Tears From Children With Chronic Hepatitis B Virus (HBV) Infection Are Infectious Vehicles of HBV Transmission: Experimental Transmission of HBV by Tears, Using Mice With Chimeric Human Livers

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(See the editorial commentary by Heiberg and Hogh, on pages 464–5.)

Background. Body fluids such as saliva, urine, sweat, and tears from hepatitis B virus (HBV) carriers are potential sources of HBV transmission.

Methods. Thirty-nine children and 8 adults who were chronically infected with HBV were enrolled. Real-time polymerase chain reaction was used for the quantification of HBV DNA.

Results. HBV DNA was detected in 73.7% of urine samples (14 of 19), 86.8% of saliva samples (33 of 38), 100% of tear samples (11 of 11), and 100% of sweat samples (9 of 9). Mean HBV DNA levels (±SD) in urine, saliva, tears, and sweat were 4.3 ± 1.1 log copies/mL, 5.9 ± 1.2 log copies/mL, 6.2 ± 0.7 log copies/mL, and 5.2 ± 0.6 log copies/mL, respectively. A statistically significant correlation was observed between the HBV DNA level in serum specimens and HBV DNA levels in saliva and tear specimens (r = 0.88; P < .001). Tear specimens from a child were injected intravenously into 2 human hepatocyte-transplanted chimeric mice. One week after inoculation, both chimeric mice had serum positive for HBV DNA.

Conclusions. The levels of HBV DNA in tear specimens from young children were high. Tears were confirmed to be infectious, using chimeric mice. Strict precautions should be taken against direct contact with body fluids from HBV carriers with high-level viremia.

Hepatitis B virus (HBV) infection causes acute and chronic liver diseases. Fortunately, HBV infection is a vaccine-preventable disease, and as of 2008, 177 countries (92%) have integrated HBV vaccine into routine infant immunization programs. However, Japan and northern European countries, where the endemicity of HBV is low, continue to implement an HBV immunization strategy that targets high-risk groups, rather than a universal vaccination program [1]. Nonetheless, HBV infection by sexual contact and household contact does occur in Japan [2–5]. Children with chronic HBV infection are usually asymptomatic and have high-level viremia. Therefore, it is believed that children with chronic HBV infection may be a major reservoir for spreading HBV to other close susceptible individuals [6–8]. This scenario would especially threaten the countries that adopt an “at-risk” immunization strategy [6, 9–13].

Body fluids such as saliva, semen, urine, sweat, and tears are also potential sources of HBV transmission. Several studies have reported that HBV DNA in these body fluids can be detected by polymerase chain reaction (PCR) [9–18]. Of these body fluids, however, only serum,
saliva, and semen have been demonstrated to be infectious in humans or experimental animal models [19–21].

In this study, HBV DNA levels in urine, saliva, tears, and sweat were quantified by real-time PCR. Body fluid samples were collected from HBV-carrier children and HBV-carrier mothers. After quantification of HBV DNA levels for each specimen type, we evaluated the infectivity of tears from HBV carriers. Mice with severe combined immunodeficiency, carrying a urokinase-type plasminogen activator transgene controlled by an albumin promoter (uPA/SCID), and with transplanted human hepatocytes have recently been used as an appropriate animal model for studying viral hepatitis due to HBV and hepatitis C virus [22–24]. Using these mice, we evaluated whether tears from HBV-carrier children were infectious.

MATERIALS AND METHODS

Patients and Materials
Eligible patients were chronic HBV carriers who attended our outpatient clinic. Their chronic HBV infection status was routinely evaluated by blood examination. All of the patients were asymptomatic. Serum, urine, saliva, tears, and sweat samples were collected when possible from each patient.

Serum samples were collected in preparation tubes. Each urine sample was collected in a sterile plastic tube. Saliva, tear, and sweat samples were collected using an indicating FTA Micro Card (Whatman, GE Healthcare, Tokyo, Japan) and sterile foam-tipped applicators (Whatman). When children shed tears spontaneously, we collected tear samples using the FTA cards. Serum, urine, saliva, tear, and sweat specimens were collected on the same day. Informed consent was obtained from all patients or all patients’ parents. This study was approved by the Research Ethics Committee of Eastern Yokohama Hospital.

HBV DNA Extraction and Real-Time PCR
HBV DNA in serum was measured by COBAS TaqMan HBV DNA test, version 2.0 (Roche Diagnostics, Tokyo, Japan). HBV DNA was extracted from 200 µL of urine, using the QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany). HBV DNA was extracted from saliva, tear, and sweat specimens that were spotted on FTA cards, using QIAamp DNA Mini kit (QIAGEN). Three circles were punched from the FTA card by use of a single-hole paper puncher (Harris Micro Punch 3.00 mm, GE Healthcare) and were used for HBV DNA extraction. The extracted DNA was dissolved in 100 µL of elution buffer.

