A mutation specific polymerase chain reaction for detecting hepatitis B virus genome mutations at nt551

Chun-Ling Ma, De-Xing Fang, Hua-Biao Chen, Fa-Qing Li, Hui-Ying Jin, Su-Qin Li, Wei-Guo Tan

AIM: The hepatitis B surface antigen (HBsAg) is considered to be one of the best markers for the diagnosis of acute and chronic HBV infection. But in some patients, this antigen cannot be detected by routine serological assays despite the presence of virus. One of the most important explanations for the lack of detectable HBsAg is that mutations which occur within the “a” determinant of HBV S gene can alter expression of HBsAg and lead to changes of antigenicity and immunogenicity of HBsAg accordingly. As a result, these mutants cannot be detected by diagnosis assays. Thus, it is essential to find out specific and sensitive methods to test the new mutants and further investigate their distribution. This study is to establish a method to investigate the distribution of the HBsAg mutant at nt551.

METHODS: A mutation specific polymerase chain reaction (msPCR) was established for amplifying HBV DNA with a mutation at nt551. Four sets of primer pairs, P551A-PPS, P551G-PPS, P551C-PPS and P551T-PPS, with the same sequences except for one base at 3’ terminus were designed and synthesized according to the known HBV genome sequences and the popular HBV subtypes, adr and adw, in China. At the basis of regular PCR method, we explored the specific conditions for amplifying HBV DNAs with a mutation at nt551 by regulating annealing temperature and the concentration of these primers. 126 serum samples from patients of hepatitis B were collected, among which 16 were positive for HBV S DNA in the nested PCR amplification. 14 of them were positive for nt551A, and the other one was positive for nt551T. The results were confirmed by nucleotide sequencing.

RESULTS: When the annealing temperature was raised to 71 °C, nt551A and nt551G were amplified specifically by P551A-PPS and P551G-PPS; At 72 °C and 5 pmole of the primers (each) in reaction of 25 µl volume, nt551C and nt551T were amplified specifically by P551C-PPS and P551T-PPS. 16 of HBV S gene fragments were characterized by using this method. 14 of them were positive for nt551A, one was positive for nt551G, and the other one was positive for nt551T. The results were confirmed by nucleotide sequencing.

CONCLUSION: The mutation specific polymerase chain reaction is a specific and sensitive method for detecting the mutations of HBV genome at nt551.
provided by Department of Laboratory Diagnosis, Nanjing Jinling Hospital. The viral markers were tested by using the enzyme immune assay (ELISA) methodology. All of the samples were positive for anti-HBs and negative for HBsAg. The ALT level was considered to be abnormal to all of them.

**Methods**

**Extraction of HBV genome DNAs**

DNAs was isolated and purified from 100 µL of serum samples. Proteinase K (20 mg/ml) and SDS (10 %) were added into 100 µL of sera and their concentrations in reaction were 2.5 mg/ml and 0.5 %, respectively. After a brief vortex, the mixture was heated at 70 °C for 3 hours. Then the same volume of phenol: chloroform was added to the mixture to extract DNA. The DNA pellet was obtained with 100 % ethanol precipitation, and was washed with 70 % ethanol. The dried DNA pellet was then resuspended in 20 µL of H2O and stored at -20 °C. All of 126 sera were prepared in this way.

**Amplification of HBV S DNAs**

The amplification of HBV S gene was carried out by using the nested PCR method. In the first-round PCR, the 50 µL reaction solution including 5 µL DNA template and 40 pmole (each) of the first-round primers. The mixture was heated to 94 °C for 5 min, followed by 30 PCR cycles consisting of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 60 s in a thermal cycler. And then 1.25 µL of the first-round PCR product served as the template for the second-round of PCR amplification which consisting of the same cycles except that the annealing temperature was raised to 65 °C. Positive PCR product, a DNA band of 1.2kb as expected, was detected on agarose gel electrophoresis. After amplification, we achieved 16 HBV S DNAs.

