Detection of hepatitis B virus DNA and HBsAg from postmortem blood and bloodstains

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Received: 10 March 2017 / Accepted: 29 October 2017 / Published online: 2 December 2017
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Abstract
A large number of accidental virus infections occur in medical and non-medical workers exposed to infectious individuals and materials. We evaluated whether postmortem blood and bloodstains containing hepatitis B virus (HBV) are infectious. HBV-infected blood and bloodstains were stored for up to 60 days at room temperature and subsequently screened for hepatitis B surface antigen (HBsAg) and HBV DNA. In addition, HBV-positive postmortem blood was added to a cell line and the production of HBV virions was examined over a period of 7 days. HBsAg and HBV DNA were detected in all samples stored for 60 days at room temperature. HBV-positive postmortem blood successfully infected the cell line and progeny viruses were produced for up to 6 days. Thus, it is crucial that due care is taken when handling not only living material infected with HBV, as well as other harmful viruses, but also blood or body fluids from cadavers or medical waste.

Introduction
At scenes of large-scale disasters or terrorist attacks, where there are a considerable number of casualties, many non-medical specialists, including police officers and firefighters, must work together with medical teams to save the survivors and investigate the cause of the incident.

During the Ebola virus outbreak of 2015, not only the medical teams assisting the patients but also many members of the public were secondarily infected with Ebola virus because of the custom of touching the deceased at their funeral [1]. In February 2015, more than 20 people, including forensic doctors at the University of Tokyo and police officers, were infected with tuberculosis during the transfer and autopsy of an infected corpse [2].

Corpses with unknown medical history are often examined in the field of forensic medicine. During the outbreak of Ebola virus and Middle East respiratory syndrome coronavirus, the Japanese government enacted a number of measures to prevent the transmission of secondary infections from travelers. However, these measures focused on living individuals, and infection from corpses was not considered. Unfortunately, it is more difficult to identify infection in a cadaver than it is in a living individual, such as by checking travel records or symptoms. Therefore, it is important to analyze the risk of infection from infected corpses.

Excessive preventative measures when dealing with potentially infected corpses are not adequate from a cost-benefit point of view, and unnecessary sterilization may result in environmental pollution. It is also unknown for how long a virus remains infectious in a corpse or bloodstain. To the best of our knowledge, no reports have clearly examined this issue.

In our previous study, as a representative harmful virus, we examined if hepatitis C virus (HCV) can be detected in blood or bloodstains that were stored at room temperature for up to 60 days [3]. HCV-RNA was found to be detectable from blood and bloodstains for up to 60 days. Anti-HCV antibody (HCV-Ab) was also detectable for up to 60 days, so HCV-Ab screening can also be used to evaluate postmortem blood and bloodstain samples.

However, even when the genome of a virus is detected, it is still not certain whether the virus capsid is also intact.
In addition, if the virus capsid is intact, it is still unclear whether this virus is still infectious. HCV is very difficult to culture in cells in vitro, and culturing of HCV isolates directly from patient sera is as yet unattainable [4].

Hepatitis B virus (HBV) is a partially double-stranded, enveloped DNA virus classified within the Hepadnaviridae as well as a member of the hepatitis virus grouping with HCV. Despite the availability of a vaccine HBV infection is still a global health problem, since over 240 million people are estimated to be chronically infected by HBV [5, 6] and more than 300,000 die annually from cancer or liver dysfunction associated with HBV infection [7]. HBV can be grown easily in cell culture [8, 9]. Therefore, in this study, we selected HBV as a representative ‘harmful virus’ for analysis. We stored HBV-infected blood and bloodstains for up to 60 days at room temperature and examined if HBV DNA and hepatitis B surface antigen (HBsAg) could be detected. In addition, HBV-infected postmortem blood was added to a cell line and we examined if this HBV-infected cell line could produce progeny virus.

**Materials and methods**

**Samples**

HBV-infected blood samples were obtained with informed consent from 6 patients (4 men and 2 women; mean age, 35.6 ± 9.0 years; range, 26–44 years) at the University Hospital, Kyoto Prefectural University of Medicine and Aiseikai Yamashina Hospital for serological analysis and clinical diagnosis (Table 1).

**Measurement of HBV in blood samples**

Prior to our experiments, the HBV DNA titer in all clinical samples was determined using the COBAS TaqMan HBV DNA Assay (Roche Molecular Systems, Pleasanton, CA). Titers ranged from 4.2 to 9.1 log IU/mL (average, 6.51 ± 2.45 log IU/mL). The limit of detection was 1.3 log IU/mL. All samples were stored at -80 °C until use.

