別紙様式第２－１

医薬品　研究報告 調査報告書

識別番号・報告回数		報告日	第一報入手日	新医薬品等の区分	総合機構処理欄
一般的名称	赤血球、血小板	研究報告の公表状況	Transfusion (United States) Aug2008, 48 (8) p1602-8	公表国：米国	
販売名(企業名)	－				

研究報告の概要

供血時点にはB型肝炎に関する血清検査で陰性であったが、その後HBV DNAが検出された供血者から血液成分(赤血球、血小板)の輸注を受けた2例の免疫不全患者について報告する。
供血者は39歳男性で、供血時点は血清検査陰性であったが、6週間後に採取した検体では抗HBc抗体陽性(HBs抗原、抗HBs抗体は未検出)となり、その後の検査でHBV DNAが検出された。
1例は化学療法により免疫不全状態にあった重症な急性リンパ性白血病の9歳女児で、HBVワクチンにより低レベルの抗HBs抗体を獲得していたが、赤血球輸注から13ヵ月後に急性B型肝炎を発症した(発症までの間、全てのHBVマーカーは陰性)。感染原因は、供血血液中にHBV DNAが存在し、供血者と受血者の遺伝子配列(pre-S/SとBCP/PC)の同一性から、輸血による感染と確認された。
もう1例は化学療法による免疫不全状態にあった骨髄異形性症候群の65歳女性(HBs抗原、HBc抗体陰性、抗HBs抗体低レベル陽性)で、先の症例と同じ供血者から得た血小板の輸注を受けていたが、感染はしなかった。
両受血者の当該輸血前の低レベル抗HBs抗体の存在は、ウイルス量がHBsAg検出限界であると考えられる1000～3000コピー/mL未満と低いため、保護的役割(感染成立の阻害)を果たした可能性がある。また、両受血者は偶然に別の供血者からの高力価抗HBs抗体を含有する血漿又は血小板を輸注されており、HBVの受動抗体を受けていた。両受血者の違いは、1例目が輸血当日に受動抗体を受けたのに対し、2例目は感染源の血小板を投与されてから3日後に受動抗体を受けたことであった。
比較的少量のHBVに曝露した両受血者では、HBVワクチン接種または受動免疫化による予防、あるいは逆に化学療法や免疫抑制による易感染性などの人的介入により、ウイルス感染の早期段階を古典的に定義する変数がかなり修飾されることが示された。この結果、本報告のように複雑な条件下では、輸血、再燃および院内要素を分けて考える必要があり、その解明には進歩した分子方法が最も有用である。

使用上の注意記載状況・その他参考事項等

重要な基本的注意
(1) 本剤の原材料となる(献血者の)血液については、HBs抗原、抗HCV抗体、・・・・陰性で、かつALT(GPT)値でスクリーニングを実施している。さらに、プールした試験血漿については、HIV-1、HBV及びHCVについて核酸増幅検査(NAT)を実施し、適合した血漿を本剤の製造に使用しているが、当該NATの検出限界以下のウイルスが混入している可能性が常に存在する。

報告企業の意見	今後の対応
後にHBV DNA陽性と判明した血液によるB型肝炎感染の報告である。 当社血漿分画製剤の製造工程におけるHBVのモデルウイルスに対するウイルスクリアランス指数は9以上である。なお、原料血漿はミニプール血漿における NAT 検査で HBV DNA 陰性を確認しており、最終製品においても HBV DNA 陰性を確認している。	今後ともにB型肝炎ウイルス感染に関する安全性情報に留意していく。

TRANSFUSION COMPLICATIONS

A probable case of hepatitis B virus transfusion transmission revealed after a 13-month-long window period

Silvano Wendel, José E. Levi, Silvana Biagini, Daniel Candotti, and Jean-Pierre Allain

BACKGROUND: Transfusion-transmitted hepatitis B virus (HBV) infection in recipients with drug-related immunodeficiency is rarely described in endemic areas. Hepatitis B surface antigen (HBsAg)–negative infectious donor blood can be identified by sensitive nucleic acid testing (NAT). Two immunodeficient patients who received blood components from a single seronegative blood donor subsequently found to contain HBV DNA are described.

MATERIALS AND METHODS: Multiple samples from the implicated donor and the two recipients were tested for HBV serologic and molecular markers. HBV genome fragments were amplified, sequenced, and phylogenetically analyzed.

RESULTS: The implicated donation had low-level HBV DNA due to the donor being in the window period before the donor's seroconversion. Recipient 1 had been vaccinated to HBV and carried anti-HBs but remained negative for all other HBV markers until she developed acute hepatitis B (viral load 2.7×10^6 IU/mL and alanine aminotransferase [ALT] level 1744 IU/L) 13 months after transfusion of red cells. Identical HBV sequences from both donor and recipient provided evidence of transfusion-related infection. Recipient 2, who received platelets from the same donation while receiving major chemotherapy, remained uninfected.

CONCLUSIONS: In unusual circumstances, HBV incubation time can be considerably prolonged. Both active and passive neutralizing antibodies to HBV likely delayed, but did not prevent, acute infection when the immune system was impaired. HBV NAT may have interdicted the infectious unit, although the donation viral load could not be quantified and odds of detection calculated.

Among blood-borne viruses of major concern in transfusion, hepatitis B virus (HBV) presents the highest residual risk,[1] despite several serologic markers available for screening. HBV DNA testing is routinely performed in Germany[2] and Japan[3] and, more recently, in several additional European countries.[4] HBV DNA testing is an expensive alternative to anti-HBc in place for years in several low-prevalence countries but remains cost-prohibitive in areas of higher prevalence to avoid blood shortage. Genomic screening can be performed on individual donations or in plasma pools ranging between 6 and 96, although it was shown that pooling reduces significantly the yield of DNA-containing donations.[4,5] In Brazil, despite relatively high prevalence of the marker, anti-HBc screening is mandatory and a few blood banks also routinely test blood donations for both hepatitis C virus (HCV) and human immunodeficiency virus (HIV) RNA but not for HBV DNA.[6] A fundamental limitation of anti-HBc screening is the inability to detect window-period, highly infectious, donations. The preseroconversion window period has been extensively studied in serial plasma donor samples and typically ranges between 37 and 87 days (median, 59 days).[7] Post-transfusion infection was not systematically investigated but the early stages were assumed to be of similar or shorter duration due to the large volume of the inoculum. The protective effect of anti-HBs has been well established as well as the increased susceptibility to HBV infection of

ABBREVIATIONS: BCP = basic core promoter; PC = precore.

From the Blood Bank, Hospital Sírio Libanês, São Paulo, Brazil; the National Health Service Blood and Tissue, Cambridge Blood Center, and the Department of Haematology, University of Cambridge, Cambridge, UK.

Address reprint requests to: S. Wendel, Blood Bank, Hospital Sírio Libanês, Rua Adma Jafet 91, São Paulo, Brazil 01308-050; e-mail: snwendel@uninet.com.br.

Received for publication December 18, 2007; revision received February 4, 2008, and accepted February 4, 2008.
doi: 10.1111/j.1537-2995.2008.01723.x
TRANSFUSION 2008;48:1602-1608.