The primers for the nested PCR were designed according to the known HBV genome sequences and the main popular subtypes, adr and adw, in China, as follows:

The primers for first-round:
P1' : 5'ACATCATCTGTGGAAGGC 3', nt2756-nt2773, the upstream primer;  
P6' : 5'TATCCCATGAAGTTAAGG 3', nt884-nt867, the downstream primer;  
The primers for second-round:  
PESC: 5'CGGAATTACCATATTTCTTGGGAAACAG 3', nt838-nt816, the upstream primer;  
PPS: 5'GCTGCAAGTTAAGTTACCCAAGAC 3', nt838-nt816, the downstream primer;  
An EcoRI or a Pst I sites was originally added at 5'-end respectively for cloning purpose.

**Amplification of HBV DNA fragments for control**

To achieve the HBV DNA fragment with an A at nt551, the wild-type HBV S DNA as template was amplified by using the primer pair P551A-PPS under the condition of regular PCR. The HBsAg mutant with a G at nt551 as template was amplified by using the primer pair P551G-PPS to achieve the HBV DNA fragment with a G at nt551. The HBV DNA fragment with an A at nt551 was achieved by using introducing mutation in a PCR. The PCR primer sequences were as follows:

P551A: 5'TCTTGCTCAAGAGAACCCTCTTA 3', nt532-nt551, upstream primer;  
P551G: 5'TCTTGCTCAAGAGAACCCTCTTG 3', nt532-nt551, upstream primer;  
P551C: 5'TCTTGCTCAAGAGAACCCTCTCT 3', nt532-nt551, upstream primer;  
P551T: 5'TCTTGCTCAAGAGAACCCTCTTT 3', nt532-nt551, upstream primer;  
PPS: See the above.

P551C-PPS and P551T-PPS were used respectively to amplify HBV DNA fragments with a C or T at nt551, which are 314 bp long. Additionally, two upstream primers had been designed respectively by introducing mutation in order to achieve the controls of HBV DNA with a C or T at nt551.

**Establishment of msPCR**

In order to amplify HBV DNA specifically, the annealing temperature of PCR was regulated according to the Tm. The reaction volume of PCR was 25 µL. The PCR reaction was carried out by using P551A-PPS, P551G-PPS, P551C-PPS and P551T-PPS as primer pairs to amplify HBV S DNA templates with an A, G, C, or T at nt551, respectively.

**Application of msPCR**

Under the condition of the msPCR method, the 16 of samples which were positive for HBV S DNA as templates were amplified by using P551A-PPS, P551G-PPS, P551C-PPS and P551T-PPS as primer pairs respectively.

**HBV S DNA sequencing**

In order to confirm the validity of the msPCR, the purified PCR products of HBV S gene fragments from selected samples according to the results of msPCR, NO.2, NO.5, NO.8 and NO.57 were sequenced by using the primer PPS.

**RESULTS**

**HBV DNA fragments for control**

The HBV S DNA with an A, G, C or T at nt551 was amplified respectively for control. This result is shown in Figure 1.

![Figure 1](image1.png)

**Figure 1** Amplification of Control HBV DNA. Lane 1: DNA Marker; Lane 2: HBV DNA with an A at nt551 amplified by P551A-PPS; Lane 3: HBV DNA with a G at nt551 amplified by P551G-PPS; Lane 4: HBV DNA with a C at nt551 amplified by P551C-PPS; Lane 5: HBV DNA with a T at nt551 amplified by P551T-PPS. The amplified DNA fragments are 314 bp long.

![Figure 2](image2.png)

**Figure 2** Establishment of the msPCR for nt551A and nt551G Lane 1: DNA Marker; Lane 2-5: P551A-PPS amplified HBV DNAs of control (nt551A in Lane 2, nt551G in Lane 3, nt551C in Lane 4, nt551T in Lane 5), Lane 6-9: P551G-PPS amplified HBV DNAs of control (nt551A in Lane 6, nt551G in Lane 7, nt551C in Lane 8, nt551T in Lane 9).

![Figure 3](image3.png)

**Figure 3** Establishment of the msPCR for nt551C and nt551T Lane 1: DNA Marker; Lane 2-5: P551C-PPS amplified HBV DNAs of control (nt551A in Lane 2, nt551G in Lane 3, nt551C in Lane 4, nt551T in Lane 5), Lane 6-9: P551T-PPS amplified HBV DNAs of control (nt551A in Lane 6, nt551G in Lane 7, nt551C in Lane 8, nt551T in Lane 9).
Establishment of msPCR

When the annealing temperature was set from 65 °C to 70 °C, the results of amplification were not specific to the four primer pairs. In other words, the templates were amplified by no-matching primers. Then the annealing temperature was raised to 71 °C, the specific amplified results were found for P551A-PPS and P551G-PPS; When the annealing temperature was raised to 72 °C and the concentration of the primers (each) were at 5 pmole in reaction of 25 µl volume, P551C-PPS and P551T-PPS amplified HBV DNAs with C or T at nt551 specifically. The specific results to establish msPCR method are shown in Figure 2 and Figure 3.

