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ORIGINAL ARTICLE

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

Junpei Hara[1] · Yuka Tanaka[1] · Hiroto Kaneko[2] · Yoshito Itoh[3] · Hiroshi Ikegaya[1]

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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.

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✉ Hiroshi Ikegaya
ikegaya@koto.kpu-m.ac.jp; ikegaya-tky@umin.ac.jp

[1] Department of Forensic Medicine, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, 465 Kajiicho, Kamigyo, Kyoto 602-8566, Japan

[2] Molecular Gastroenterology and Hepatology, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, 465 Kajiicho, Kamigyo, Kyoto 602-8566, Japan

[3] Aiseikai Yamashina Hospital, 19-4 Takehanashichoyacho, Yamashina, Kyoto 607-8086, Japan

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 M deoxynucleoside triphosphate, 1.5 mM $MgCl_2$, and 0.6 µM primers. The sense primer 5′-GTCTAGACTCGTGGTGGACTTCTCTC-3′ and antisense primers 5′-AAGCCAAACAGTGGGGGAAAGC-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/

Table 1 Clinical blood samples used in this study

Case No.	1	2	3	4	5	6
Age (years) and sex	33 M	26 M	37 M	44F	44 M	30 M
Virus titer (log IU/mL)	5.9	9.1	4.2	4.3	6.9	8.7
Genotype	C	C	A	C	C	C

M: male, F: female

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 ($3.0 \times 10e^5$ cells/well) was plated in 2-cm tissue culture dishes. Blood samples containing 5.0 log copies/mL HBV ($1.79 \times 10e^3$ 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 4^{th} to 8^{th} 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).

Table 2 HBsAg and HBV DNA detection in blood and bloodstain samples

Test sample type		Number of positive sample among tested samples after *n* days of storage			
		3 days	9 days	27 days	60 days
HBsAg	Bloodstain	6/6	6/6	6/6	6/6
	Blood	6/6	6/6	6/6	6/6
HBV DNA	Bloodstain	6/6	6/6	6/6	6/6
	Blood	6/6	6/6	6/6	6/6

N = 6

Table 3 Detection of HBsAg and HBV DNA within the medium of the cell line cultured with HBV-positive whole blood

Day	4	5	6	7	8
Control	-/-	-/-	-/-	-/-	-/-
×1	+/+	+/+	+/+	CD	CD
×10	-/-	-/-	-/-	-/-	-/-
×100	-/-	-/-	-/-	-/-	-/-

CD: Tests were not performed because of cell death

+/+: Both HBsAg and HBV DNA were positive

-/-: Both HBsAg and HBV DNA were negative

No sample was positive for HBsAg or HBV DNA

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 $\times 10^3$ 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.

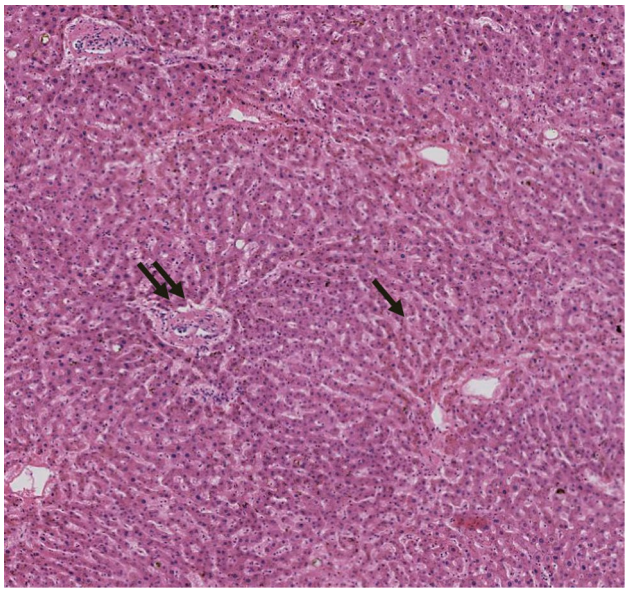

Fig. 1 Liver tissue from the clinical postmortem case (X400). Centrilobular necrosis (single arrow) and slight fibrosis of Glisson's sheath were seen (double arrows); however, no active infiltration of inflammatory cells was seen

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.

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Compliance with ethical standards

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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.