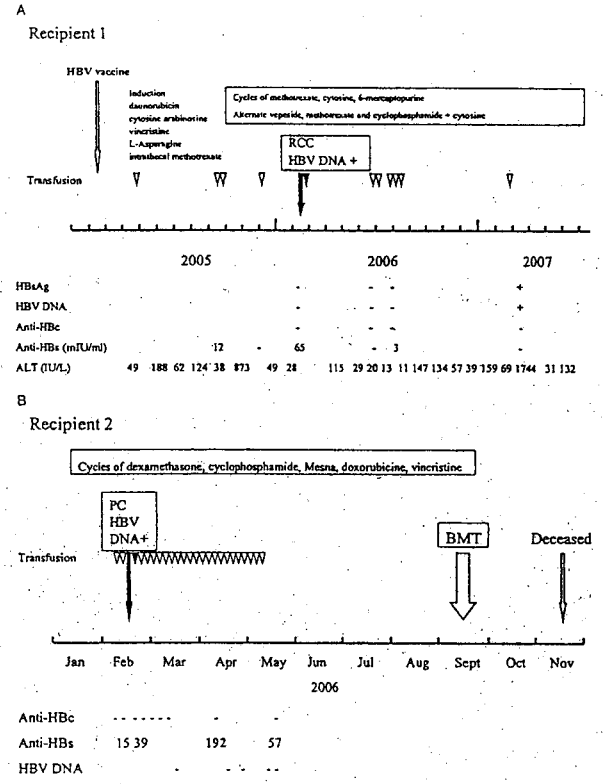

Fig. 1. Case description. (A) Summary of Recipient 1 clinical history. The implicated transfusion of RBCs is indicated by a full arrow. Other transfusions received are indicated as open triangles. The filled triangle indicates the blood product containing high titer of anti-HBs. Bolded ALT levels indicate values above 5 times upper normal level. The HBV infectious component and the PLTs containing high anti-HBs level were transfused on the same day. (B) History of Recipient 2. Symbols are as in Recipient 1 (A). This patient received a PLT concentrate (PC). The interval between receiving the infectious PC and the PC containing high anti-HBs was 3 days. BMT = bone marrow transplantation.

immunodeficient recipients of organs from anti-HBs–carrying donors.

Here are presented two cases of immunodeficient recipients of blood components from a single unit containing very low levels of HBV DNA. One of these recipients developed acute HBV infection 13 months after transfusion despite carrying vaccine-induced anti-HBs while the other was not infected.

CASE REPORT

On March 6, 2007, the hospital notified the blood center that a 9-year-old female child suffering from a high-grade acute lymphoblastic leukemia (Recipient 1), diagnosed in April 2005, was experiencing a clinical episode of acute hepatitis B. Serologic tests confirmed this diagnosis: the presence of hepatitis B surface antigen (HBsAg) and anti-HBc immunoglobulin M (IgM) and an alanine aminotransferase (ALT) level of 1744 IU per L later supported by an HBV DNA load of 2.7×10^8 IU per mL. The patient history revealed 24 transfusions including 13 units of red cell (RBC) and 11 apheresis platelet (PLT) concentrates between April 26, 2005, and August 13, 2006 (Fig. 1A). During this period, she received chemotherapy according to the PROPII-97 protocol consisting of induction by daunorubicin, cytosine arabinosine, vincristine, dexamethasone, and L-asparaginase as well as intrathecal methotrexate/dexamethasone/cytosine-arabinoside. Maintenance treatment consisted of alternate cycles of high-dose methotrexate and cytosine with 6-mercaptopurine, followed by alternate cycles of vepesid plus methotrexate and cyclophosphamide plus cytosine.

Records from the implicated donors were examined and most were excluded as the source of HBV infection because at least one subsequent donation was negative for the presence of HBsAg and anti-HBc. One donor, however, whose RBCs were transfused to the child on February 23, 2006, also donated PLTs by apheresis on March 30, 2006, and subsequent testing results indicated a seroconversion to anti-HBc, without detectable HBsAg, anti-HBs, or HBV-DNA.

A plateletpheresis concentrate prepared from the index automatic blood donation of February 23 (Trima, Gambro BCT, Lakewood, CO) was transfused to a second patient (Recipient 2), a 65-year-old female diagnosed with high-risk myelodysplastic syndrome evolving to biphenotypic leukemia. At the time of the suspect transfusion, she was receiving Hyper-CVAD (ondosetin, dexamethasone, cyclophosphamide, Mesna, doxorubicine, and vincristine) plus intrathecal QT (methotrexate and aracytin). She was negative for the presence of HBsAg and anti-HBc but had a low level of anti-HBs (13 mUI/mL). In September 2006, she received marrow transplantation in another hospital where no clinical or laboratory evidence of HBV infection was observed. She died of sepsis in November 2006.

Unfortunately, when retrospective investigation was initiated, the archive sample of the implicated donation had already been discarded from the repository according to the national policy mandating the storage of a sample from nonreactive donations for 1 year. Two separate aliquots of 230 µL of plasma, however, had been archived for potential investigation, allowing us to perform polymerase chain reaction (PCR) amplification and DNA sequencing for comparison with recipient data.

TABLE 1. HBV markers in samples from the implicated donor

Date of sample collection	HBsAg	Anti-HBc* sample OD/cut-off	Anti-HBs	HBV DNA
February 17, 2006	Negative	Negative	ND	ND
Repository samples February 17, 2006	Negative	Negative (0.866/0.407)	ND	Positive
March 31, 2006	Negative	Reactive (0.142/0.382)	Negative	Negative

* Hepanostika anti-HBc Uniform, BioMerieux, Boxtel, the Netherlands. Specificity in package insert is 99.85 percent.
OD = optical density; ND = not done.

MATERIALS AND METHODS

Serologic testing

Anti-HBc (Abbott/Murex, Delkenheim, Germany), HBsAg (Axsym MEIA, Abbott Laboratories, Abbott Park, IL), and anti-HBs (Axsym MEIA, Abbott) testing was performed according to the manufacturer's instructions. Anti-HBs levels are expressed in mIU per mL.

Molecular testing

DNA was extracted from 200 µL of serum and/or plasma with a DNA blood mini kit (QIAamp, Qiagen, Hilden, Germany) in Brazil and either tested locally or shipped to the UK in dry ice. HBV DNA was detected initially by one-step PCR using 7 µL of extract DNA submitted to a fast PCR protocol (Applied Biosystems, Foster City, CA) in the presence of 1 µmol per L of each primer OY1 sense (5'-CAAGGTATGTTGCCCGTTTG-3') and OY2 antisense (5'-AAAGCCCTGACCACTGA-3'),[8] in a final volume of 25 µL. Nested PCR was performed on 12.5 µL of DNA in a 25-µL reaction (final volume) as previously described.[9] All PCR procedures were performed in a thermocycler (Model 9700, Applied Biosystems). Two nested PCR procedures were used to amplify a 276-bp fragment located in the basic core promoter (BCP) and precore (PC) regions and a 1434-bp fragment spanning the whole pre-S/S gene, as previously described.[10] Sequences of BCP/PC and pre-S/S regions were obtained by direct sequencing of amplicons. Sequences were aligned with reference HBV genotype A to H sequences using computer software (Clustal W software implemented in Mac Vector Version 7.2, Accelrys, San Diego, CA), and the alignments were confirmed by visual inspection. Phylogenetic analysis was performed using computer software (PAUP 4.0b10, Sinauer Associates, Inc., Sunderland, MA) after exclusion of positions containing an alignment gap from pairwise sequence comparisons. Nucleotide distances were analyzed by neighbor-joining algorithm based on Kimura two-parameter distance estimation. To confirm the reliability of the phylogenetic trees, bootstrap resampling was performed for each analysis (1000 replicates).