Application of msPCR

16 samples were tested by using the msPCR method. This result is shown in Table 1.

HBV S DNA sequencing

According to the results of msPCR, four DNA samples, No.2, No.5, No.8 and No.57 were selected to sequence. The sequencing results showed that No.2 was a T at nt551, both No.5 and No.8 were an A at nt551, and No.57 was a G at nt551, confirming the results of the msPCR. The sequencing results are shown in Figure 4.

DISCUSSION

In the recent years, mutant HBsAg have caused great academic interest[19,20] and many analyses and researches have been made for the emergence of HBV mutants with mutations in the “a” determinant of HBsAg which result in immune escape[13,16,21]. According to these research results, it is very important to further investigate mutant distributions and clarify mutations in HBV S gene which could cause the changes of antigenicity and immunogenicity of HBsAg[3,12,22,23]. It is well known that the mutants of HBsAg were able to cause infection and horizontal transmission despite the presence of anti-HBs[25-29]. The increasing use of HBV vaccine has had overwhelming positive influence on the prevention of hepatitis B viral infection, but have no effective impact to those mutants[30]. The mutations in the coding region of “a” determinant of HBsAg could not be detected in some routine assays[31,32].

This study was to obtain a specific and sensitive method for monitoring the HBsAg mutant with a mutation at nt551. The method of msPCR is different from immunoassays that are based on the antigen-antibody reaction[33,34]. To detect mutant HBsAg, the unique specific monoclonal antibodies are required. But these kinds of antibodies are limited or not available commercially. Because HBV is a double-stranded DNA virus, its genome is fairly stable in the blood and tissue, the PCR amplification of HBV DNA is relatively easy[35]. The msPCR is actually a method to detect the specific site mutation. This method was firstly developed to detect site mutation of allele-special genes of β-globin genome DNA for sickle cell anemia[36]. And then it was used in virological studies. This mechanism was used in our study.

The msPCR was established at the basis of the known HBV DNA. The primers were only one-base different from each other at 3’ terminus, thus the non-specific DNA should not be amplified at regular value of Tm. However, we considered the variability of annealing temperature and set it as high as possible. When it was 71 °C and 72 °C, the ideal result was found respectively for different primer pairs. The process confirmed that the annealing temperature is the key factor to establish msPCR method of nt551. Additionally, the concentration of the primers is also an important factor for this msPCR. In short, different primers amplify HBV DNA specifically with different conditions.

The aim of this study was to detect the mutation of the known HBV S gene at nt551. All of 126 serum samples were collected from hepatitis B patients in hospital. After the nested PCR amplification, 16 samples were positive for HBV S gene. The msPCR detection showed that 14 of them were an A at nt551, one was a G at nt551, and the other was a T at nt551. The reliability of msPCR was confirmed by sequencing analysis of four samples. A special attention should be paid to No.2. It is a T at nt551, resulting in a Met (ATG) to Leu (TTG) change at aa133 of HBsAg. Whether this mutation caused the change of antigenicity need further identification. In addition to these mutations in HBsAg “a” determinant, there were several other mutations in S gene. These results further confirmed the diversity and popularity of HBV S gene mutation.

In conclusion, this msPCR is a sensitive and specific approach for the detection of mutations in HBV S gene, and will have tremendous potential in screening HBsAg mutants and further investigating their distribution in patients of hepatitis B.

