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Serological Survey for Hepadnavirus in Long-tailed Macaques (*Macaca fascicularis*) at their ex-situ Habitats in Indonesia

Intan Citraningputri[a], Diah Iskandriati[b*], Rachmitasari Noviana[c], Uus Saepuloh[d], R P Agus Lelana[e], Joko Pamungkas[f]

[a]*Primatology, Graduate School of Bogor Agricultural University, Dramaga Bogor, Indonesia*
[b,c,d,f]*Primate Research Center, Bogor Agricultural University, Jalan Lodaya II no 5 Bogor 16151, Indonesia*
[e,f]*Faculty of Veterinary Medicine, Bogor Agricultural University, Dramaga Bogor, Indonesia*
[a]*Email: iccitraningputri@gmail.com*
[b]*Email: atie@indo.net.id*
[c]*Email: jpi-pssp@indo.net.id*

Abstract

The discovery of hepatitis B virus (HBV) infection among long-tailed macaques from Mauritus Island in 2013 is a new finding that showed HBV could infect non-human primate family *Cercopithecidae*. The aim of this study is to investigate the prevalence of Hepadnavirus among *Macaca fascicularis* that are living outside of their natural habitat in Indonesia. Hepatitis B surface antigen (HBsAg) screening test was performed on 95 plasma and serum samples collected from the different sources captivity, confiscated long-tailed macaques, and performance monkey. DNA detection was carried out on seropositive samples to HBsAg. Screening test showed that 11 of 95 (11.6%) samples were reactive to HBsAg. Prevalence of HBsAg is higher in confiscated animals and performance monkey (55%) compared with captive *M. fascicularis* (45%). However, no HBV DNA could be detected in HBsAg samples that were tested. HBsAg positive result indicate that the long-tailed macaques could be infected by hepatitis B virus naturally, although HBV DNA could not be detected in this study.

Keywords: hepatitis B virus; long-tailed macaques; ex-situ habitat.

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* Corresponding author.

1. Introduction

Hepatitis is a general term meaning inflammation of the liver and can be caused by a viral infection [1]. Viral hepatitis disease is a major cause of cirrhosis and liver cancer [2]. One of the viruses that causes hepatitis in humans, leading to a major global impact is hepatitis B virus (HBV) [1]. Hepatitis B virus comes from the family *Hepadnaviridae*. *Hepadnaviridae* can be found in aves (avihepadnavirus), and mammals (orthohepadnavirus) especially primates [3]. Hepatitis B infection have a high degree of endemicity in Indonesia, with positive 9.4% HBsAg prevalence of the 10391 serum samples, it showed that among 10 people in Indonesia there is 1 people infected by Hepatitis B virus [4]. Among the member countries of SEAR WHO (World Health Organization South-East Asian Region) Indonesia is the second country with the most cases of Hepatitis B after Myanmar [4]. Although HBV is generally known as a disease in the human population, only few studies have published the prevalence of HBV in primate species, particularly primates in captivity and infected by humans [5, 6]. In the natural habitat, non-human primate HBV infection has been identified in gibbon (GiHBV), orangutan (OuHV), chimpanzees (ChHBV), gorilla (GoHBV), and woolly monkey (WMHBV) [7, 8]. Various studies suggest that hepadnavirus in non-human primate species are genetically distinct from human HBV [7, 9]. Among the high prevalence of hepatitis B infection in apes and *Atelidae* (woolly monkey), there have been no reports of HBV infection that have occured in small primates *Cercopithecidae*. Until 2013, there are reports findings of hepadnavirus that are genetically close to human HBV in long-tailed macaque (*Macaca fascicularis*) from Mauritius Island [10]. More recently, a study in performance monkeys conducted to survey various pathogenic disease agents include hepatitis B, in the area of Jakarta, West Java and Central Java. The results showed that antibodies against hepatitis B were detected as much as 8.9% (4/45), but from all of the seropositive samples, DNA hepatitis B virus is undetectable [11]. In addition to living in the primary and secondary forests, long-tailed macaques can be found coexisting with humans in many habitats, such as in the agricultural areas, villages, tourist areas, religious sites, and also used as a pet, and entertainment commodity such as performance monkey [12, 13]. Long-tailed macaques are known to live sympatric with other primates such as gibbons, orangutans, and Thomas's langur [14]. The possibility of physical contact with the long-tailed macaques to humans and other primate species allows the transmission of various agents of diseases, including viral hepatitis B. The hepatitis B virus can be transmitted through direct contact with blood and body fluids contaminated with HBV [1]. Detection of HBV infection can be done through serological examination of the surface antigen and antibodies to HBV by ELISA, but despite of that HBV viral DNA can also be detected through the polymerase chain reaction (PCR) [5, 10] . Hepatitis B chronic disease management is generally a long, costly, only partially effective and often lead to the emergence of resistant variants [15]. Currently, the approach in immunotherapy are expected to restore humoral immune responses that are specific to this case, but in terms of biomedical research, small primate species suitable as an animal model of hepatitis B virus are still not discovered yet [16, 10]. The authors in [10, 11] noted that HBV infections have been reported in long-tailed macaques. However, a lot of data and information is needed in identifying and proving that the long-tailed macaques in Indonesia can become infected with HBV. Long-tailed macaque widely distributed in natural habitats and around civil society, the research about the presence of HBV in the long-tailed macaque is indispensable. The purpose of this study was to investigate and determine the prevalence Hepadnavirus on a long-tailed macaques. In this study, samples taken from ex-situ institutions that