RESULTS

Analysis of the implicated donation sample and donor

Upon retesting, the repository sample gave the same serologic results as in the screening (anti-HBc and HBsAg nonreactive) but HBV DNA was detected by two distinct PCR methods, both single-step and nested PCR. The first assay has a limit of detection of 500 IU per mL and the second of 100 IU per mL, and both showed clear amplicons, suggesting that, although not properly quantified, the viral load was above 500 IU per mL. Viral load, however, could not be quantified due to the limited sample availability. Of note, the patient and the donor samples were processed 3 weeks apart, the donor sample first, and were kept in different freezers, limiting considerably the possibility of cross-contamination. On the basis of phylogenetic analysis of the pre-S/S gene, the sample was classified as genotype A1. Translation of the "a" region of the S gene indicated a wild-type amino acid sequence when compared to the genotype consensus sequence. The BCP/PC region was also wild type without mutation in either the 1762 to 1764 doublet or the 1896 nucleotide of PC codon 18 or in any of the start codons for PC or core sequences.

When retested from a sample collected 6 weeks after the index donation, the donor plasma showed clear anti-HBc seroconversion but no HBsAg or anti-HBs detectable (Table 1). Other HBV serologic markers such as IgM anti-HBc could not be tested for lack of available sample volume.

The donor was a 39-year-old male who denied risk factors. He was of mixed race, partly of African origin. His donation did not react for anti-HIV and anti-HCV.

Recipient 1

A summary of the Recipient 1 data is presented in Fig. 1A. Before transfusion of the implicated component, anti-HBs was present at low levels on two occasions as expected in a child previously vaccinated to HBV. ALT levels were fluctuating around upper normal levels except on two occasions in May and October 2005 and 2006 when levels reached 188 and 873 IU per L. In the subsequent absence of markers of HBV infection, these high ALT levels could be attributed to the underlying disorder and the chemotherapy. In the period after the transfusion of the implicated component, HBV DNA or serologic markers were never detected until the acute HBV infection 13 months later. During this period, as in the preceding year, ALT levels fluctuated but did not exceed four times upper normal levels. Between transfusion in February 2006 and the acute episode in March 2007, the patient received seven blood components. A single dose of PLT concentrate obtained from a double unit of PLTs prepared by apheresis containing an anti-HBs titer of greater than 1000 mIU per mL was transfused on February 23, 2006, the same day as the implicated HBV DNA containing RBCs. The amount of plasma transfused with the PLTs was approximately 125 mL.

Seven samples collected from Recipient 1 between February 2006 and August 2006 did not contain detectable HBV DNA. After a period of 7 months without transfusion, a sample collected on March, 30, 2007 contained a viral load of 2.7×10^4 IU per mL. This strain was sequenced in the BCP/PC and pre-S/S regions. The latter sequence was phylogenetically analyzed and revealed a genotype A1. When these sequences were aligned with the corresponding sequences obtained from the suspected donation, the 276- and 1202-nucleotide-long sequences, respectively, were identical except for one ambiguity. Within the pre-S/S region, Sample SL167648 (donor) showed a sequence ambiguity (adenosine/guanine) at nucleotide 231 starting from the ATG of the S protein. This suggested the presence of quasispecies in the donor while at position 231 only guanine was detected in the recipient sequence. Phylogenetic analysis of the pre-S/S region showed that recipient and donor sequences clustered with HBV genotype A1 reference sequences of African origin, supported by bootstrap values of 100 percent over 1000 replicates. On that basis, the relationship between donor and recipient HBV infection was clearly established. Since HBV genotype A1 in Brazil is essentially found in Brazilians with African ancestry, racial origins of donor and recipient were examined. The donor was of mixed African origin and the recipient was Caucasian.

Recipient 2

Recipient 2 received the PLT concentrate prepared from the same donor and donation transfused to Recipient 1. Follow-up samples collected up to June 2006 (3 months after transfusion) did not reveal the presence of any serologic or molecular marker of HBV infection (Fig. 1B). Before receiving the PLT concentrate from the suspected blood unit, a low titer of anti-HBs was detected acquired either from active or from passive immunity to HBV. The elevation of anti-HBs titer to 192 mIU per mL observed in April 2006 was probably related to passive immunization since, coincidentally, the second unit of a double-plateletpheresis concentrate collected from the same strongly anti-HBs–reactive donation (>1000 mIU/mL) whose PLTs were transfused to Recipient 1 was transfused to Recipient 2. This concentrate contained approximately 125 mL of plasma and was transfused 3 days after the implicated PLT concentrate. Overall, despite receiving PC from an infectious blood donation, no evidence of HBV infection was found in this immunosuppressed adult patient to date.

DISCUSSION

Posttransfusion viral infection has been the focus of considerable scrutiny after the occurrence of HIV infections related to transfusion. Although receiving considerably less attention, reporting of HBV posttransfusion infection has been limited by screening for specific HBV markers such as HBsAg and anti-HBc in some low-prevalence countries. More recently, genomic screening for HBV has become available and was implemented in several countries either in pools of plasma from blood donations or in individual donations. Most anti-HBc screening countries, however, do not feel that it is necessary to screen for HBV DNA and hence do not address the risk of window period. Countries where HBV infection is relatively high (European Mediterranean countries or Poland) as well as some relatively affluent countries with high infection prevalence (Southeast Asia) started screened for DNA to avoid deferring a number of donors that would endanger the blood supply to patients.

Few studies describe the duration of the window period in humans. Most investigate blood donors where the origin of the infection was mostly unknown or post-transfusion. The latter situation had the peculiarity of a large volume of inoculum (100-250 mL) compared to no more than 5 mL in the situation of intravenous drug use, nosocomial infection, or vertical or sexual transmission. In a study conducted in the 1950s, inmates were inoculated with Australian antigen-positive serum; the interval between infection and detection of HBV antigen was 45 to 92 days (mean, 77 days) but longer when the inoculum was diluted 1:1000 (92-130 days).[11] The infectious dose seems therefore to influence the duration of the window

period. Other elements possibly interfering in the time interval between viral contact and seroconversion to HBsAg (window period) such as the state of the immune system of the infected individual or the presence of specific neutralizing antibodies to HBsAg have not yet been systematically examined. Only in the situation of transplantation of organs from donors carrying anti-HBs with or without detectable HBV DNA was evidence of infection provided in patients receiving immunosuppressive drugs for liver transplantation.[12] In contrast, experiments conducted in chimpanzees indicated that, in immunocompetent animals, low levels of HBV in the presence of anti-HBs were not infectious.[13] It has also been well known for many years that the risk of developing chronic HBV infection was inversely proportional to the immunocompetence of children.[14] In none of these circumstances, however, was the duration of the window period or the level of preseroconversion viral load addressed.

