Detection of HBV Genome by Gene Amplification Method in HBsAg Negative Blood Donors

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Abstract

The incidence of post-transfusion hepatitis has been reduced greatly by screening blood donors for hepatitis B surface antigen (HBsAg). However, hepatitis B virus infection still accounts for a certain number of cases of post-transfusion hepatitis. The purpose of this study was to detect HBV DNA in the HBsAg negative blood samples by using nested PCR with two primer pairs specific to core region. Two hundreds blood samples from HBsAg negative donors, and 14 samples from HBsAg positive donors were provided by the blood bank of Ramathibodi Hospital. The results showed that HBV DNA was detected in all 14 HBsAg positive blood samples and in 7 (3.5%) of 200 HBsAg negative blood samples.

This study showed that the absence of HBsAg in otherwise apparently healthy individuals may not be enough to ensure lack of circulating HBV. The more sensitive ELISA technique is still in need. Otherwise, the safety of blood transfusion can be enhanced by careful selection of blood donors and careful consideration of risks and benefit of the patients who need blood transfusions.

Key word : HBsAg, PCR, Blood Donor, HBV DNA, ELISA

The routine screening of donated blood for presence of hepatitis B surface antigen (HBsAg) was introduced in 1971 and the more sensitive third generation testing for HBsAg was introduced in 1975[1,2]. Even though the HBsAg test has significantly reduced the incidence of post-transfusion hepatitis caused by hepatitis B virus (HBV), HBV associated post-transfusion hepatitis still occurs in 0.3-1.7 per cent of the recipients of HBsAg negative blood and accounted for 7-10 per cent of all post-transfusion hepatitis cases[3]. A few reports have documented occasional post-transfusion hepatitis B infection in recipients of HBsAg negative blood[4,5]. By using the polymerase chain reaction

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(PCR) technique, HBV DNA was detected in 4 per cent of HBsAg negative blood donors in Taiwan[6] and in 6.9 per cent of anti-HBc positive blood donors in Japan[7]. The purpose of our study was to use the PCR technique which amplified the regions in C gene for assessment of the prevalence of HBV DNA in 200 blood donors who were HBsAg negative by screening with the third generation ELISA assay.

MATERIAL AND METHOD

Blood units

A total of 200 HBsAg negative and 14 HBsAg positive plasma samples were obtained from the blood bank of Ramathibodi Hospital. Routine screening of blood donors for presence of HBsAg was performed by the third generation enzyme immunoassay (Abbott laboratories, Illinois, U.S.A.).

Preparation of DNA samples

Viral DNA was purified from plasma samples as described previously by Chomcznyski[8]. Briefly, a 200 µl of plasma was incubated with 100 µl of saturated phenol and 100 µl chloroform/isoamyl alcohol (24:1) and 200 µl of 4 M Guanidium isothiocyanate solution for 1-2 minutes, then centrifuged at 10,000 rpm for 15 minutes. The aqueous phase was harvested and mixed with 50 µl 3M Sodium acetate (pH 5.2) plus 1,000 µl of cold absolute ethyl alcohol and incubated for 2 hours or over night at -20°C. The DNA pellet was collected by spinning the solution at 10,000 rpm for 10 minutes, and then, dried and redissolved in TE (Tris-EDTA) buffer. This DNA was used as a template for PCR.

Amplification of HBV core region by nested PCR

DNA samples were amplified by a nested PCR technique using two primer sets from a highly conserved core region of the HBV genome (11). The sequence of the forward strand outer primer, 5'-GCTTTGGGGCATGGACATTGACCCG TATAA-3' was between map position 1763-1793 of the HBV genome. The complementary or reverse (R) outer primer, strand 5'-CTGACTACTAATTCCCT GGATGCTGGGTCT-3' was at map position 2032-2002. The sequence of forward strand inner primer, 5'-GACGAATTCCATTGACCCGTATAAAGA ATT-3' was at map position 1778-1808, and it was synthesized to contain the EcoRI restriction endonuclease recognition cleavage site at the 5' end. The complementary or reverse (R) strand inner primer, 5'-ATGGGATCCCTGGATGCTGGGTCTTCCA AA-3' was at map position 2017-1987, and was synthesized to contain the BamHI recognition cleavage site at the 5' end. A DNA sample was amplified in a 100 µl reaction volume containing sterile water, 2.5 unit of Taq DNA polymerase, 200 µM of each d NTPs, 1 µM of each primer, 2.5 mM of $MgCl_2$, and 1 x PCR buffer solution. The amplification cycles were as the followings : denaturing at 94°C for 5 minutes, annealing at 45°C for 1 minute, and extending at 72°C for 1 minute for the first 1 cycle : 94°C for 1 minute, 45°C for 1 minute, 72°C for 1 minute, for 33 cycles : 94°C for 1 minute 45°C for 1 minute, 72°C for 5 minutes, for the last cycle. For reamplification, a 10 µl aliquot of the primary PCR product was amplified as described above using an inner primer pair. To eliminate the source of DNA contamination, all reagents were prepared and stored in new disposable containers. Reagents were aliquoted, and reaction mixtures were prepared with the use of disposable syringes and pipettes. All reagents were assayed for the presence of HBV DNA, and all experiments were performed in parallel with positive and negative control sera.