handle long-tailed macaques, both in breeding facilities and wildlife rehabilitation centers.

2. Materials and methods

2.1. Sample collection and processing

Total of 91 of serum and plasma samples were collected from two primate facilities in Bogor, West Java and 4 of plasma samples were obtained from performance monkeys in Solo, Central Java. Blood samples were taken during routine medical examination in those facilities except for samples from performance monkeys were collected during pathogen surveillance for its population. Blood samples were collected in plain tubes or EDTA-tubes and the serum or plasma was separated by centrifugation at 3000 x*g* for 20 min and then analyzed or stored at -80 °C until serological tests were performed. Processing and laboratory analysis were conducted at the Laboratory of Biotechnology, and Laboratory Microbiology Immunology of PRC-IPB.

2.2. Serology test for HBsAg

Detection of Hepatitis B surface antigen (HBsAg) was carried out using commercial ELISA kit (HBsAg ™, Human®, Human für Biochemica Gesellschaft und Diagnostica mbH, Germany) according to manufacturer's instruction manual. Briefly 50 ul serum or plasma samples, positive and negative controls were added into antibody-coated plates and Horseradish peroxidase-conjugated anti-HBsAg (Guinea pig) was then incubated for 80 minutes at 37^{o}C. Tetramethyl benzidine (TMB) substrate was added for 30 min at room temperature for color development. The reaction was stopped by adding Sulphuric acid and optical density was measured at 450 nm using ELISA reader. The Cut-off value (COV) were calculated by adding 0.025 to the mean value of the negative control

2.3. DNA extraction

Total DNA was extracted from serum or plasma samples using the QIAamp DNA blood mini kit (Qiagen, US) according to the manufacturer's instructions. The quality of DNA were further evaluated by performing the PCR amplification to GAPDH housekeeping gene.

2.4. Procedure Polymerase Chain Reaction (PCR) and electrophoresis

Polymerase Chain Reaction were performed in Thermal cycler Applied Biosystem 9700 using primer sequences developed by author in [7]. Primers was designed to amplify the Pre-S1 region and were used to detect HBV in orangutans and the Javan gibbon species [7, 17, 18]. The master mix for PCR consisted of 12.5 µL KAPA Taq HotStart ™ Readymix (containing 1U KAPA Taq DNA polymerase, KAPA Taq buffer, 0.2 mM dNTPs, and 1.5mM $MgCl_2$) (KAPA Biosystem, Boston, USA), 1 µL of each primer (10 pmol/ µL), 5.5 µL ddH_2O, and 5 µL DNA template. PCR amplification was performed by following protocol: denaturation for 5 min at 94^{o}C, amplification for 45 cycles consisting of 30 sec at 94^{o}C, 30 sec at 52^{o}C and 30 sec at 72^{o}C and post extension at 72^{o}C for 7 min. The amplified DNA then analyzed by 1.8% agarose gel electrophoresis and visualized using GelDoc (BioRad). Expected target product for this primer pair is ± 455 base pairs.