In the complicated and discrepant cases presented here, several areas of uncertainty require discussion. First is the authentication of the donation as source of Recipient 1 infection and as a window-period donation. This implication is based on two main elements: 1) the presence of HBV DNA in the donation and 2) the identity of pre-S/S and BCP/PC sequences between donor and recipient. The presence of HBV genome in the implicated donation was found in two separate laboratories in Brazil and in England using different amplification methods and targeted regions. These positive results are strongly supported by obtaining sequences from two such regions. The hypothesis of laboratory contamination is unlikely because the prevalence of chronic hepatitis is 0.2 percent in blood donors in the São Paulo blood center (limiting the possibility of sample to sample cross-contamination) and amplification of HBV in the donor and recipient samples was performed 3 weeks apart from samples stored in different freezers. Finally, being of genotype A1 in a donor of partial African origin is the most plausible since in an unpublished study of 33 strains of HBV from the same blood center, 52 percent of strains were of genotype A1 (J.P. Allain and M. Premnath, unpublished). This dominance of genotype A1 was confirmed by several other studies in Brazil.[15,16] The donor seroconversion to anti-HBc 42 days after the implicated donation without anti-HBs or HBsAg is not totally convincing (Table 1). While HBV DNA as sole evidence of HBV recent infection strongly suggests being in the window period, the negativity of HBV DNA, HBsAg, and anti-HBs in the second sample is unexpected, unless the stage of infection in the follow-up sample corresponds to the second window period, after disappearance of HBsAg and possibly DNA before the occurrence of anti-HBs. Unfortunately, no further sample was obtained from this donor.

While the identical sequence of more than 1500 cumulated bases between donor and recipient HBV strains leaves little doubt about the donor being responsible for the infection, once contamination of the donor sample has been excluded, the discrepancy of the outcome of HBV contact between the two recipients raises multiple questions. Although both patients received chemotherapy accompanied with assumed substantial immunosuppressive effects and similar volumes of HBV DNA–containing plasma (110 and 180 mL for Recipients 1 and 2, respectively), only Recipient 1 developed infection. Neither age nor volume of the inoculum could significantly affect the ability to develop an immune response since, at age 9, the maturity of the immune system is comparable to that of an adult. The presence of low levels of anti-HBs before the implicated transfusion in both recipients might have played a protective role, particularly as the blood component viral load was low, below 1000 to 3000 copies per mL, which is considered the limit of detection for HBsAg.[17,18] Coincidentally, both recipients received passive antibodies to HBV in the form of 125 mL of plasma containing high-titer anti-HBs from the same double-plateletpheresis donation. One difference between the two patients was that Recipient 1 received 125 mL of this plasma the day of transfusion with the implicated product while Recipient 2 received the same volume of plasma 3 days after being in contact with the implicated PLT concentrate. Since the suspected viral strain was wild type in the S region, there is a high likelihood that anti-HBs either raised by vaccine or passively transmitted was neutralizing the circulating virus.

Recipient 1 did not receive any transfusion during the 7 months preceding the episode of acute hepatitis B and, therefore, no reinforcement of her low level of anti-HBs. During the same period of time, the immunosuppressive effect of the chemotherapy accumulated and one can speculate that at one point, the precarious protection offered by low-level neutralizing antibodies became insufficient to contain the virus that started actively replicating.

Posttransfusion HBV infection window period typically ranges between 37 and 87 days in HBV-only infection and between 80 and 110 days when HCV coinfection was present.[7] The prolongation of the interval between infectious contact and evidence of active viral replication in Recipient 1 was unexpected and remains difficult to explain. Conflicting factors are at play. First the chemotherapy received by the patient to treat leukemia had likely some immunosuppressive effect, which was expected to shorten the window period and facilitate viral replication. In contrast, prior HBV vaccination and passive immunization was expected to prevent or at least delay the clinical expression of the infection. One hypothesis to explain the evidence is that most of the virus received by transfusion was complexed by neutralizing antibodies either actively acquired by vaccination or passively transmitted. Some free virus, however, may have persisted in the liver, escaping the immune system until the level of immunodefi-

ciency was such that viral replication could take place. This hypothesis is compatible with the surprising absence of detectable HBV DNA in the middle of this long window period in two samples collected in July and August 2006, 5 and 6 months after the infectious contact. Typically, after the eclipse period of approximately 2 weeks during which no evidence of viral DNA is found, low levels of HBV DNA without detectable HBsAg are detectable during the window period.[17,19,20] Recently a very similar case to ours was published, reporting a 19-week window period in a leukemia patient receiving unspecified chemotherapy regimen and carrying anti-HBs passively transmitted by PLT transfusion (58 mIU/mL) at the time of receiving the low-viral-load window-period donation.[21]

In view of these inconsistencies, the hypothesis of an HBV reactivation from a previously recovered HBV infection can be formulated. Strains mutated in the antigenic "a" region of the S gene, however, are usually found together with anti-HBc.[22] In this case, the absence of detectable anti-HBc and the wild-type genotype A1 (Recipient 1 was Caucasian) of the sequenced strain are strong argument against such hypothesis.

These two recipients in contact with a relatively low amount of HBV illustrated that human intervention, whether preventive such as HBV vaccination or passive immunization or to the contrary facilitating infection such as chemotherapy or immunosuppression can considerably modify the variables classically defining the early stages of a viral infection. As a result, in complicated situations such as described here, advanced molecular methods can be most helpful to resolve cases where transfusion, reactivation, and nosocomial elements may need to be separated.

ACKNOWLEDGMENT

We acknowledge Dr Vicente Odone Filho for referring Recipient 1 for investigation.