References

1. Victory KR, Coronado F, Ifono SO, Soropogui T, Dahl BA, Centers for Disease Control and Prevention (CDC) (2015) Ebola transmission linked to a single traditional funeral ceremony—Kissidougou, Guinea, December 2014-January 2015. MMWR Morb Mortal Wkly Rep 64:386–388
2. Yanagi M (2016) The group infection of tuberculosis. Doctors in The University of Tokyo were also infected. The Mainichi (**In Japanese**)
3. Takasaka T, Itoh Y, Kaneko H, Ikegaya H (2011) Detection of hepatitis C virus and antibodies in postmortem blood and bloodstains. J Clin Microbiol 49:1122–1123
4. Ramirez S, Mikkelsen LS, Gottwein JM, Bukh J (2016) Robust HCV genotype 3a infectious cell culture system permits identification of escape variants with resistance to sofosbuvir. Gastroenterology 151:973–985
5. Ott JJ, Stevens GA, Groeger J, Wiersma ST (2012) Global epidemiology of hepatitis B virus infection: new estimates of age-specific HBsAg seroprevalence and endemicity. Vaccine 30:2212–2219
6. Lavanchy D (2005) Worldwide epidemiology of HBV infection, disease burden, and vaccine prevention. J Clin Virol 34(Suppl 1):S1–S3
7. Coppola N, De Pascalis S, Onorato L, Calò F, Sagnelli C, Sagnelli E (2016) Hepatitis B virus and hepatitis C virus infection in healthcare workers. World J Hepatol 8:273–281
8. Sells MA, Chen ML, Acs G (1987) Production of hepatitis B virus particles in Hep G2 cells transfected with cloned hepatitis B virus DNA. Proc Natl Acad Sci USA 84:1005–1009
9. Ladner SK, Otto MJ, Barker CS, Zaifert K, Wang GH, Guo JT, Seeger C, King RW (1997) Inducible expression of human hepatitis B virus (HBV) in stably transfected hepatoblastoma cells: a novel system for screening potential inhibitors of HBV replication. Antimicrob Agents Chemother 41:1715–1720
10. Mizokami M, Nakano T, Orito E, Tanaka Y, Sakugawa H, Mukaide M, Robertson BH (1999) Hepatitis B virus genotype assignment using restriction fragment length polymorphism patterns. FEBS Lett 450:66–71
11. Nannini P, Sokal EM (2016) Hepatitis B: changing epidemiology and interventions. Arch Dis Child. https://doi.org/10.1136/archdischild-2016-312043 **[Epub ahead of print]**
12. Thomas DL, Factor SH, Kelen GD, Washington AS, Taylor E Jr, Quinn TC (1993) Viral hepatitis in health care personnel at The Johns Hopkins Hospital. The seroprevalence of and risk factors for hepatitis B virus and hepatitis C virus infection. Arch Intern Med 153:1705–1712
13. Murokawa H, Yoshikawa A, Ohnuma H, Iwata A, Katoh N, Miyamoto M, Mine H, Emura H, Tadokoro K, Japanese Red Cross NAT Screening Research Group (2005) Epidemiology of blood donors in Japan, positive for hepatitis B virus and hepatitis C virus by nucleic acid amplification testing. Vox Sang 88:10–16
14. Zanetti AR, Mariano A, Romanò L, D'Amelio R, Chironna M, Coppola RC, Cuccia M, Mangione R, Marrone F, Negrone FS, Parlato A, Zamparo E, Zotti C, Stroffolini T, Mele A, Study Group (2005) Long-term immunogenicity of hepatitis B vaccination and policy for booster: an Italian multicentre study. Lancet 366:1379–1384
15. US Public Health Service (2001) Updated US public health service guidelines for the management of occupational exposures to HBV, HCV, and HIV and recommendations for postexposure prophylaxis. MMWR Recomm 50 (RR-11), pp 1–52
16. Zanetti AR, Van Damme P, Shouval D (2008) The global impact of vaccination against hepatitis B: a historical overview. Vaccine 26:6266–6273
17. Mele A, Tosti ME, Mariano A, Pizzuti R, Ferro A, Borrini B, Zotti C, Lopalco P, Curtale F, Balocchini E, Spada E, National Surveillance System for Acute Viral Hepatitis (SEIEVA) Collaborating Group (2008) Acute hepatitis B 14 years after the implementation of universal vaccination in Italy: areas of improvement and emerging challenges. Clin Infect Dis 46:868–875
18. Yan H, Zhong G, Xu G, He W, Jing Z, Gao Z, Huang Y, Qi Y, Peng B, Wang H, Fu L, Song M, Chen P, Gao W, Ren B, Sun Y, Cai T, Feng X, Sui J, Li W (2012) Sodium taurocholate cotransporting polypeptide is a functional receptor for human hepatitis B and D virus. Elife 1:e00049
19. Kullak-Ublick GA, Ismair MG, Kubitz R, Schmitt M, Häussinger D, Stieger B, Hagenbuch B, Meier PJ, Beuers U, Paumgartner G (2000) Stable expression and functional characterization of a Na^+-taurocholate cotransporting green fluorescent protein in human hepatoblastoma HepG2 cells. Cytotechnology 34:1–9
20. Neurath AR, Kent SB, Strick N, Parker K (1986) Identification and chemical synthesis of a host cell receptor binding site on hepatitis B virus. Cell 46:429–436
21. Petit MA, Dubanchet S, Capel F, Voet P, Dauguet C, Hauser P (1991) HepG2 cell binding activities of different hepatitis B virus isolates: inhibitory effect of anti-HBs and anti-preS1 (21–47). Virology 180:483–491
22. Qiao M, Macnaughton TB, Gowans EJ (1994) Adsorption and penetration of hepatitis B virus in a nonpermissive cell line. Virology 201:356–363
23. Glebe D, Urban S (2007) Viral and cellular determinants involved in hepadnaviral entry. World J Gastroenterol 13:22–38