Analysis of amplified DNA

A 2.5 µl of the amplified DNA product was fractionated by gel electrophoresis in 2 per cent NuSeive® agarose, and the DNA band was visualized over the UV light after staining with ethidium bromide solution. DNA marker was Gelmarker™ (Research Genetics Inc. Huntsville, U.K.). Gelmarker™ contains linear double stranded DNA bands of 1000, 700, 525, 500, 400, 300, 200, 100 and 50 base pairs.

RESULTS

Detection of HBV DNA by amplification of core region using nested PCR

Fourteen HBsAg positive plasma were amplified by nested PCR using a set of primers specific to core region. Amplified DNA at expected size of 270 bp was detected in all HBsAg positive plasmas. (Fig. 1)

DNA extracted from 200 HBsAg negative plasma samples were tested and it was found that 7 (3.5%) of 200 samples were positive for HBV DNA.

The results of the investigation of all plasma samples are shown in Table 1.

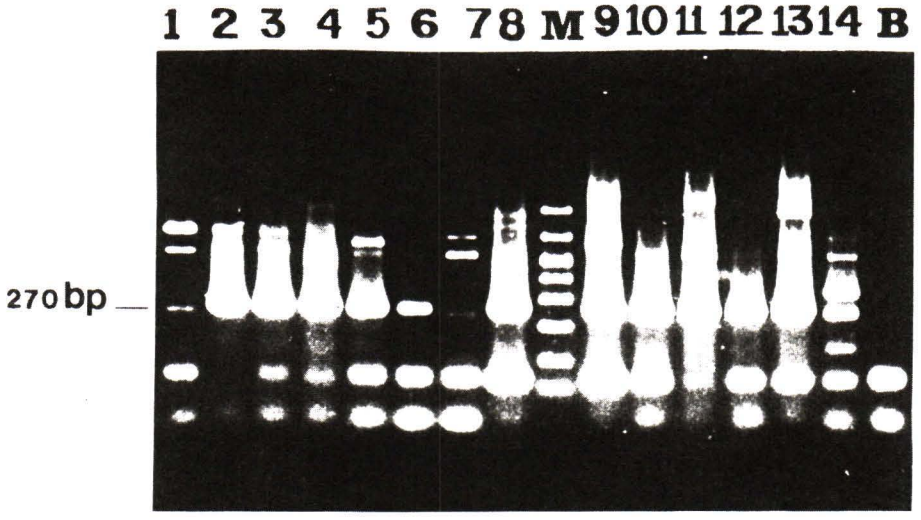

Fig. 1. Amplified products by nested PCR (C gene) from 14 HBsAg positive blood samples
Lane 1-8 and 9-14, HBsAg positive;
Lane M, DNA molecular weight marker ranging from 50 - 1000 bp;
Lane B, Blank

Table 1. Investigation for presence of HBV DNA in plasma samples as determined by amplification of C regions.

Samples	No. tested	Number % of samples positive by DNA amplification of C region
HBsAg negative plasma	200	7 (3.5)
HBsAg positive plasma	14	14 (100)

Detection of anti-HBc in HBV DNA positive serum samples

The seven plasma samples which were positive for HBV DNA but negative for HBsAg were further investigated for presence of anti-HBc by ELISA (Abbot laboratories, Illinois, U.S.A.) The result showed that only 4 samples were positive for anti-HBc.