**Blood and bloodstain preparation**

Bloodstain samples were prepared by soaking cotton buds in 0.1 mL of HBV-infected whole blood samples (n = 6) for 1 min and then drying at room temperature for up to 60 days. HBV-infected whole blood samples (n = 6) were placed in sealed 2-mL test tubes and kept at room temperature (20 °C) for up to 60 days. The prepared blood and bloodstain samples were analyzed at 3, 9, 27, and 60 days after preparation.

**Detection of HBsAg**

HBsAg from the bloodstain and whole blood samples was detected using immunochromatography with an Ortho Quick Chaser HBsAg Kit (Ortho Clinical Diagnostics, Tokyo, Japan). Before testing, the bloodstain samples were soaked in 400 µL saline; 100 µL of the extracted solution was then analyzed using immunochromatography. The limit of detection was 20 ng/mL.

**Detection of HBV genome**

DNA was extracted from 200 µL diluted whole blood and 200 µL solution extracted from bloodstained materials with a QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany). The extracted DNA was eluted in 50 µL elution buffer and used for genome amplification of the HBV S gene using PCR with AmpliTaq Gold DNA Polymerase (Applied Biosystems LLC, Foster City, CA, USA) in 25 µL aliquots containing 2.5 µL 10 × Gold buffer, 500 µM deoxynucleoside triphosphate, 1.5 mM MgCl2, and 0.6 µM primers. The sense primer 5′-GTCTAGACTCGTGTTGACCTCTCTC-3′ and antisense primers 5′-AAGCCAAAAGTCGGGGGAAGC-3′ were used as previously [10].

DNA polymerase was initially activated at 95 °C for 11 min for PCR. PCR amplification was performed for 35 cycles at 94 °C for 15 s, 55 °C for 5 s, and 72 °C for 30 s, followed by a final step at 72 °C for 10 min. Amplification was carried out in a PC-320 thermal cycler (ASTEC, Fukuoka, Japan). PCR products were mixed with 6 × loading buffer Orange G and subjected to electrophoresis on a 1.5% agarose gel at 100 V for 30 min. The electrophoresed agarose gel was stained with ethidium bromide (0.5 µg/
mL). The image from the agarose gel was captured under UV transillumination on a LAS 4000 mini camera system (Fujifilm, Tokyo, Japan). The limit of detection was 2.6 log copies/mL.

**HBV-infected postmortem case**

In August 2016, a body was found floating in the sea by a fisherman, about 700 m from the coast. A rescue helicopter arrived at the scene soon after the emergency call. However, the victim was found to be in cardiopulmonary arrest and was pronounced dead at 14:08 pm. He was unidentified and the cause of death was unknown. Therefore, the body was sent for autopsy the next day and the cause of death was determined as drowning. Subsequent police investigation revealed that he was a 56-year-old male textile manufacturer living in the neighboring city. On the previous day, he had gone fishing at around 10:00 am. His medical history was never found.

**HBV infection of a cell line with postmortem blood**

A sample of whole blood was taken from the autopsy case and immediately separated; HBV copy number was measured as 5.0 log copies/mL. The sample components were stored at -80 °C until use. Human hepatocyte carcinoma-derived HepG2 cells were obtained from the RIKEN BioResource Center through the National BioResource Project of the Ministry of Education, Culture, Sports, Science and Technology (MEXT) Japan. The experiment was started 2 weeks after the autopsy. The HepG2 cell line (3.0 × 10^5 cells/well) was plated in 2-cm tissue culture dishes. Blood samples containing 5.0 log copies/mL HBV (1.79 × 10^3 IU/mL) were diluted from ×1 to ×100. Each 1 mL of diluted whole blood sample was added to individual dishes containing the cell line. These were incubated with an additional 1 mL DMEM, 10% fetal bovine serum, and 1% streptomycin at 37 °C for 24 h. The dishes were washed with fresh medium and incubated for an additional 72 h. Subsequently, the medium was changed in each dish and samples of the spent medium were sent for HBV analysis (Day 4). From the 4th to 8th days, the medium was changed every 24 h in each dish and the spent medium samples were sent for HBV analysis. HBV analysis was performed by HBsAg detection and PCR amplification of the HBV genome using the aforementioned methods.

HBV-infected blood samples were obtained with informed consent. This study was approved by the Institutional Review Board of Kyoto Prefectural University of Medicine (G-52).