REFERENCES

1. Koyanagi T, Nakamura M, Sakai H, Sugimoto R, Enjoji M, Koto K, Iwamoto H, Kumazawa T, Mukaide M, Nawata H. Analysis...
of HBs antigen negative variant of hepatitis B virus: unique substitutions, Glu129 to Asp and Gly145 to Ala in the surface antigen gene. M ed Sci M etod 2000; 6: 1156-1169

2 Bruno M R, Rodriguez UA, Bonino F. Hepatitis B virus mutants. Intervirology 1999; 42: 69-80

3 Kfoury Baz EM, Zheng J, Mazuruk K, Van Le A, Peterson DL. Characterization of a novel hepatitis B virus mutants demonstration of mutation-induced hepatitis B virus surface antigen group specific “a” determinant conformation change and its application in diagnosis assays. Transfus M ed 2001; 11: 355-362

4 Dong J, Cheng J, Wang Q, Huangfu J, Shi S, Zhang G, Li L, Li C. The study on heterogeneity of hepatitis B virus. Zhonghua Yixue Zazhi 2002; 82: 81-85

5 Kreutz C. Molecular, immunological and clinical properties mutated hepatitis B viruses. J Cell M ed 2002; 6: 113-143

6 Zhong S, Chan JY, Yee W, Tam JS, Johnson PJ. Hepatitis B envelope protein mutants in human hepatocellular carcinoma tissues. J Viral Hepat 1999; 6: 195-202

7 Weinberger KM, Zoulek G, Bauer T, Bohm S, Jilg W. A novel deletion mutant of hepatitis B virus surface antigen. J M ed Virol 2000; 58: 105-110

8 Roznovsky L, Harrison T, Fang ZL, Ling R, Lochman I, Orsagova I, Pliskova L. Unusual hepatitis B surface antigen variant in a child immunized against hepatitis B. J M ed Virol 2000; 61: 11-14

9 Carman WF, Zanetti AR, Karayiannis P, Waters J, Tanzi E, Zuckerman AJ, Thomas HC. Vaccine-induced escape mutant of hepatitis B virus. Lancet 1990; 336: 325-329

10 Yang X, Lei J, Zhang Y, Luo H, Huang L, Zhang Y, Tang X. A novel stop codon mutation in S gene: the molecular basis of a patient with cryptogenic cirrhosis. Zhonghua Yixue Zazhi 2002; 82: 400-402

11 Zhu Q, Lu Q, Xiong S, Yu H, Duan S. Hepatitis B virus S gene mutants in infants infection despite immunoprophylaxis. Chin Med J 2001; 114: 321-324

12 Fang DX, Li FQ, Tan WG, Chen HB, Jin HY, Li SQ, Lin HJ, Zhou ZX. Transient expression and antigenic characterization of HBsAg of HBV nt551 A to G mutant. Antiviral Res 1999; 14: 179-185

13 Santantonio T, Gunther S, Sterneck M, Rendina M, Messner M, Launois F, Francavilla A,Pastore G, Will H. Liver graft infection by HBV S-gene mutants in transplant patients receiving long-term HBig prophylaxis. Hepatogastroenterology 1999; 46: 1848-1854

14 Rodriguez-Frias F, Bult M, Jardi R, Vargas V, Quer J, Cotrina M, Martel M, Esteban R, Guardia J. Genetic alterations in the S gene of hepatitis B virus in patients with acute hepatitis B, chronic hepatitis B and hepatitis B liver cirrhosis before and after liver transplantation. Liver 1999; 19: 177-182

15 He C, Nomura F, Itoga S, Isobe K, Nakai T. Prevalence of vaccine-induced escape mutants of hepatitis B virus in the adult population in Chinese prospective study in 176 restaurant employees. J Gastroentero Hepatol 2001; 16: 1373-1377

16 Cooreman MP, Leroux-Roels G, Paulij WP. Vaccine and hepatitis B immune globulin-induced escape mutations of hepatitis B virus surface antigen. J Biomed Sci 2001; 8: 237-247

17 Komatsu H, Fujisawa T, Sogo T, Isozaki A, Inui A, Kobata M, Ogawa Y. Acute self-limiting hepatitis B after immunoprophylaxis failure in an infant. J M ed Virol 2002; 66: 28-33

18 Heijstink RA, Van Bergen P, Van Roosmalen MH, Paulij WP, Schalm SW, Osterhaus AD. Anti-HBs after hepatitis B immunization with plasma-derived and recombinant DNA-derived vaccines binding to mutant HBsAg. J Vaccine 2001; 19: 3671-3680

