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Primary *Tupaia javanica* Hepatocytes Culture as In Vitro Replication System for Ape Hepatitis B Viruses

MARYATI SURYA[1,2]*, DIAH ISKANDRIATI[1,2], SILMI MARIYA[2], UUS SAEPULOH[1,2], PERMANAWATI[2], DONDIN SAJUTHI[1,2,3], AND JOKO PAMUNGKAS[1,2,3]

[1]*Primatology Program, Graduate School, Institut Pertanian Bogor, Jalan Lodaya II/5, Bogor 16151, Indonesia;*
[2]*Primate Research Center, Institut Pertanian Bogor, Jalan Lodaya II/5, Bogor 16151, Indonesia;*
[3]*Faculty of Veterinary Medicine, Institut Pertanian Bogor, Jalan Lodaya II/5, Bogor 16151, Indonesia*

Hepatitis B virus (HBV) is a DNA virus with liver as primary target organ. This virus caused chronic infection that can progress to cirrhosis, liver cancer and even death. *In vitro* model system of hepatocyte cultures is important and widely used to study a variety aspects of hepatitis B. Development of small animal *Tupaia* sp. for the *in vitro* model system is an alternative to the existing hepatocyte cultures. The specific purpose of the study is to develop *Tupaia javanica* hepatocytes culture for HBV replication, and in a broader spectrum to answer the need for *in vitro* model of hepatocytes. Primary *T. javanica* hepatocytes (PTH) culture was successfully maintained for 14 days to reach 80% confluence, and infection of Javan gibbon HBV (GiHBV) and orangutan HBV (OuHBV) onto the culture on day 15 showed viral replication for up to eight days as measured by polymerase chain reaction (PCR). PCR quantification indicated that the highest copy number of DNA virus was detected on day two and decreased until day 8 after infection. Cell receptor for HBV attachment, known as sodium taurocholate cotransporting polypeptide (NTCP) was expressed on the surface of PTH and shown as green luminenscent when observed by immunofluorescence assay. Sequence of partial S gene from the apes HBVs after the viruses have been infected to the PTH showed amino acid identity to their wildtype as high as 99.29% for GiHBV and 95.71% for OuHBV. This study suggested that the primary *T. javanica* hepatocytes culture can support the replication of GiHBVand OuHBV.

Key words: hepatitis B virus, hepatocytes culture, *in vitro* model, *Tupaia javanica*

Virus hepatitis B (VHB) adalah virus DNA dengan organ hati sebagai target utama. Virus ini menyebabkan infeksi kronis yang dapat berkembang menjadi sirosis, kanker hati bahkan kematian. Kultur hepatosit adalah model *in vitro* yang penting dan banyak digunakan dalam mempelajari berbagai aspek terkait dengan hepatitis B. Pengembangan model *in vitro* dari hewan kecil *Tupaia sp.* merupakan alternatif kultur hepatosit yang sudah ada. Tujuan dari penelitian ini adalah mengembangkan kultur hepatosit *Tupaia javanica* (PTH) untuk replikasi VHB sehingga secara luas dapat menjawab kebutuhan akan model *in vitro* hepatosit. Kultur hepatosit primer *T. javanica* berhasil mencapai kepadatan 80% pada hari ke-14, dan infeksi VHB *Javan gibbon* (GiHBV) dan VHB orangutan (OuHBV) ke dalam kultur pada hari ke-15 menunjukkan replikasi virus hingga hari ke-8 berdasarkan pemeriksaan *polymerase chain reaction* (PCR). Kuantifikasi PCR mengindikasikan bahwa jumlah kopi DNA virus tertinggi terdeteksi pada hari ke-2 dan menurun hingga hari ke-8 setelah infeksi. Pada pewarnaan imunofluoresensi, reseptor sel tempat pelekatan VHB, yaitu *sodium taurocholate cotransporting polypeptide* (NTCP) terekspresi pada permukaan hepatosit yang terlihat sebagai pendaran hijau. Penjajaran sebagian gen S dari VHB kera setelah virus diinfeksikan ke dalam kultur primer hepatosit menunjukkan kesamaan asam amino terhadap virus *wildtype* sebesar 99.29% untuk GiHBV dan 95.71% untuk OuHBV. Penelitian ini mengindikasikan bahwa kultur hepatosit primer *T. javanica* mampu mendukung replikasi GiHBV dan OuHBV.