Quantification of HBV DNA in urine, saliva, tear, and sweat samples was performed using an in-house TaqMan real-time assay. The real-time PCR was performed using a genotype-independent method described previously [25]. PCR was performed in an MX3000P (Stratagene), and the results were analyzed with MxPro software (version 3.0). The lower limit of detection was >100 copies/mL. All assays were performed in duplicate with negative control samples. This assay was standardized using HBV DNA samples of known concentrations measured by the COBAS TaqMan HBV DNA test and recombinant plasmid controls. In this study, the standard of qualification is based on the result of COBAS TaqMan HBV DNA test. Therefore, the conversion factor between HBV copies/mL and HBV IU/mL is considered to be 5.82 copies/ IU. Genotyping of HBV was determined by the PCR-Invader assay [26].

Tear Specimen for Experimental Transmission
For experimental transmission, a tear specimen was collected from a 10-month-old girl with chronic HBV infection. The source of her HBV infection was mother-to-child transmission due to the failure of prophylactic treatment. A total of 200 µL of tears were gently collected from her face when she cried, using a 1.0-ml syringe. The 200-µl tear specimen was diluted with 1300 µL of sterile saline, yielding a total volume of 1500 µL. The specimen underwent filter sterilization with a 0.2-µm filter.

Inoculation of Chimeric Mice With Livers Repopulated by Human Hepatocytes
Three male chimeric mice were purchased from PhoenixBio (Hiroshima, Japan). Human hepatocytes were imported from BD Bioscience (Woburn, MA). Of the 3 mice, 2 (mouse 101 and mouse 102) were inoculated once intravenously with 100 µL of the sterilized tear sample. The remaining mouse (mouse 103) was orally inoculated with 100 µL of the sterilized tear sample every 4 weeks. After inoculation, blood samples for real-time PCR assay were collected from the chimeric mouse every week.

HBV DNA Extraction From Mice Samples and Real-Time PCR
A total of 50 µL of whole blood samples were collected from the mice every week after inoculation, and serum was separated. Saliva and tear specimens were collected from chimeric mice, using FTA cards. HBV DNA was extracted from 20 µL of mouse serum, using SMI-TEST EX-R&D (Medical Biological Laboratories, Aichi, Japan). The extracted DNA was dissolved in 20 µL of nuclease-free water. HBV DNA was quantitatively measured using real-time PCR with the TaqMan PCR Core Reagent kit (Applied Biosystems, Foster City, CA). Real-time PCR was performed in a 25-µL reaction mixture containing 0.125 µL Ampli Taq Gold with 0.2 µM primers (forward primer: 5′-CACATCAGATTTCTAGGAGC-3′ [nucleotides 166–186]; reverse primer: 5′-AGTTTTGTAGTGAATTTGGA-3′ [nucleotides 325–344]), 0.3 µM probe (5′-FAM-CAGAGTTCTAGAGTCGAGTGGAC-TAMRA-3′ [nucleotides 242–267]), and 5 µL extracted DNA. The nucleotide position was based on GenBank accession number AB300361 (genotype C). After incubation for 2 min at 50°C and for 10 min at 95°C, the PCR cycling program underwent
n = 18 (16 were HBV e antigen [HBVeAg] positive); 6 HBV carriers fell into the following age groups: 0–5 years, and the median age was 9 years (range, 0–21 years), n = 11 (9 were HBeAg positive); and 20–27 years: n = 9 (7 were HBcAg positive). Of the 47 patients with chronic HBV infection, 39 were positive for HBcAg. In addition, 39 patients had serum HBV DNA levels of >6 log copies/mL. One, 6, and 40 patients were infected with genotype A, genotype B, and genotype C, respectively. Serum samples were collected from all patients. From the 47 patients, we collected 19 urine samples, 38 saliva samples, 11 tear samples, and 9 sweat samples. One subject provided urine, saliva, and tears only; 3 provided urine, saliva, and sweat only; 10 provided urine and saliva only; 10 provided saliva and tears only; 1 provided urine and sweat only; 1 provided saliva and sweat only; 4 provided urine only; 13 provided saliva only; and 4 provided sweat only. Samples were collected individually at the same time. The characteristics of body fluid samples are shown in Table 1. There were no significant differences in sex, the number of patients with a serum HBV DNA level of ≥6 log copies/mL, and the prevalence of genotype C among patients supplying different types of samples. However, there was a significant difference in the age of patients supplying the different kinds of samples.