REFERENCES

1. Schreiber GB, Busch MP, Kleinman SH, Korelitz JJ. The risk of transfusion-transmitted viral infections. N Engl J Med 1996;334:1685-90.
2. Roth WK, Weber M, Petersen D, Drosden C, Buhr S, Sireis W, Weichert W, Hedges D, Seifried E. NAT for HBV and anti-HBc testing increase blood safety. Transfusion 2002; 42:869-75.
3. Meng Q, Wong C, Rangachari A, Tamatsukuri S, Sasaki M, Fiss E, Cheng L, Ramankutty T, Clarke D, Yawata H, Sakakura Y, Hirose T, Impraim C. Automated multiplex assay system for simultaneous detection of hepatitis B virus DNA, hepatitis C virus RNA, and human immunodeficiency virus type 1 RNA. J Clin Microbiol 2001;39:2937-45.
4. Brojer E, Grabarczyk P, Liszewski G, Mikulska M, Allain J-P, Letowska M. Characterization of HBV DNA positive/HBsAg negative blood donors identified in the Polish NAT screening program. Hepatology 2006;44:1666-74.
5. Owusu-Ofori S, Temple J, Sarkodie F, Anokwa M, Candotti D, Allain JP. Pre-donation screening of blood donors with rapid tests: implementation and efficacy of a novel approach to blood safety in resource-poor settings. Transfusion 2005;45:133-40.
6. Wendel S, Levi JE, Takaoka DT, Silva IC, Castro JP, Torezan-Filho MA, Ghaname J, Gioachini R, Durigon EL. Primary screening of blood donors by NAT testing for HCV-RNA: development of an "in-house" method and results. Rev Inst Med Trop Sao Paulo 2007;49:177-85.
7. Mimms L, Mosley JW, Hollinger FB, Aach RD, Stevens CE, Cunningham M, Vallari DV, Barbosa LH, Nemo GJ. Effect of concurrent acute infection with hepatitis C virus on acute hepatitis B virus infection. BMJ 1993;307:1095-7.
8. Yokosuka O, Tagawa M, Omata M. PCR detection of hepatitis B virus. In: Persing D, editor. Diagnostic molecular microbiology, principles and applications. Washington, DC: ASM Press; 1993. p. 2-3.
9. Allain JP, Candotti D, Soldan K, Sarkodie F, Phelps B, Giacherti C, Shyamala V, Yeboah F, Anokwa M, Owusu-Ofori S, Opare-Sem O. The risk of hepatitis B virus infection by transfusion in Kumasi, Ghana. Blood 2003;101: 2419-25.
10. Candotti D, Opare-Sem O, Rezvan H, Sarkodie F, Allain JP. Molecular and serological characterization of hepatitis B virus in deferred Ghanaian blood donors with and without elevated alanine amino transferase. J Viral Hepat 2006;13: 715-24.
11. Barker LF, Murray R. Relationship of virus dose to incubation time of clinical hepatitis and time of appearance of hepatitis-associated antigen. Am J Med Sci 1972;263: 27-33.
12. Roche B, Samuel D, Gigou M, Feray C, Virot V, Schmets L, David MF, Arulnaden JL, Bismuth A, Reynes M, Bismuth H. De novo and apparent de novo hepatitis B virus infection after liver transplantation. J Hepatol 1997;26:517-26.
13. Prince AM, Lee DH, Brotman B. Infectivity of blood from PCR positive, HBsAg negative, anti-HBs-positive cases of resolved hepatitis B infection. Transfusion 2001;41:329-32.
14. Hyams KC. Risks of chronicity following acute hepatitis B virus infection: a review. Clin Infect Dis 1995;20:992-1000.
15. Motta-Castro AR, Martins RM, Yoshida CF, Teles SA, Panago AM, Lima KM, Gomes SA. Hepatitis B virus infection in isolated Afro-Brazilian communities. J Med Virol 2005;77:188-93.
16. Araujo NM, Mello FC, Yoshida CF, Niel C, Gomes SA. High proportion of subgroup A' (genotype A) among Brazilian isolates of hepatitis B virus. Arch Virol 2004;149:1383-95.
17. Biswas R, Tabor E, Hsia CC, Wright DJ, Laycock ME, Fiebig EW, Peddada L, Smith R, Schreiber GB, Epstein JS, Nemo GJ, Busch MP. Comparative sensitivity of HBV NATs and HBsAg assays for detection of acute HBV infection. Transfusion 2003;43:788-98.
18. Kuhns MC, Kleinman SH, McNamara AL, Rawal B, Glynn S, Busch MP. Lack of correlation between HBsAg and HBV DNA levels in blood donors who test positive for HBsAg and anti-HBc: implications for future HBV screening policy. Transfusion 2004;44:1332-9.
19. Yoshikawa A, Gotanda Y, Itabashi M, Mineshigi K, Kanemitsu K, Nishioka K. Hepatitis B NAT virus-positive blood donors in the early and late stages of HBV infection: analyses of the window period and kinetics of HBV DNA. Vox Sang 2005;88:77-86.
20. Yoshikawa A, Gotanda Y, Minegishi K, Taira R, Hino S, Tadokoro K, Ohnuma H, Miyakawa K, Tachibana K, Misoguchi H. Lengths of hepatitis B viremia and antigenemia in blood donors: preliminary evidence of occult (hepatitis B surface antigen-negative) infection in the acute stage. Transfusion 2007;47:1162-71.
21. Gerlich WH, Wagner FF, Chudy M, Harrisho] LH, Lattermenn A, Wienzek S, Glebe D, Sanievski M, Schuttler CG, Wend UC, Willems WR, Bauerfeind U, Jork C, Bein G, Platz P, Ullum H, Dickmeiss E. HBsAg non-reactive HBV infection in blood donors: transmission and pathogenicity. J Med Virol 2007;79:S32-S36.
22. Tabor E. Infections by hepatitis B surface antigen gene mutants in Europe and North America. J Med Virol 2006;78:S43-S47. □

別紙様式第2-1

No. 37

医薬品 研究報告 調査報告書

識別番号・報告回数			報告日	第一報入手日	新医薬品等の区分	総合機構処理欄
				2008. 10. 17	該当なし	
一般的名称	人全血液		研究報告の公表状況	Satoh K, Iwata-Takakura A, Yoshikawa A, Gotanda Y, Tanaka T, Yamaguchi T, Mizoguchi H. Vox Sang. 2008; 95(3): 174-80.	公表国 日本	
販売名(企業名)	人全血液-LR「日赤」(日本赤十字社) 照射人全血液-LR「日赤」(日本赤十字社)					

研究報告の概要

○B型肝炎ウイルス(HBV)DNAおよびHBV表面抗原の新規濃縮方法:オカルトHBV感染検出方法への応用

背景:輸血後B型肝炎ウイルス(HBV)感染のリスクは、HBV核酸増幅技術(NAT)の導入後減少したが、HBV DNA陽性かつ表面抗原(HBsAg)陰性オカルトHBV感染の問題は未解決である。その理由の一つは、オカルトHBV感染はミニプールNATにより検出するにはHBV DNA量が少なすぎることである。HBVコア抗体(HBcAb)の検査は、オカルトHBV感染を完全には排除していない。そのため、検出感度を上げるために、HBV DNAとHBsAgを同時に濃縮する新規方法を開発した。

方法:二価金属存在下でpoly-L-lysineを使用し、ウイルス凝集反応を増強させ、ウイルスを濃縮する。濃縮処理時間を短縮するためにpoly-L-lysineでコートした磁気ビーズ法を用いる。HBcAb陽性およびHBsAg陰性供血血液77本について、酵素免疫法(EIA;AxSYM、Abbott社)および赤血球凝集阻害検査(日本赤十字社)により、HBsAgおよびHBcAbをそれぞれ調べた。

結果:HBV DNAとHBsAg量は、最高4〜7倍に濃縮された。この方法により、HBcAb陽性およびHBsAg陰性供血者77名のうち35名は個別NATにてHBV DNA陽性となり、更に供血者5名はHBVの濃縮によりHBV DNA陽性となった。オカルトHBV感染者40名のうち27名は、HBsAgの濃縮によりHBsAg陽性となった。

結論:HBV DNAおよびHBsAgを濃縮する我々の新しい方法により、EIAとHBV NATの感度が上昇し、HBsAg EIAを用いてオカルトHBV感染者40名のうち27名を検出することができた。

使用上の注意記載状況・その他参考事項等

人全血液-LR「日赤」
照射人全血液-LR「日赤」

血液を介するウイルス、細菌、原虫等の感染
vCJD等の伝播のリスク

報告企業の意見	今後の対応
HBV DNAとHBsAg量を、同時に最高4〜7倍に濃縮することで、EIAとHBV NATの感度が上昇し、HBV DNA量が少ないオカルトHBV感染者40名のうち27名を検出することができたとの報告である。	日本赤十字社では、HBs抗原検査及びHBc抗体検査を実施することに加えて、HBVについて20プールでスクリーニングNATを行い、陽性血液を排除している。また、これまでの凝集法と比べて、より感度の高い化学発光酵素免疫測定法(CLEIA)及び精度を向上させた新NATシステムを導入した。HBV検査に関する新たな知見等について今後も情報の収集に努める。

MedDRA/J Ver.11.0J

(2)