Kata kunci: kultur hepatosit, model *in vitro*, *Tupaia javanica*, virus hepatitis B

Hepatitis B virus (HBV) infection is globally distributed and infected approximately two billion people in the world population (WHO 2009). An estimation of 240 million people chronically infected with HBV(WHO 2015) in the world. It is the most common type of hepatitis found in developing countries, including Indonesia which served as the third highest prevalence country in the world. The viral infection can cause acute and chronic hepatitis, cirrhosis, hepatocellular carcinoma (HCC) and can lead to death. In the United States and Europe, approximately 10% of patients infected with HBV lead to a chronic disease with the consequences of development into liver cancer. While an effective vaccine is available for preventing HBV infection, there is no effective therapy for patients worldwide that are already chronically infected by HBV. Therefore, we need a treatment strategy to reduce deaths (CDC 2015).

The primary obstacle to conduct research for drug

*Corresponding author; Phone: +62-251-8320417, Email: atie@indo.net.id

development and gene-based therapy for HBV infection is the lack of good in vitro as well as in vivo systems using small animal model, which can be infected by the virus and assure the replication occur in the system (Guha *et al*. 2004). In the development of in vitro model, hepatocytes cultures have been demonstrated using hepatocytes of human origin, both primary cell cultures or hepatoma cells cultures. However, the use of hepatocytes culture of human origin is limited due to ethical factors. This limitation led researchers to develop hepatocyte cultures of small animal origin. The primary hepatocytes cultures of woodchuck and peking duck have been successfully developed, but these cultures were only capable in supporting woodchuck hepatitis virus (WHV) and duck hepatitis B virus (DHBV) replication (Tuttleman *et al.* 1986; Theze *et al*. 1987; Witt-Kehati *et al*. 2016).

Tupaia belangeri, a small mammal, can be infected by HBV experimentally although so far there is no data found regarding natural infection with HBV in *Tupaia* sp. (Guha*et al*. 2004). *T. belangeri* have been used as an animal model to study the effectiveness of the vaccine, the safety of blood product, the effectiveness of chemotherapy and disinfectants against viral hepatitis. Human HBV can infect hepatocytes of *T. belangeri* both *in vitro* and *in vivo*. In addition, primary hepatocytes of *T. belangeri* can also be infected with woolly monkey's HBV (WMHBV). This virus was able to synthesize mRNA and cccDNA, and release Hepatitis B surface Antigen (HBsAg) and HBeAg into the culture medium (Walter *et al*. 1996; Kock*et al*. 2001; Glebe *et al.* 2003). The suceptibility of *T. belangeri* against human HBV infection in the *in vivo* and *in vitro* systems making these animals good model of liver cancer caused by HBV and aflatoxin B1 (Cao *et al*. 2003).

T. belangeri is found in South-East Asia, north of the Isthmus of Kra, including Thailand, Bangladesh, Burma, far North-Eastern India and Nepal, Southern China, Cambodia, Lao PDR, Viet Nam, and associated coastal islands, including Hainan (Han *et al.* 2008a). The limitations to use these animals out of their habitat is an obstacle for the development of antiviral drugs and vaccine research in Indonesia. It is necessary to find an attempt to replace it with other species of animals that have a genetic relationship with *T. belangeri. Tupaia javanica*, one of several Indonesian *Tupaia,* is distributed in Bali, Java, Nias, and western Sumatera (Han *et al*. 2008b; Roberts *et al*. 2011). Until now there has been no report of the use of this animal in biomedical research. *T. javanica* has genetic similarity to *T. belangeri* up to 91 % (analyzed using Basic Local Alignment Search Tool). It becomes an opportunity to explore the ability of *T. javanica* hepatocytes to support the replication of HBV as occurs in hepatocytes *T. belangeri*. The purpose of this study was to developed *T. javanica* hepatocytes cultures to assess the ability of the hepatitis B virus replication in cell culture. It is expected to answer the need for hepatocytes *in vitro* model for biomedical research related to hepatitis B.