3. Result

A total of 95 serum and plasma samples have been collected from the two facilities. The number and type of sample are adjusted to the availability of existing sample collections at the facilities. The origin of the samples are categorized into three types: 1) the samples from breeding facility at the PRC-IPB (Bogor 1, Bogor 2, Bogor 3, Tangerang), 2) samples from performance monkeys (PRC's collection), and 3) samples from confiscated monkeys (wildlife rescue center's collection). The results of HBsAg and HBV DNA are presented in Table 1. Based on ELISA test for HBsAg, HBsAg was detected in 11 out of 95 long-tailed monkeys (11.6%) although none of those animal showed the presence of HBV DNA (Figure 1).

Table 1: The prevalence of Hepatitis B infection in samples collected from the PRC-IPB and wildlife rescue center Bogor on a June 2014 until Februari 2015.

Sample Origin	Type of Sampel	Number of Sampel	Hepatitis B virus examination	
			HBsAg	VHB DNA
PRC-IPB				
Bogor 1	Serum	8	0/8	-
Bogor 2	Plasma	11	3/11	0/3
Bogor 3	Plasma	25	2/25	0/2
Tangerang	Serum	30	0/30	-
Performance monkey	Serum	4	1/4	0/1
Wildlife Rescue Center				
Collection I	Serum	4	0/4	-
Collection II	Plasma	13	5/13	0/5
Total %			**11,6%**	**0%**

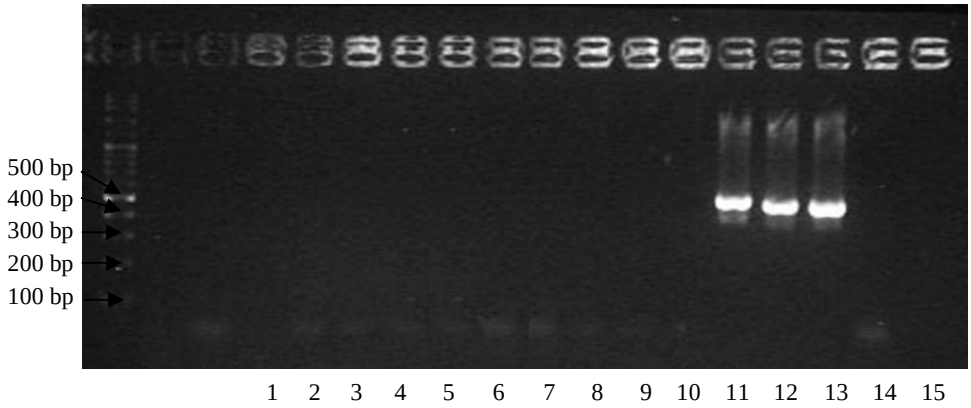

Figure 1: The results of PCR amplification of viral hepatitis B Regio Pre-S1 in HBsAg seropositive samples. (1) individu T3336, (2) T3199, (3) T3331, (4) J190111B, (5) J300511C, (6) Nala, (7) Matu, (8) Pandu, (9) Brahma, (10) Awi, (11) TM SL3, (12) Positive control VHBHu, (13) Positive control VHBGi, (14) Positive control VHBOu, (15) Negative control.

4. Discussion

HBsAg test was performed using commercial ELISA kits (HBsAg ™, Human®, Human für Biochemica Gesellschaft und Diagnostica mbH, Germany) that utilized monoclonal antibody (mAb, mice) to human HBsAg. Monoclonal antibody is monospecific antibody that are identical because they are produced by one type of immune typel that are all clones of a single parent cell. Monoclonal antibodies have monovalent affinity, in that they bind to the same epitope, and has only one epitope [19]. From these definitions it can be said that the ELISA test used in this study are specific to binding human HBV surface antigen protein, so the hepatitis B virus were detected in *M. fascicularis* indicated has same surface antigen protein (gene S) with human HBV.

Some cross-reaction possibility of monoclonal antibody on the ELISA test, that monoclonal antibody is specific for a single epitope, but may cross-react with epitopes having a similar three-dimensional structure. Monoclonal antibodies can also react with two different multiepitopic antigens if they share the particular epitope recognized by the monoclonal antibody. Detection of HBsAg by ELISA in *M. fascicularis* is have done reported, but can not detected HBsAg in Asian origin *M. fascicularis* [20], and reported have been detected in 4 of 21 *M. fascicularis* Mauritius Island origin with positive HBV DNA [10].