DISCUSSION

Hepatitis B infection is a major health problem in Southeast Asia. Although serologic hepatitis B markers are convenient for detecting HBV infection. The seronegativity of HBsAg or other HBV markers does not absolutely mean the absence of HBV DNA and infectivity[12,13]. It was reported that a small number of donors who are negative for all hepatitis B serologic markers may still harbor HBV DNA and be potentially infectious[14]. Wang et al[6] detected HBV DNA in 9 (4%) of 206 HBsAg negative blood donors in Taiwan by using PCR assay. One study[15] used PCR to document the presence of HBV DNA in a patient who had no serologic markers for hepatitis B but whose blood was shown to transmit HBV infection to a chimpanzee. The detection of HBV DNA in 7 (3.5%) of 200 blood donors who were negative for HBsAg in our study raised an interesting epidemiological and public health issues which is particularly relevant to the blood banks. Presence of HBV DNA in sera of HBsAg - negative individuals could be explained by several possibilities such as HBsAg being present in undetectable levels; or it was masked by anti-HBs in the form of HBsAg-anti-HBsAg complexes, and

thus became undetectable by regular immunoassays(16).

Our study has demonstrated the presence of HBV DNA in 7 of 200 HBsAg negative plasma samples, and anti-HBc was detected in 4 of these 7 samples. This finding suggested that these 4 blood donors might be chronic healthy carriers with an undetectable level of HBsAg. In addition, the absence of anti-HBc in the remaining 3 cases could be explaned in such a way that the stage of HBV infection is very early, i.e. before the occurrence of anti-HBc synthesis. The events may be common in a hyperendemic area like Thailand. Our finding was also supported by Sun C-F et al(17), who reported that two of seven HBsAg negative but HBV DNA positive sera were negative for all HBV serological markers.

Although PCR is the most sensitive method in detecting HBV DNA, the expense of reagents such as Taq polymerase and the tedious work may make it not suitable for routine screening of large numbers of samples at present. Therefore, the current serologic screening method remains the best, considering the cost and effectiveness. However, it is impossible to exclude all infectious donors by serologic screening of HBV markers. The safety of blood transfusion can also be enhanced by careful selection of blood donors and careful consideration of risks and benefit of patients who need blood transfusions. To further avoid post-transfusion hepatitis B, perhaps, with future improvement in technology, PCR may be an effective screening test not only for HBV but for other transfusion transmitted pathogens as well.

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การตรวจหาสารพันธุกรรมของไวรัสตับอักเสบ บี โดยวิธีเพิ่มปริมาณยีนในเลือดของผู้บริจาคโลหิตที่มีผลลบต่อ เอชบีเอส แอนติเจน

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จากการตรวจกรองหา เอช บี เอส แอนติเจน (HBsAg) ของเลือดที่บริจาคก่อนให้ผู้ป่วย ทำให้จำนวนผู้ติดเชื้อโรคไวรัสตับอักเสบ บี (HBV) หลังการได้รับเลือดลดจำนวนลงมาก แต่ก็ยังมีรายงานการติดเชื้อไวรัสตับอักเสบหลังการได้รับเลือดที่มี เอช บี เอส เป็นลบอยู่เป็นครั้งคราว ความประสงค์ของการศึกษานี้เพื่อตรวจหาสารพันธุกรรมของไวรัสตับอักเสบ บี ในเลือดที่มี เอช บี เอส เป็นลบ โดยวิธี nested PCR ซึ่งใช้ primers จำเพาะต่อยีน ซี (Core region) ผลของการวิจัยพบสารพันธุกรรมของไวรัสตับอักเสบ บี ในผู้บริจาคเลือด 7 ราย (3.5%) จากจำนวนเลือดที่มี เอช บี เอส แอนติเจนเป็นลบ 200 ราย การศึกษานี้แสดงว่าเลือดที่มี เอช บี เอส แอนติเจนเป็นลบ มีโอกาสที่จะทำให้เกิดโรคติดเชื้อไวรัสตับอักเสบ บี ได้ อย่างไรก็ตามปัจจุบันการตรวจกรองเลือดโดยวิธี เซโรโลยี ยังคงเป็นวิธีที่ดีที่สุด โดยอาศัยการคัดเลือกผู้บริจาคและคำนึงถึงความเสี่ยงและความจำเป็นของผู้ป่วยด้วย จนกว่าจะมีการพัฒนาเทคนิคทาง PCR ให้ง่ายและเหมาะสมกว่านี้

คำสำคัญ : เอชบีเอส แอนติเจน, PCR, เลือดบริจาค, สารพันธุกรรมของไวรัสตับอักเสบบี, ELISA

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