MATERIALS AND METHODS

Isolation and Primary Culture of Tupaia Hepatocytes. Three wild adult *T. javanica* Horsfield 1822 (identified by the Indonesian Institute of Science Number 269/IPH.I.03/KS.02/IX/2013, Fig 1) were maintained in the quarantine facility of Primate Research Center, Bogor Agricultural University (IPB PRC). All animals procedures were performed by veterinarians at the IPB PRC, and animals protocols have been evaluated and approved by IPB PRC Institutional Animal Care and Use Committee (IACUC) Number PRC-IPB-13-D004. Procedure of hepatocytes isolation was refered to Glebe *et al*. (2003) with the following modification: pre-perfusion washing solution (Hanks Balanced Salt Solution from Invitrogen, USA; cat# 14170-112, 5 mM EGTA, 0.25 μg mL^{-1} amphotericin B), and perfusion solution (DMEM, 100× $CaCl_2$, 1% colagenase II) was used to remove red blood cells from the liver.This step was done using 50 mL syringe until the liver was semi-solid and pale. Livers then were minced and incubated at 37 °C for 5-10 min, centrifugated at 300-500 g for 5 min, and washed using saline buffer to obtain cell pellets. Cells were plated in 24-well culture plate at 10^4 cells per well using growth media (HBM and HCM hepatocytes media from Lonza USA supplemented with 20% Fetal Bovine Serum) at 37 °C and 5% CO_2.

Immunofluorescence of NTCP. The assay was performed after the cells reach 80% of its confluence. The cells were washed with saline buffer followed by fixation with methanol and acetone. Cells were then stained with NTCP polyclonal antibody (H-42, from Santa Cruz Biotechnology, Inc USA) and incubated at 37 °C for 1 h. The cells were washed with saline buffer and incubated with anti-rabbitIgG Fluorescein Isothiocyanate (FITC) (Sigma USA) for 1 h at 37 °C. The cells images were captured with Nikon fluorescence microscope. NTCP unstained cells were served as negative control.

Virus Infection on the Cells. OuHBV and GiHBV

Fig 1 *Tupaia javanica* Horsfield 1822 (identified by the Indonesian Institute of Science Number269/IPH.I.03/KS.02/IX/2013), Photographed by Walberto Sinaga.

isolates were obtained from the viruscollection of IPB PRC. HBsAg titers (IU mL^{-1}) of the isolates were 60 000 IU mL^{-1} (GiHBV), and 125 000 IU mL^{-1} (OuHBV).Virus suspension from both isolates were added to PTH on day 15 after the cells reach 80% confluence followed by 18 h of incubation at 37 °C with 5% CO_2. The cells were washed with saline buffer to removed excess virus and later growth media were added. The released viruses were collected from infected cells media on days 1 to 8 after infection.

Detection and Quantification of HBV Replication. Detection of released HBV in the media was performed using Polymerase Chain Reaction (PCR) and the number of DNA virus were calculated using real time PCR (qPCR). DNA was extracted using QiAmp Blood DNA Mini Kit (Qiagen, USA) according the manufacture's procedures. The primer set used in this method was described by Warren *et al.* (1999). This primer set amplified the partial S gene of HBV and yielded aproximately 456 bp fragment of this gene. PCR amplification conducted using conventional PCR (Applied BioSystem 9700, USA) at the conditions of 94 °C for 10 min, followed by 40 cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 2 min, with a final extension at 72 °C for 10 min. PCR reaction at 25 µL total reaction containing 10 pMol mL^{-1} of each primer, 12.5 uL Kappa HotStartReadyMix (KAPA Biosystems, USA), 5 µL DNA and adjusted with nuclease free water to 25 µL. Amplicons (5 µL) were visualized on 1% agarose gel and sized against a 100-bp DNA ladder (Invitrogen, USA). For HBV replication quantification, we used iQ5 Real Time PCR (BIO-RAD, USA). Real time PCR amplification were performed by following protocol: denaturation for 2 min at 98 °C, amplification for 40 cycles consisting of 5 sec at 98 °C and 10 sec at 55 °C. The reactions were 10 pMol mL^{-1} of each primer, 12.5 µL SsoFastEvaGreenSupermix (BIO-RAD, USA), 5 µL DNA and adjusted with nuclease free water to 20 µL.