19 Francois G, Kew M, van Damme P, Mphahlele M, Meheus A. Mutants hepatitis B virus: a matter of academic interest only or a problem with far-reaching implications? Vaccine 2001; 19: 3799-3815

20 Burda M R, Gunther S, Dandri M, Will H, Petersen J. Structural and functional heterogeneity of nature occurring hepatitis B virus variants. Antiviral Res 2001; 52: 125-138

21 Coleman PF, Chen YC, Mushahwar IK. Immunoassay detection of hepatitis B surface antigen mutants. J M ed Virol 1999; 59: 19-24

22 Torresi J. Earned-Silveira L, Civitico G, Wales T, Levin SR, Fyfe J, Locarnini SA, Manns M, Trautwein C, Bock TC. Restoration of replication phenotype of lamivudine-resistant hepatitis B virus mutants by compensatory changes in the “fingers” subdomain of the viral polymerase selected as a consequence of mutations in the overlapping S gene. Virology 2002; 299: 88

23 Cooreman MP, van Roosmalen MH, te Morsche R, Sunnen CM, Van EM, Jansen JB, Tyt tgat GN, de Wit PL, Paulij WP. Characterization of the reactivity pattern of monomucosal antibodies against wild-type hepatitis surface antigen to GI45R and other naturally occurring loop escape mutations. Hepatology 1999; 30: 1287-1292

24 Wu L, Yuan ZH, Liu F, Waters JA, Wen YM. Comparing the immunogenicity of hepatitis B virus gene variants by DNA immunization. J Viral Immunol 2001; 14: 359-367

25 Oon CJ. C, Wen, Kew MC. Molecular analysis of hepatitis B virus genomes isolated from black African patients with fulminant hepatitis B. J M ed Virol 2001; 65: 405-492

26 Nanter j K, Gupta RC, Bisht R, Sarin SK, Khandekar P. Identification of a novel surface mutant of hepatitis B virus in a seronegative chronic liver disease patient. Virology 1999; 65: 103-109

27 Chen WN, Oon CJ. Hepatitis B virus surface antigen mutation in Singapore adults and vaccinated children with high anti-HBs virus antibody levels but negative for HBsAg. J Clin Microbiol 2000; 38: 2793-2794

28 Oon CJ, Chen WN, Goo KS, Goh KT. Intra-familial evidence of horizontal transmission hepatitis B virus surface antigen mutant GI45R. J Infect 2001; 40: 261-264

29 Chen WN, Oon CJ, Koh S. Horizontal transmission of a hepatitis B virus surface antigen mutant. J Clin Microbiol 2000; 38: 938-939

30 Schories M, Peter T, Rasenack J. Isolation, characterization and biological significance of hepatitis B virus mutants from some of a patient with immunologically negative HBV infection. J Hepatol 2000; 33: 799-811

31 Weinberger KM. Bauer T, Bohm S, Jilg W. High genetic variability of the group-specific adeterminant of hepatitis B virus surface antigen (HBsAg) and the corresponding fragment of the viral polymerase selected as a consequence of mutations in the overlapping S gene. J Hepatol 2000; 32: 1165-1174

32 Chen WN, Oon CJ, Moh MC. Detection of hepatitis B virus surface antigen mutants in paraffin-embedded hepatocellular carc tissues. Virus Genes 2000; 20: 263-267

33 Ijaz S, Torre R, Teedder RS, Williams R, Naoumov NV. Novel immunoassay for the detection of hepatitis B virus escape mutants and its application in transplant recipients. J Med Virol 2001; 63: 210-216

34 Jolivet-Reynaud C, Lesenechal M, O Donnell B, Becquart L, Fousadier A, Forge F, Bataille-Poiriot N, Carman W, Jolivet M. Localization of hepatitis B surface antigen epitopes present on variants and specifically recognised by anti-hepatitis B surface antigen monoclonal antibodies. J M ed Virol 2001; 65: 241-249

35 Wormman JF, Feng L, Mamiya N. Molecular biology and the diagnosis and treatment of liver diseases. World J Gastroentero 1998; 4: 185-191

36 Wu DY, Ugozzoli L, Pall BK, Wallace RB. Allele-specific enzymatic amplification of β-globin genomic DNA for diagnosis of sickle cell anemia. Proc Natl Acad Sci USA 1989; 86: 2757-2760