and age of those HBV-positive patients. However, no significant difference was observed.

**Primers, serum DNA extraction, enzymes, plasmids, bacteria, cells and chemicals.** Primers were designed according the HBV genotype B and C for cloning of HBsAg, introducing mutations to nt 349 and nt 353 and sequencing of MHR. All primers were synthesized by Invitrogen. The sequence of primers used in this study are listed in Table 1.

Blood donors’ serum samples were used for HBV DNA extraction. The DNA extraction was conducted using QIAamp DNA Mini kit (Qiagen) following the manufacturer’s instruction.

Table 1. Primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
<th>Target genes</th>
<th>Plasmid construct</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clone_F</td>
<td>TTAGCTAGCGCCGACCACATGGAGAACCACCAGG</td>
<td>Cloning of HBsAg isolated from mutated HBV</td>
<td>pIRES-Neo3-HBsAg-M</td>
</tr>
<tr>
<td>Clone_R</td>
<td>CTCGGATCTTTA AATGTATACCCAAAGAC</td>
<td>HBV isolated from blood donor</td>
<td></td>
</tr>
<tr>
<td>117(Ser)_F</td>
<td>ACATCAACTACCTCAATGGAGACCACGAG</td>
<td>Introducing A to T mutation in nt 349 (Thr to Ser for aa 117)</td>
<td></td>
</tr>
<tr>
<td>117(Ser)_R</td>
<td>CTTGACATTGTCGCCATGGAGTATTGTATGT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>118(Thr)_F</td>
<td>ACATCAACTACCCACCCAGGGACCACAG</td>
<td>Introducing T to A mutation in nt 353 (Met to Thr in aa 118)</td>
<td>pIRES-Neo3-HBsAg117S118M</td>
</tr>
<tr>
<td>118(Thr)_R</td>
<td>CTTGACATTGTCGCCATGGAGTATTGTATGT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>117–118(Ser-Thr)_F</td>
<td>ACATCAACTACCCACCCAGGGACCACAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>117–118(Ser-Thr)_R</td>
<td>CTTGACATTGTCGCCATGGAGTATTGTATGT</td>
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Cloning of target DNA fragments was conducted using Phusion high fidelity DNA polymerase (New England Biolabs). PCR products were purified using Gel purification kit (Takara) and digested with BamHI (Takara), HindIII (Takara) and Nhel (Takara), before being ligated into pIRE5-Neo3 vector (Takara). The insertions were confirmed by DNA sequencing.

HEK293T cells were maintained in Dulbecco’s minimal essential medium supplemented with 10 % FBS (Life Technology). The transfection reagent polyfection (Qiagen) was used for plasmid transfection according the manufacturer’s instructions. At 48 h after the transfection, the cell culture supernatant containing recombinant protein expressed from the plasmids was collected and used for ELISAs.

HBsAg diagnosis ELISA kits and standard samples (quality control). A total of 10 commercial ELISA kits from different companies were used in this study for HBsAg diagnosis. The full names of these companies are not listed here due to conflict of interest between these manufacturers. The HBsAg standard samples (quality control) were purchased from the Clinical Testing Center, China Ministry of Health. All tests were repeated at least four times.

HBsAg neutralization assay. For the HBsAg neutralization assay, 5 μl anti-HBV immune globulin (SichuanYuanda Shuyang Pharmaceutical) was added to 50 μl serum sample and incubated at 37 °C for 1 h. The mixture was then examined by the indicated ELISA kits according to manufacturers’ instructions. Serum samples with PBS added were used as blank controls. The neutralization ratio was defined as the S/N value difference between the immune globulin group and blank control divided by the S/N value of blank control.

Western blot analysis. HEK293T cells transfected with different plasmids were lysed using the Laemmli sample buffer, as previously described (Nan et al., 2012; Patel et al., 2010). SDS-PAGE and Western blots were conducted for these cell lysate as previously described (Nan et al., 2012). Briefly, proteins were separated by SDS-PAGE, transferred onto PVDF membrane and probed with mouse mAbs against HBsAg (Jingtiancheng Biotech) and tubulin (Sigma). Specific reactions were detected using goat anti-mouse IgG conjugated to HRP (Sigma) and revealed by a chemiluminescence substrate. The chemiluminescence signal was digitally recorded using the ChemiDoc XRS imaging system (Bio-Rad).

DNA sequencing of MHR from HBV-positive blood donors. A total of 134 blood samples from HBV-positive donors collected from 2008 to 2009 were used for DNA extraction. The MHR region from all samples was cloned by PCR and subjected to DNA sequencing.

Statistical analysis. Difference in indicators between samples was assessed by Student’s t-test. A two-tailed P value of less than 0.05 was considered significant.

ACKNOWLEDGEMENTS

This study was sponsored by a grant from Young Physicians Overseas Training Project from Nanjing Medical Science and Technique Development Foundation of Jiangsu Province (grant no. QRX11240), which was awarded to Y. Y. The authors declare no conflict of interest.

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