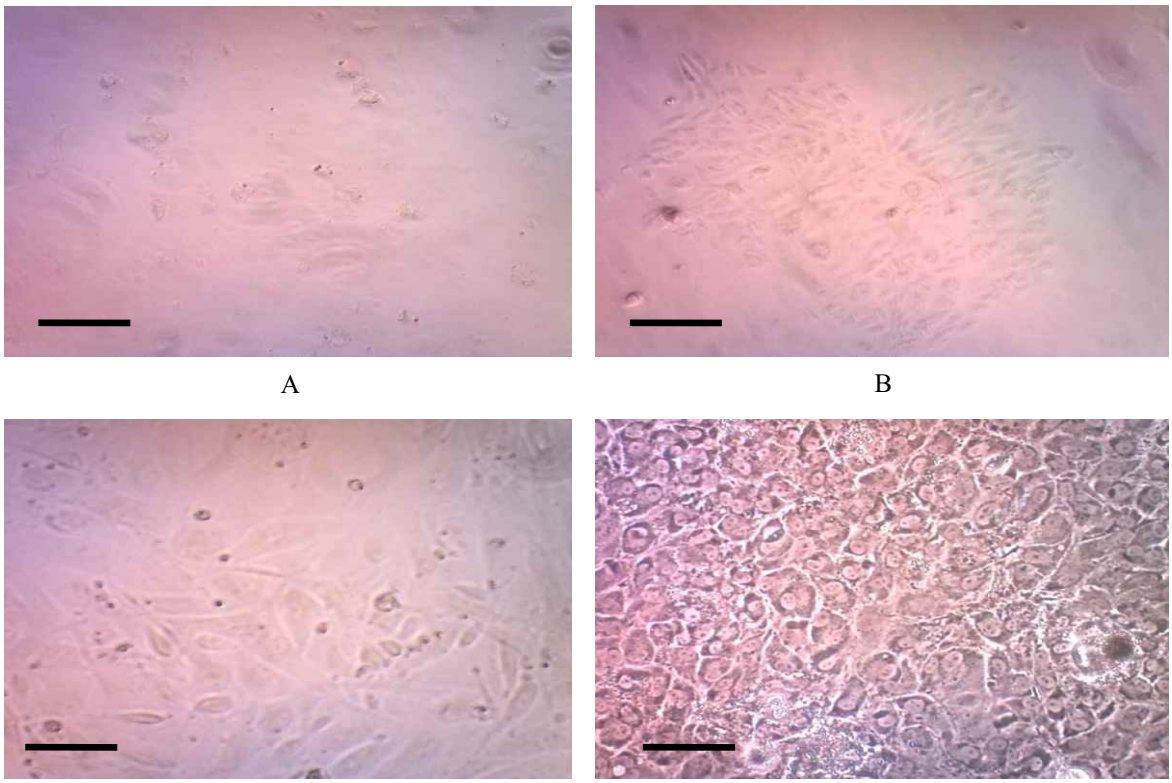
Sequencing and Alignment of Wildtype and Post-infection HBVs. HBV DNA from wildtype and post-infection were extracted using DNA purifying kit (Qiagen, USA). Sequencing of nucleotides were carried out in Applied Biosystems at 1st BASE Malaysia using the primer set mentioned above. Alignment of the nucleotide sequences was done with CLUSTALW (EMBL-EBI).

RESULTS

The liver organ was collected from adult male *T. javanica* Horsfield, 1822 (Fig 1) and prepared for primary hepatocytes culture. On day 1, cells showed individual elongated-shaped for 3 d and then the cells transformed into polygonal shape and began to form colony. From the observation the stage of hepatocytes proliferation on day 1, 4, 7, and 14. Cells reached 80% confluence on day 14 (Fig 2).