JRC2008T-064

Vox Sanguinis (2008) 95, 174–180
© 2008 The Author(s)
Journal compilation © 2008 Blackwell Publishing Ltd.
DOI: 10.1111/j.1423-0410.2008.01091.x

ORIGINAL PAPER

A new method of concentrating hepatitis B virus (HBV) DNA and HBV surface antigen: an application of the method to the detection of occult HBV infection

K. Satoh,[1] A. Iwata-Takakura,[1] A. Yoshikawa,[1] Y. Gotanda,[1] T. Tanaka,[2] T. Yamaguchi[3] & H. Mizoguchi[1]

[1]Japanese Red Cross Saitama Blood Center, Hidaka, Saitama, Japan
[2]Tokyo Women's Medical University, International Research and Educational Institute for Integrated Medical Sciences (IREIMS), Kawada-cho, Shinjuku-ku, Tokyo, Japan
[3]The National Institute of Health Sciences, Division of Biological Chemistry and Biologicals, Kamiyoga, Setagaya-ku, Tokyo, Japan

Vox Sanguinis

Received: 29 May 2008,
revised 17 July 2008,
accepted 18 July 2008,
published online 5 August 2008

Background The risk of post-transfusion hepatitis B virus (HBV) infection has been reduced after the implementation of HBV nucleic acid amplification technology (NAT). However, the problem of HBV DNA-positive and HBV surface antigen (HBsAg)-negative occult HBV infections remains to be solved. This is in part due to the HBV DNA load being too low to detect these occult HBV infections using mini-pool NAT. In Japan, the assay for the antibody against the HBV core antigen (anti-HBc) has not completely excluded occult HBV infection. To solve this problem, we have developed a new method of concentrating HBV DNA and HBsAg simultaneously to increase the sensitivity of detection tests.

Methods Virus concentration is achieved by the enhancement of the agglutination of viruses using poly-L-lysine in the presence of a bivalent metal. Poly-L-lysine-coated magnetic beads are used to shorten the time of each step of the concentration procedure. Seventy-seven anti-HBc-positive and HBsAg-negative donations were examined. HBsAg and anti-HBc were tested by enzyme immunoassay (EIA) (AxSYM; Abbott) and haemagglutination inhibition test (Japanese Red Cross), respectively.

Results HBV surface antigen and HBV DNA levels were concentrated up to four- to sevenfold. Using this method, 35 of the 77 anti-HBc-positive and HBsAg-negative donors were HBV DNA-positive by individual NAT and a further five donors became HBV DNA-positive by HBV concentration. Twenty-seven of 40 occult HBV infections became HBsAg-positive by HBsAg concentration.

Conclusion Our new method of concentrating HBV and HBsAg increased the sensitivities of EIA and HBV NAT, and enabled us to detect 27 of 40 occult HBV infections by HBsAg EIA.

Key words: anti-HBc, concentration of HBV DNA, concentration of HBsAg, occult HBV infection, poly-L-lysine-coated magnetic beads.

Introduction

More than 350 million people worldwide are chronically infected with hepatitis B virus (HBV) [1]. HBV is one of the most important viral infections transmitted by transfusion. Nucleic acid amplification technology (NAT) screening has widely been introduced for hepatitis C virus (HCV) and human immunodeficiency virus, and has greatly reduced the risk of transfusion-transmitted infection by these viruses. In contrast, HBV NAT has not been widely implemented, in part due to assay sensitivity issues. HBV therefore remains a source of post-transfusion infection. The risk of post-transfusion HBV infection has been reduced after the implementation of

Correspondence: Akira Yoshikawa, Japanese Red Cross Saitama Blood Center, 1370-12 Takahagi, Hidaka-shi, Saitama-ken, 350-1213, Japan
E-mail: yoshikawa@saitama.bc.jrc.or.jp

HBV NAT in Japan, and other countries reduce the risk of transmission by using assays with increased sensitivity for the detection of HBV surface antigen (HBsAg) [2–8]. These approaches have reduced the window period in the early stage of infection. The problem of occult HBV infection, recently defined as individuals who are HBsAg-negative and HBV NAT-positive regardless of the presence or absence of antibody to hepatitis B core antigen (anti-HBc) and antibody to hepatitis B surface antigen (anti-HBs), however, remains to be solved. Anti-HBc screening of blood donations has reduced the risk of occult HBV infection [9–13]. However, in HBV endemic areas such as Asia, anti-HBc screening is not generally utilized, because the rate of positivity is so high that many blood products would be discarded. One possible solution to this problem is to modify the cut-off value of the anti-HBc test and also to take into account the titre of anti-HBs. Using this approach, the Japanese Red Cross (JRC) has succeeded in reducing the frequency of post-transfusion HBV infections, particularly post-transfusion fulminant HBV infection [14,15]. However, the problem of occult HBV infection has not been completely removed and each year a number of cases of transfusion-associated HBV continue to be reported [16,17]. In an attempt to address this, the cut-off value of anti-HBc has been decreased and the sensitivity of HBV NAT testing increased by reducing the pool size from 50 to 20 and also increasing the input volume for the NAT assay from 0·2 ml to 0·85 ml [15]. However, there are limitations for the strategy from the view point of cost-effectiveness.

We have developed a new method of concentrating HBsAg and HBV, which could improve the detection of occult HBV infection. The principle of virus concentration is to induce the agglutination of viruses and poly-L-lysine in the presence of a bivalent metal. Poly-L-lysine-coated magnetic beads are used to shorten each step in the concentration procedure.

Materials and methods

Samples

Hepatitis B virus surface antigen-positive and/or anti-HBc-positive donations that did not meet standard JRC requirements were collected with the cooperation of blood centres in the eastern part of Japan from March 2003 to June 2006. None of these donations were used for transfusion purposes. Two hundred and fifty-nine donations were available. These were subdivided into 2·5-ml tubes and stored at –20°C. The remaining plasma from the donation was also stored at –20°C. Of the 259 donations, 182 were HBsAg-positive by enzyme immunoassay (EIA) (AxSYM®; Abbott Laboratories, North Chicago, IL, USA) and 77 were anti-HBc-positive [$\geq 2^5$ by haemagglutination inhibition assay (HI), JRC in-house], HBsAg-negative (EIA; AxSYM®) and anti-HBs-negative [$< 2^4$ (less than 200 mIU/ml)] by passive haemagglutination assay (JRC in-house). An anti-HBc titre $\geq 2^5$ by HI is equal to $\geq 2^7$–2^8-fold diluted sample that is positive (≥ 50% inhibition) by anti-HBc EIA (AxSYM®).

The 77 anti-HBc-positive donations were used to study the efficacy of the HBV DNA and HBsAg concentration techniques.

Preparation of poly-L-lysine-coated magnetic beads

COOH magnetic beads (125 mg/2·5 ml) (IMMUTEX-MAG™; Japanese Synthetic Rubber, Tokyo, Japan) were added to 0·1 M 2-morphorinoethansulphate (MES) (Wako Pure Chemical, Tokyo, Japan) solution (final volume, 5·0 ml; pH 5·0) and were incubated for 10 min. Activated magnetic beads (25 mg/ml) were suspended in a coupling buffer [5 ml of 100 mM MES (pH 5·0), 50 µl of 100 mg/ml poly-L-lysine (Wako) and 1·2 ml of distilled water] and mixed by continuous inversion at room temperature for 15 min. Then 1·25 ml of 1-ethyl-3-(3-dymethyl-aminopropyl)-carbodiimido (Wako) solution was added to the mixture and mixed by continuous inversion at 10°C for 20 h. Then the solution was replaced with 1 M ethanolamine (Wako) to block reactions at 4°C overnight. Poly-L-lysine-coated magnetic beads were washed five times with phosphate-buffered saline (PBS) and stored at 4°C at a concentration of 50 mg/ml.