**Immunostaining for HBV Surface Antigen (HBsAg) and HBV Core Antigen (HBcAg)**

Immunostaining for HBsAg and HBcAg was performed on frozen sections, using the Ventana i VIEW DAB detection kit (Ventana Medical Systems, Tucson, AZ) and the Dako Envision kit (Dako, Tokyo, Japan), respectively. Primary monoclonal antibodies to HBsAg (Santa Cruz Biotechnology, CA), at a 1:100 dilution, and polyclonal antibodies to HBcAg (Dako), at a 1:500 dilution, were used. Liver tissue was taken from mice after they were euthanized, and the tissue was stored at −80°C.

**Statistical Analysis**

Categorical variables were compared between groups, using the Yates corrected χ² test or the Fisher exact test. Noncategorical variables were compared between groups by the Mann–Whitney U test. For analysis of the correlation between log HBV DNA level in serum and in saliva and tears, we used the Pearson correlation coefficient. All tests were 2-sided, and a P value of ≤0.05 was considered to indicate statistical significance. All statistical analyses were performed with StatMate IV for Windows (Advanced Technology for Medicine & Science, Tokyo, Japan) and Microsoft Office Excel 2007.

**RESULTS**

**Patients and Materials**

Between August 2009 and September 2010, 39 children and 8 adults who were chronically infected with HBV were randomly enrolled in this study. Twenty-six subjects were male, and 21 were female; the mean age (±SD) was 12.4 ± 12.0 years, and the median age was 9 years (range, 0–47 years). The 47 HBV carriers fell into the following age groups: 0–5 years, n = 18 (16 were HBV e antigen [HBVeAg] positive); 6–10 years, n = 11 (9 were HBeAg positive); 11–19 years, n = 9 (7 were HBcAg positive); and 20–27 years: n = 9 (7 were HBeAg positive). Of the 47 patients with chronic HBV infection, 39 were positive for HBcAg. In addition, 39 patients had serum HBV DNA levels of ≥6 log copies/mL. One, 6, and 40 patients were infected with genotype A, genotype B, and genotype C, respectively. Serum samples were collected from all patients.
respectively. Levels of HBV DNA in urine were significantly lower than those in other body fluids. Levels of HBV DNA in body fluids from patients who had a high viral load (ie, >9 log copies/mL) in serum are shown in Figure 2. Mean levels (±SD) of HBV DNA in urine (n = 10 specimens), saliva (n = 23), tears (n = 8), and sweat (n = 8) were 4.4 ± 0.9 log copies/mL,

6.4 ± 0.9 log copies/mL, 6.4 ± 0.9 log copies/mL, and 5.3 ± 0.6 log copies/mL, respectively. Even after the HBV load in serum was well matched, the HBV DNA levels in urine specimens were significantly lower than those in saliva and tear specimens.

Although there was no significant difference in HBV DNA levels between saliva, tears, and sweat specimens from patients with high viral load in serum, the quantification of HBV DNA in saliva and tear specimens showed almost the same levels (Figure 2). Levels of HBV DNA in the 11 pairs of saliva and tear specimens are shown in Figure 3. Mean HBV DNA levels (±SD) in saliva and tear specimens were 6.1 ± 1.0 log copies/mL and 6.2 ± 0.8 log copies/mL, respectively. The levels of HBV DNA in tear specimens were as high as those in saliva specimens.

The association between the levels of HBV DNA in serum specimens and in saliva and tear specimens was evaluated. Because the upper detection limit of the COBAS TaqMan HBV DNA test was >9 log copies/mL, we used data from patients in whom the levels of HBV DNA in serum ranged from 2.9 to 8.8 log copies/mL. Data from 15 patients (15 serum samples, 15 saliva samples, and 3 tear samples) were available for the correlation analysis. A significant correlation was observed in the levels of HBV DNA between serum specimens and saliva and tear specimens (r = 0.88; P < .001) (Figure 4A). The relationship between HBV DNA in serum specimens and HBV DNA in saliva and tear specimens was described as follows: [log HBV DNA load in saliva and tear specimens] = −3.23 + 1.06 × [log HBV DNA load in serum specimens]. On the other hand, there was no significant

Figure 2. To adjust serum hepatitis B virus (HBV) DNA levels among groups, we show the HBV DNA levels in urine, saliva, tear, and sweat samples from patients whose levels of HBV DNA in serum were ≥9 log copies/mL. Although a significant difference in HBV DNA levels between urine and sweat specimens was not present, HBV DNA levels in urine specimens were significantly lower than those in saliva and tear specimens (P < .05). The bar indicates the mean of the levels of HBV DNA. SDs are indicated by vertical bars.