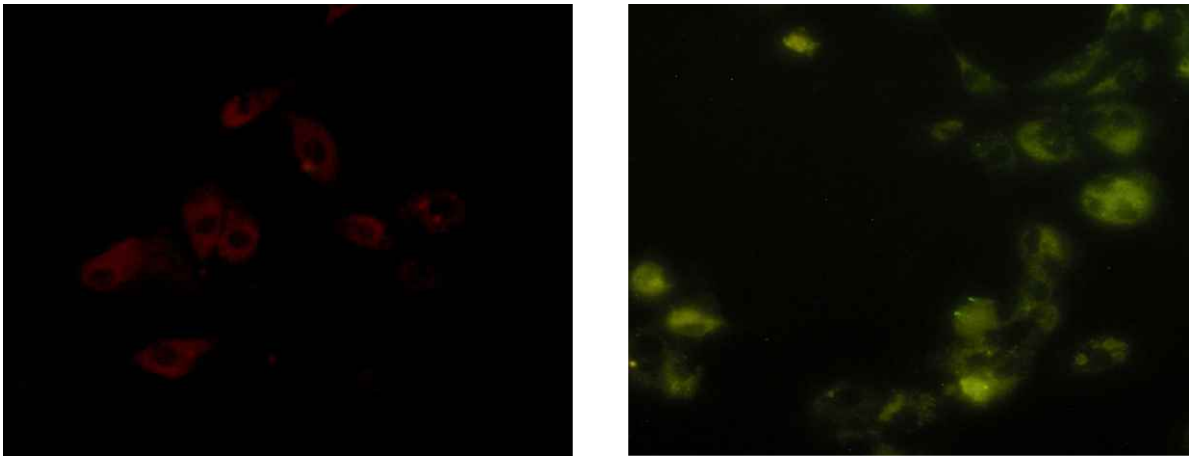
Immunofluorescence assay was carried out to confirm that *T. javanica* hepatocytes has NTCP receptor for HBV entry into the cells. Fig 3 showed the expression of NTCP indicated by the greenish fluoresceinated color under fluorescence microscope while the unstained cells showed no fluorescein coloring. The greenish fluoresceinated cells resulted from the reaction of NTCP protein bound to its antibody which later reacted with anti-rabbit IgG conjugated to FITC. The result showed 70% field view NTCP.

The presence of virus did not indicate any change in cells morphology or cytophatic effect of the cells (Fig 4). There were no differences in hepatocytes



A B C D

Fig 2 Cells morphology of PTH cultures: (A) Day 1, individual elongated shape cells, (B) Day 4, colony of polygonal shape cells at approximately 30% confluence, (C) Day 7, colony of polygonal shape cells at approximately 50% confluence;(D) Day 14, colony of polygonal shape cells at approximately 80% confluence. Bar = 200 µm.



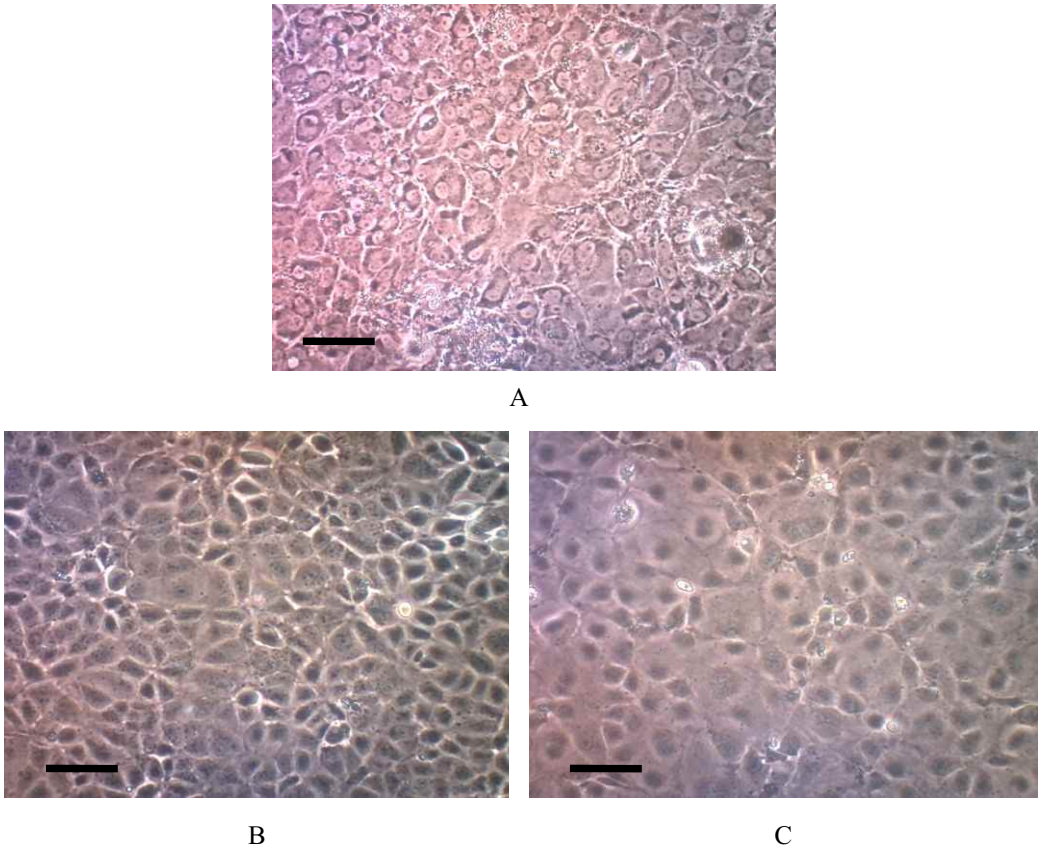
A B

Fig 3 Detection of NTCP receptors in PTH culture using immunofluorescence assay; (A) Negative control (without NTCP antibody),(B) Hepatocyte NTCP(green objects, 70% field view). Bar = 100 µm.

morphology between the infected and uninfected PTH. The cells morphology was colonized polygonal-shaped.