It takes 3 days to prepare the poly-L-lysine-coated magnetic beads. Initially, the poly-L-lysine-coated magnetic beads were manufactured in house as described above. Subsequently they have been purchased from JSR.

Concentration of HBsAg and HBV DNA

Poly-L-lysine-coated magnetic beads were added to 2 ml of plasma at a final concentration of 1 mg/ml. Then, 30 µl of 1·1 M $Zn(COOH)_2$ was added to the sample. The resulting mixture was mixed and left to stand for 5 min. The agglutinated HBsAg/HBV DNA and magnetic beads were trapped in a magnetic field (MagicalTrapper®, Toyobo, Tokyo, Japan) and washed twice with PBS to remove impurities. The concentrated HBsAg was eluted with 0·25 ml of 0·4 M ethylenediaminetetraacetic acid (EDTA) solution. The whole volume of the sample was eluted for EIA testing (AxSYM®, Abbott) (effective eightfold concentration). HBV DNA was eluted with 100 µl of 0·4 M EDTA solution and 50 µl or 100 µl was used for individual NAT (10- or 20-fold concentration, respectively). The concentration and elution process takes 30 min.

HBV DNA extraction and quantification

Hepatitis B virus DNA was extracted using an Ex-R&D kit® (Sumitomo Chemical, Tokyo, Japan). HBV DNA was detected quantitatively as described previously [3]. Briefly, to quantify

the HBV DNA, nucleic acid extracts were amplified and titrated by using a sequence-detection system (TaqMan, ABI Prism 7700 Sequence Detector; PE Applied Biosystems, Foster, CA, USA). Quantification of the HBV DNA was calculated from the working curve (10^7, 10^6, 10^5, 10^4, 10^3 and 10^2 copies/ml) produced by domestic standard samples that were prepared based on the international standard (NIBSC: National Institute for Biological Standards and Control). Calculation was carried out using Sequence Detector version 1·7 (PE Applied Biosystems). The qualitative detection limit was assumed to be 60 copies/ml (95% confidence interval) and quantitative detection limit was assumed to be 100 copies/ml (95% confidence interval).

The AxSYM® HBsAg assay was used for detection of HBsAg. Tests were carried out in accordance with the manufacture's instructions. A positive result is defined as a signal/noise (s/n) ratio ≥ 2. Samples with different concentrations of HBsAg were used to assess the effectiveness of HBsAg concentration. High-titre HBsAg samples (AxSYM®; s/n ratio 266) were sequentially diluted 10-fold up to a final dilution of 10 000-fold using normal plasma. Lower low-titre HBsAg samples (AxSYM®; s/n ratio 12) were diluted up to a final dilution of 1000-fold. Samples known to have HBsAg below the level of detection in the AxSYM assay (s/n ratio 1·7) were diluted to a final dilution of 100-fold. The respective diluted samples were then concentrated eightfold as described above.

The parallel translation of linear line of dilution curves caused by HBsAg dilution and concentration was studied, plotting the s/n ratio of the EIA on the vertical axis to the dilution fold of the samples on the horizontal axis in both logarithm scales.

The effect of anti-HBs on HBV DNA concentration was studied by adding anti-HBs obtained from immunized horse serum. The titre of purified anti-HBs was 51 200 IU/l. The volumes of anti-HBs added to the samples were 0 µl, 20 µl (1024 mIU/l) and 35 µl (1792 mIU/l).

The effects of other viruses on HBsAg and HBV DNA concentrations were studied in the presence of parvovirus B19 (non-enveloped DNA virus) or HCV (enveloped RNA virus).

Data shown in the tables represent the average of the results of two or three experiments.

Results

Hepatitis B virus was concentrated quantitatively by our new method in a broad range of HBV DNA loads. However, the efficacy of concentration varied from sample to sample. The efficacy of concentration (measured value/expected value: original × concentration times) is shown in Table 1. The efficacy of the concentration process decreased from 0·76 to 0·49 as the HBV DNA load increased from 10^3 to 10^6 copies/ml (Table 1).

Table 1 Effect of the concentration method on concentration of HBV DNA samples

Sample no.	Original (copies/ml)	10-fold concentration (copies/ml)	Efficacy of concentration*
1	1·6 E + 06	7·8 E + 06	0·49
2	4·2 E + 05	2·1 E + 06	0·50
3	9·0 E + 04	5·7 E + 05	0·63
4	2·2 E + 04	1·6 E + 05	0·73
5	4·6 E + 03	3·5 E + 04	0·76

*Efficacy = 10-fold concentration (copies/ml)/original × 10 (copies/ml).

Table 2 Effect of hepatitis B surface antibody (HBsAb) on concentration of HBV DNA

Original sample		10-fold concentration		
HBV DNA (copies/ml)	HBsAb (mIU)	HBV DNA (copies/ml)	Efficacy of concentration
120	0	860	0·72
	1024	1400	1·17
	1792	1300	1·08

The efficacy of HBsAg concentration is shown in Fig. 1. For the high-titre HBsAg samples (s/n ratio 266·03), 100-fold dilution samples were more than limit for detection (s/n ratio 4·88) and 1000-fold dilution samples were less than the limit for detection (s/n ratio 1·16). Following eightfold concentration of HBsAg, the 1000-fold dilution sample was found positive (s/n ratio 3·24). Similarly, in the low-titre sample the undiluted sample was above the detection limit (s/n ratio 11·91). The 10 times dilution sample (s/n ratio 1·69) was negative but became positive following eightfold concentration (s/ratio 4·36). The negative samples (s/n ratio 1·66) became positive by eightfold concentration (s/n ratio 3·49). Based on the parallel translation of linear line shown in Fig. 1, the relative efficacy of concentration was about 0·64(5·1/8) in high-titre samples and 0·56(4·5/8) in low-titre samples.

The effects of anti-HBs and other viruses on HBsAg/HBV DNA concentration were determined. The effect of anti-HBs on HBV DNA concentration is shown in Table 2. The efficacy of HBV DNA concentration in the presence of anti-HBs was superior to that in the absence of anti-HBs. However, in the presence of anti-HBs (antigen–antibody coexistence samples), anti-HBs prevented the detection of HBsAg.