Figure 3. Hepatitis B virus (HBV) DNA levels in saliva and tear samples that were paired. Both groups showed the same HBV DNA levels. The bar indicates the mean of the levels of HBV DNA. SDs are indicated by vertical bars.
association between HBV DNA loads in serum and urine specimens (HBV DNA levels in 9 serum specimens ranged from 2.1 to 8.6 log copies/mL; $r=0.39; P=.30$) (Figure 4B).

Transmission of HBV by Tears
The level of HBV DNA in tear specimens collected from a 10-month-old girl (genotype C; serum HBV DNA load, >9.0 log copies/mL) were 7.1 log copies/mL. The final concentration of HBV DNA in filter-sterilized tear specimens was 6.1 copies/mL. A total of 100 µL of the filter-sterilized tear specimen was injected intravenously into 2 chimeric mice. One week after inoculation, both chimeric mice became positive for HBV DNA in serum (no. 101 had an HBV DNA level of 5.2 log copies/mL, and no. 102 had an HBV DNA level of 5.1 log copies/mL). The levels of HBV DNA in serum from the chimeric mice gradually increased with time. Seven weeks after inoculation, the levels of HBV DNA in serum from the chimeric mice increased to 9 log copies/mL and remained at this level thereafter (Figure 5). Saliva and lacrimal fluids were collected using FTA cards at day 80 (for mouse 101) and day 91 (for mouse 102). Although HBV DNA was extracted from a very small spot (1 pinched-out circle from the FTA card), the levels of HBV DNA were 4.4 log copies/mL (in saliva) and 4.5 copies/mL (in lacrimal fluids) in mouse 101 and 4.0 log copies/mL (in saliva) and 4.3 log copies/mL (in lacrimal fluids) in mouse 102. The remaining chimeric mouse (mouse 103) was orally inoculated with 100 µL of the filter-sterilized tear specimen. Unfortunately, we had to discontinue oral administration because of the deterioration of the mouse’s health 35 days after inoculation. The chimeric mouse (mouse 103) had been inoculated orally twice (on days 0 and 28) before discontinuation. Real-time PCR performed 6 times (on days 0, 7, 14, 21, 28, and 35) detected no HBV DNA in serum.

Immunohistological Analysis of Liver Tissue for HBV Antigens
Immunohistochemical staining was performed on a liver specimen from the mouse with HBV viremia (no. 101). The hepatocytes were positive for HBsAg and HBeAg (Figure 6).
These findings indicated that HBV transmission from tears could be replicated in a human liver chimeric mouse model.

**DISCUSSION**

Although it has been reported that HBV DNA was detectable by PCR in tears from chronic HBV carriers [11, 17], tears have been considered to be low risk for HBV transmission. However, this study demonstrated that tears from children chronically infected with HBV were highly infectious. HBV DNA from serum could be detected in both chimeric mice 1 week after inoculation. Moreover, the levels of HBV DNA in serum continuously increased and reached the upper limit of the PCR assay 7 weeks after inoculation. A previous study showed that chimeric mice usually became positive for HBV DNA in serum 4 weeks after intravenous inoculation with serum from HBV carriers [28]. The levels of HBV DNA in tears used for this study were much higher than those in serum used in the previous study. Therefore, HBV DNA in serum from the chimeric mice became detectable quickly after inoculation.

Recent studies measuring HBV DNA in body fluids from HBV carriers have been conducted in the Netherlands, Sweden, and Denmark. Including the present study, all studies are from counties in which a selective HBV immunization program has been implemented [9–13]. Clearly, physicians from these countries are keen to know whether various body fluids might be sources of HBV transmission. Additionally, physicians are concerned that a vaccination strategy that focuses on at-risk groups is ineffective for prevention of HBV infection. Although recent studies have shown that HBV DNA in urine, saliva, tear, and sweat specimens from chronic HBV carriers was detectable by PCR, these studies did not show that body fluids from chronic HBV carriers were infectious in animal experiments. Approximately 30 years ago, the infectivity of semen and saliva from HBV carriers was proven by experimental transmission, using gibbons [20, 21]. Since then, no other body fluids have been evaluated for infectivity. This study is the first to confirm that tears are infectious sources of HBV.