Based on the origin historical data, it is known that macaques with higher positive HBsAg are derived from the samples of confiscated and pets macaques, as much as 55% (6/11) compared with samples of captive macaques, 45% (5/11). From these results, it is assumed that pet macaques are at higher risk to infection. Direct contact between NHP pets and their owners is often intimate, as pets climb about and clinging to their owners.Food sharing between pets and owners is common. These activities all have the potential to bring macaque body fluids, especially oral secretions, into contact with their owner's mucus membranes, a potential portal of entry for infectious agents [13]. Hepatitis B virus is in bloodborne pathogen or pathogenic agents present in the blood or body fluids, so that this virus is transmitted through contact with blood products / fluids / mucous. Immunity system also affects the transmission of the disease, when in a stressful environmental conditions, high density and often in poor conditions, which may compromise immunity and facilitate disease transmission [21].

Possible detection of hepatitis B virus surface antigen in *M. fascicularis* is not only caused by HBV transmission between *M. fascicularis* with humans. Some other possibilities are 1) the transmission of *M. fascicularis* with other non human primate species in their natural habitat, and 2) the possibility of indigenous/HBV natural infection. One indigenous hepatitis B have been reported in chimpanzees, where in 11 of 13 chimpanzees with chronic HBV infection looks are genetically different from human HBV genotype. Then when compared with other HBV primate species isolates, HBV chimpanzees have a typical nucleotide and different amino acid in whole genome and gene S which can quickly and accurately identify this strain [9].

Although HBV in primate species may be indigenous, but transmission to humans remains a concern. Certain publications also stated that gibbons, woolly monkey HBV strains, and human HBV can cause hepatitis B infection in chimpanzees. The potential for zoonotic disease transmission exists where blood or body fluid exposure is common, could include chronically infected animals kept as family pets, close contact with caretakers, or in situations in which chimpanzees are slaughtered and used as bushmeat [9].

Failed to detection of HBV viral DNA can also be caused by a primer pair hepSF-1 and hepSR-out used in this study can not amplifying the viral DNA sample well. In a study of HBV in *M. fascicularis* Mauritius Island origin, the primer use of areas that overlap on the surface gene region and the core gene, which is highly conserved among all human HBV genotype [10]. This primer encodes a surface area gene (S gene) of 118 bp with a forward primer base sequence (5 'GGAGTGGGCCTCAGC CCGTTTCTC'3) at position 489-512 and reverse primer (5'GCCCCCAATACCACATCATCCATA'3) [16]. When compared to the primers used in this study, the forward primer SF1 HepB-encoding gene region PreS1 in positions 912-932 and reverse primer encoding gene region PreS1 in the position from 1348 to 1367. Meanwhile, when compared to the primers used by author [10] using the method of real time-PCR, that used the primers from the DNA sequence of overlapping between the core gene at position 1186 to 1203 and position 1266 - 1283. So the possibility of this differences in gene coded region that causes HBV DNA not amplified. Based on phylogenetic analysis, showed that HBV genomes of Mauritius Island long-tailed macaques clustered on human HBV.

In addition, the positive HBsAg and negative HBV DNA can be interpreted as two possibilities: 1) the amount of virus in a sample is very low and 2) chronic infection. According to the authors in [22, 23] noted that the acute phase of infection, hepatitis B virus DNA can be detected in the blood by PCR at 3-5 weeks after exposure, and HBsAg will be detected in the blood 7- 9 weeks after exposure. HBV DNA concentration will be increased gradually with the increase of HBsAg, then will reach a peak during the acute infection. Then the concentration of serum HBsAg will decline to undetectable levels in 4-6 months, if the level of HBsAg remains persistent for more than 6 months, then it is categorized as a chronic infection.

In chronic infection, HBsAg will still be detected after more than 6 months. While HBV DNA can only be found in high levels in the serum during the phase of viral replication. But in non-replicating phase, HBV DNA only slightly or not can be detected in the serum [24]. Studies on a sample of gibbons show the results of HBsAg titers were very low, and the viral DNA with very low levels also indicate asymptomatic infection status careers with low infection [5].

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