### Results

HBV-DNA and HBsAg were detected in all blood and bloodstain samples stored at room temperature for up to 60 days, with viral loads of 4.2-9.1 log IU/mL detected (Table 2). All samples in this study were therefore positive for HBsAg and HBV-DNA.

In the postmortem case, HBsAg and HBV DNA were detected in the HepG2 cell line with HBV copies of 10.0 ×10^3 copies/mL detected up to 6 days. After 6 days, cell death occurred and the culture was discontinued. HBsAg and HBV-DNA were not detected in diluted samples of HBV-positive whole blood (Table 3).

### Discussion

In our study, all samples were positive for both HBV-DNA and HBsAg, which may indicate that the virus capsid is sustained for a considerable period of time in blood and bloodstains. In addition, the fact that HBV from postmortem blood infected the cell line indicates the need for careful handling of materials that have come in to contact with a corpse, blood, and other body fluids.
Almost 90% of HBV infections occur during the perinatal period and within 6 months after birth [11]. The HBV vaccine first went on sale in 1982. In many countries, HBV vaccination for newborn babies and medical workers started in the 1980s [11]. Therefore, the HBV-positive rate of blood donors is low, at approximately 0.1% [12]. Nowadays, the infection rate of HBV is lower than that of HCV [13]. However, despite vaccination, there are some people whose anti-HBs titer are negative or less than 10 IU/mL. Although it is said that immunological memory persists in such cases after vaccination, it is an issue that merits consideration [14].

In the United States, 6.2% of medical workers are positive for Anti-HBc, which is higher than the rate in blood donors, which is 1.8% [12], indicating that medical workers are at a high risk of infection. Approximately 75% of HBV-related transmissions in healthcare workers are via percutaneous injury with a scalpel or needle; the remaining mode of transmission in these workers is via mucosal-cutaneous exposure. When an individual is positive for both HBsAg and HBeAg, there is a 22–31% risk of hepatitis [15]. Even in a high-risk working environment, medical workers have existing knowledge about infectious diseases and the appropriate use of guards such as gloves and masks. However, there is a higher risk of infection (and becoming Anti-HBc-positive) when non-medical workers, who lack this medical expertise, attend a disaster. The risk of HBV infection has been reduced by universal vaccination in several countries [16, 17], however the infection risk, not only in the medical field but also in the general population remains high, therefore it is advisable to extend universal vaccination to the rest of the world.

In this study, although it was only with a single case, HBV in postmortem blood successfully infected the HepG2 cell line (Table 3). HepG2 cells are a human hepatoblastoma cell line derived from a 15-year-old male with a well-differentiated carcinoma. HepG2 cells differ morphologically from primary hepatocytes. Recently, the sodium taurocholate cotransporting polypeptide (NTCP) was identified as a receptor for HBV [18]; however, it is not expressed in HepG2 cells [19]. However, some reports have described binding and entry of HBV using normal HepG2 cells; furthermore, although virion production was not observed in these studies [20–23], it was following transfection of HBV DNA in related studies [8, 9]. It is therefore possible that the mechanisms of viral entry into HepG2 cells or hepatocytes has not been clearly elucidated. In our case study, we used normal HepG2 cells to observe the infection of HBV even though it is much easier to infect cells with HBV following NTCP expression. Even in these challenging conditions without NTCP expression, HBsAg and HBV DNA were detected in cultured cells. This finding does not conclude directly that HBV in postmortem materials remains infectious to humans, for instance it may be due to residual HBV in the culture dish. However, at least we can say that there is a possibility of infection.

Our single case had no significant illness or a past medical history and, in addition, did not present with any significant gross pathology within the liver tissue (Figure 1). Interestingly, even in such an inactive case, postmortem blood still had the potential to be infectious. Therefore, we should increase our preparedness and awareness concerning the possibility of infection from postmortem materials.

Acknowledgements We are grateful to the blood sample donors.

Language editing services were provided by ThinkSCIENCE, Tokyo, Japan.

Compliance with ethical standards

Funding This work was supported by a Grant-in-Aid for Scientific Research C from the Japan Society for the Promotion of Science (no. 22590641).

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This study was approved by the institutional review board of Kyoto Prefectural University of Medicine (G-52).

Informed consent Informed consent was obtained from all individual participants included in the study.

Fig. 1 Liver tissue from the clinical postmortem case (X400). Centrilobular necrosis (single arrow) and slight fibrosis of Glisson’s sheath were seen (double arrows); however, no active infiltration of inflammatory cells was seen.
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