Tears are presumed to originate from circulating blood. HBV DNA was first detected in tears in 1994 by PCR. In a previous study, tear specimens from 47.1% of HBV carriers (16 of 34) were positive for HBV DNA [17]. In 2006, a previous study measured HBV DNA in paired saliva and tear specimens. Of 7 patients with chronic HBV infection, 4 (57%) had tear specimens that were positive for HBV DNA. The levels of HBV DNA in tear specimens ranged from $0.2 \times 10^3$ to $1.4 \times 10^4$ copies/mL [11]. Compared with the previous study, the levels of HBV DNA in tears were relatively high in this study. There are 2 possible explanations for the difference in HBV DNA levels between these studies. First, the majority of the patients supplying tear samples in our study were very young children (median age, 1 year). Young children with chronic HBV infection are usually in the immunotolerant phase and have a high viral load. Second, the FTA card was effective at collecting body fluids and extracting DNA. Although the number of tear samples was small, this study demonstrates that tears, as well as saliva, contain a large amount of HBV DNA. Interestingly, HBV DNA in lacrimal fluid and saliva could also be detected in the chimeric mice. These findings suggest that tears, like saliva, have the potential to transmit HBV.

Among body fluids, the highest levels of HBV DNA are detected in blood. However, HBV DNA can also be detected in urine, saliva, tears, and sweat. In this study, HBV DNA was detected in a high proportion of body fluid samples. In addition, there was a statistically significant correlation in the

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**Figure 6.** Immunohistological staining for liver tissue with antibodies to hepatitis B virus (HBV) surface antigen (HBsAg) and HBV core antigen (HBCAg). A, HBsAg was expressed on cytoplasmic membrane (original magnification ×400). B, HBCAg were expressed in nuclei of hepatocytes (original magnification ×400). Arrows indicate the nuclei of HBCAg-positive staining.
levels of HBV DNA between tear and saliva specimens and serum specimens, in which $[\log \text{HBV DNA level in saliva and tear specimens}] = -3.23 + 1.06 \times [\log \text{HBV DNA level in serum specimens}]$. Similarly, previous studies reported that the levels of HBV DNA in saliva specimens were significantly related to the levels of HBV DNA in blood specimens. In this study, however, the levels of HBV DNA in urine specimens were not significantly associated with the levels of HBV DNA in serum specimens. The levels of HBV DNA in urine samples were significantly lower than those in saliva and tear samples. This finding is also consistent with that of a previous study [13]. We cannot provide any clear explanation why the levels of HBV DNA were lower than those in other body fluids. Further studies are required to study not only the infectivity of urine but also the mechanism of the reduction of the HBV DNA level in urine.

It has been known that the oral administration of serum from HBV carriers causes HBV infection [19]. After we confirmed the infectivity of tears through the intravenous route, tears were administered orally to a chimeric mouse. Although both transmission routes were investigated using the same sample, this study, like previous animal experiments [20, 21], failed to demonstrate that HBV infection occurred through an oral route; unfortunately, the period of observation was not sufficient to evaluate the infectivity of tears. We tried to detect HBV DNA in the liver of chimeric mouse 103 after discontinuation of oral administration of tear specimens, but HBV DNA was not detectable in the liver by real-time PCR (data not shown).

There are few studies that have measured the levels of HBV DNA in sweat specimens from chronically infected patients. A previous study quantified HBV DNA levels in Olympic wrestlers, who were negative for HBsAg but positive for HBV DNA in blood [14]. In the previous study, a statistically significant relation between the levels of HBV DNA in blood and sweat was observed. In the present study, all sweat samples were positive for HBV DNA. In addition, the levels of HBV DNA in sweat specimens were high (mean level $\pm$SD), 5.2 $\pm$ 0.6 log copies/mL. Therefore, sweat from HBV carriers might also have the potential to cause horizontal HBV infection.

The US Centers for Disease Control and Prevention considers that the risk of transmission in child-care settings is very low [29–31]. However, Ireland, Norway, and Sweden have a policy that children should be immunized if another child in a day care center is positive for HBsAg. This study showed that various body fluids from young HBV carriers have a high concentration of HBV DNA. Previous studies have reported that 10% of HBV particles are infectious [32]. Therefore, all body fluids from HBV carriers should be considered to be infectious, and HBV vaccine should be recommended for day care staff.

In conclusion, HBV DNA was detected at high proportions in urine, saliva, tear, and sweat specimens from chronic HBV carriers. The levels of HBV DNA in saliva and tear specimens from young children were extremely high. In addition, tear samples from a child with chronic HBV infection were confirmed to be infectious, using chimeric mice. Although the HBV transmission risk between young children in nurseries or day care centers may be limited, strict precautions should be taken against contact with body fluids from HBV carriers with high-level viremia, especially in counties implementing an immunizing program focused on individuals at-risk for HBV infection.

Notes

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