The effect of the coexistence of HCV or parvovirus B19 on the efficiency of HBsAg/HBV DNA concentration is shown in Table 3. HCV (10^6 copies/ml) and parvovirus B19 (2^{11} by RHA: receptor-mediated haemagglutination assay) had no

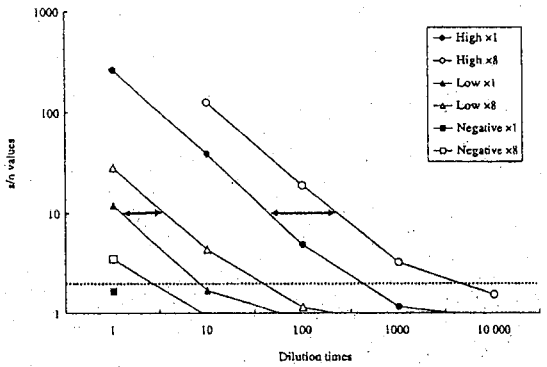

Fig. 1 Parallel translation of linear line caused by hepatitis B surface antigen (HBsAg) concentration. Vertical axis shows signal/noise (s/n) values of enzyme immunoassay (EIA) indicated by logarithm, and horizontal axis shows dilution fold of samples indicated by logarithm. The linearity was observed more than two (s/n value). Closed circle, high titre of HBsAg (×1: non-concentration); open circle, eightfold concentration of high titre of HBsAg (×8: concentration); closed triangle, low titre of HBsAg (×1:non-concentration); open triangle, eightfold concentration of low titre of HBsAg (×8: concentration); closed square, negative (s/n; < 2) titre of HBsAg (×1: non-concentration); open square, eightfold concentration of negative titre of HBsAg (×8: concentration). The dotted line shows two s/n values (cut-off values). Arrows show the distance of parallel translation by HBsAg concentration.

Data for Fig.1

		HBsAg: EIA(AxSYM: s/n^{-1}) dilution with normal plasma				
		1	10	100	1000	10 000
High	×1	266·03	38·81	4·88	1·16	0·91
	×8		126·77	18·95	3·24	1·54
Low	×1	11·91	1·69	0·86	0·77	
	×8	28·28	4·36	1·15	0·76	
Negative	×1	1·66				
	×8	3·49	0·93	0·8		

Table 3 Effect of coexistence of HCV or parvovirus B19 on efficiency of hepatitis B surface antigen (HBsAg) concentration

Plasma for dilution	AxSYM (s/n[b]) HBsAg dilution with various kinds of plasma[a]	10-fold concentration of diluted HBsAg plasma
Normal plasma	1·39	3·80
HCV-positive plasma[c]	1·18	3·47
Parvovirus B19-positive plasma[d]	1·31	3·77

[a]The original HBsAg-positive plasma titre is 6·19: EIA (AxSYM; s/n).
[b]More than 2 means positive.
[c]The titre of anti-HCV was > 2^{12} and the load of HCV RNA was 10^6 copies/ml.
[d]The titre of B19 antigen was 2^{11} by receptor-mediated-haemagglutination assay.

effects on the concentration of HBsAg/HBV DNA. Although the parvovirus B19 could not be concentrated by this method because of its lack of envelope, HCV RNA could be concentrated quantitatively (data not shown).

Seventy-seven anti-HBc positive (≥ 2^5 by HI assay by JRC criteria) and HBsAg-negative (EIA, AxSYM®) donations were selected to study the efficacy of HBsAg and HBV DNA concentrations. Of the 77 samples, 35 were positive by individual NAT and a further five became NAT positive following concentration (Table 4). Of 35 samples (Table 4; lanes d, e), 16 (Table 4; lane e) had HBV DNA loads of 120–1500 copies/ml and the other 19 samples (Table 4; lane d) had HBV DNA loads less than the quantitative detection limit (< 100 copies/ml). However, the HBV DNA loads of all these samples exceeded 100 copies/ml following concentration (Table 4; lanes d, e). Five samples (Table 4; lanes b, c) that were negative by individual NAT became positive (less than 100–510 copies/ml) following concentration.

Table 4 Detection of occult HBV by concentration of HBV DNA and hepatitis B surface antigen (HBsAg)

				HBV DNA (copies/ml)				
				a	b	c	d	e
			Original	Negative	Negative	Negative	< 100	≥ 100
			Concentration (×20)	Negative	< 100	≥ 100	≥ 100	NT
HBsAg (AxSYM)	I	Original	Negative	34	0	0	8	5
		Concentration (×8)	Negative					
	II	Original	Negative	3	1	4	11	11
		Concentration (×8)	Positive					

NT, not tested.

Of the 40 samples (Table 4; lanes b-e) that were HBV DNA-positive either before or after concentration, 13 were HBsAg-negative even following HBsAg concentration. Of these 13 samples, 5 (Table 4, lane I-e) had HBV DNA loads exceeding 100 copies/ml by conventional individual NAT, and eight (Table 4; lane I-d) were quantitatively less than 100 copies/ml on the non-concentrated sample but became NAT positive (≥ 100 copies/ml) following concentration. Of the 77 samples, 30 (Table 4; lane II) had detectable HBsAg following HBsAg concentration. Of these 30 samples, 27 were NAT positive but three (lane II-a) remained NAT-negative even after concentration. Thirty-four of the 77 samples (Table 4; lane I-a) remained negative for both HBsAg and HBV DNA following concentration for both markers.

Discussion

We have previously reported that HBV DNA could be detected in the HBsAg-negative phases of HBV infection (early window period and occult HBV infections) [2–4,18]. However, the use of HBV NAT remains limited, because the HBV viral loads seen in HBsAg-negative infected donors (occult HBV infection) are generally low [19–22]. Although the infectivity of occult HBV is low compared to that in the window phases of early infection [17], we have encountered post-transfusion HBV infection caused by both HBsAg and mini-pool NAT-negative, but individual NAT-positive donations [16].

It has previously been reported that NAT sensitivity can be increased by reducing the number of donations in the mini-pool [23], increasing the input volume of serum, and by addition of an ultracentrifugation step [24]. From the viewpoint of cost-effectiveness, an inexpensive and easy method to increase sensitivity is desirable. We have previously reported a virus concentration method using polyethyleneimine [25]. However, HBV DNA and HBsAg were not concentrated qualitatively by the method, because the combination of extracted nucleic acids of viruses and magnetic beads is difficult to dissociate in the presence of protein-degenerative reagents. We have solved this problem with the use of poly-L-lysine that coagulates with viruses in the presence of bivalent metal ions (zinc acetate).

Owing to the low concentrations of HBV DNA present in early acute infection when both mini-pool NAT and HBsAg are non-reactive, individual NAT would be the best option giving a much higher yield, an increased window period closure, and consequently greater benefit. It is also much debated whether the most sensitive HBsAg detection method is superior to mini-pool NAT, but inferior to individual NAT [21,23]. If 20-pool NAT samples are concentrated 20 times, the sensitivity of 20-pool NAT might be equal to that of individual NAT.

It is important to determine whether HBV could be concentrated in the presence of anti-HBs. In this study, HBV was much more efficiently concentrated in the presence of anti-HBs than without (Table 2). The results showing that the efficacy of concentration was more than 1·0 might be a result of the easy coagulation of antigen antibody-reacted materials with poly-L-lysine beads. However, in the case of HBsAg concentration, it is difficult to measure the efficacy of HBsAg concentration in the presence of anti-HBs, because anti-HBs inhibits the detection of HBsAg by EIA. The coexistence of other viruses would not affect the concentration of HBsAg/HBV DNA, as shown in Table 3. Moreover, the procedure is useful for concentrating coinfected enveloped viruses as HCV, although it will be difficult to concentrate non-enveloped viruses as parvovirus B19. HCV that is difficult to concentrate by ultracentrifugation because of its low density is easily concentrated quantitatively by our method.

We succeeded in concentrating HBsAg from occult HBV infection. The theoretical plasma HBsAg concentration was eightfold (2 ml of plasma/0·25 ml of elution); however, from the parallel translation of the linear line (vertical axis - s/n