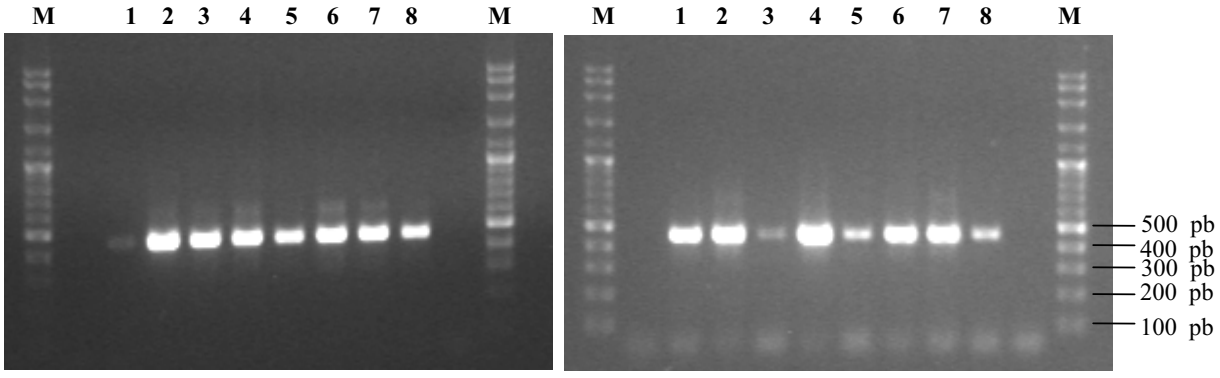
Fig 5 showed detection of amplified HBV DNA by conventional PCR assay. It showed the presence of both GiHBV and OuHBV DNA from day 1 to day 8 after infection. The 456 bp fragment was shown by DNA band with different thickness indicating the initial concentration of released HBVs in the culture medium.

The amplification of HBV partial S gene indicated that viral replication occurred from day 1 to day 8. In this study, the peak of viral DNA copy number (3 835 and 4 825 viral copy number) was on day 2 for both GiHBV and OuHBV. The viral copy number decreased after day 2 for both viruses, although OuHBV tends to increased until day 5. Fig 6 showed viral DNA copy number as measured by real time PCR.



A B C

Fig 4 Cells morphology of PTH culture pre- and post-infection with apes HBV: (A) PTH on day 14, pre-infection, (B) PTH on day 22, post-infection with GiHBV, (C) PTH on day 22, post-infection with OuHBV. Bar = 200 µm.



A B

Fig 5 Replication of HBV DNA detected in culture medium. Apes HBV were used to infect PTH. Detectionof partial S gene was done by conventional PCR and visualized on 1% agarose gel, resulted 456 bp (M= marker; 1-8=supernatant days 1-8 after infection): (A) GiHBV replication, (B) OuHBV replication.

To see if the virus infection to *Tupaia* cells causes any mutation of HBV, the sequencing for both viruses from wildtype and post-infection were carried out using Applied Biosystems. In the GiHBV partial S gene occurred one point of mutation at nucleotide position 78 (G78A) with identity up to 99.76% between wildtype and post-infection viruses, that caused amino acid changes, glycine to glutamate (Gly138Glu) with identity 99.29%. On the other hand, in OuHBVthere were 14 points of nucleotide mutation (T2875C, A2918C, A2967C, A2977C, T3022A, T3071A, T3077C, A3112G, G3116T, A7G, G10T, A20G, A28G, and A44T) resulted 96,67 % identity between wildtype and post-infection. Out of the 14 nucleotide mutations occurred, only a change of 6 amino acids had occurred (identity 95.7%): Thr40Asn, Asp43Glu, Thr75Ser, Ser90Ala, Ala119Thr, and Phe130Tyr. Fig 7 showed the aminoacid alignment

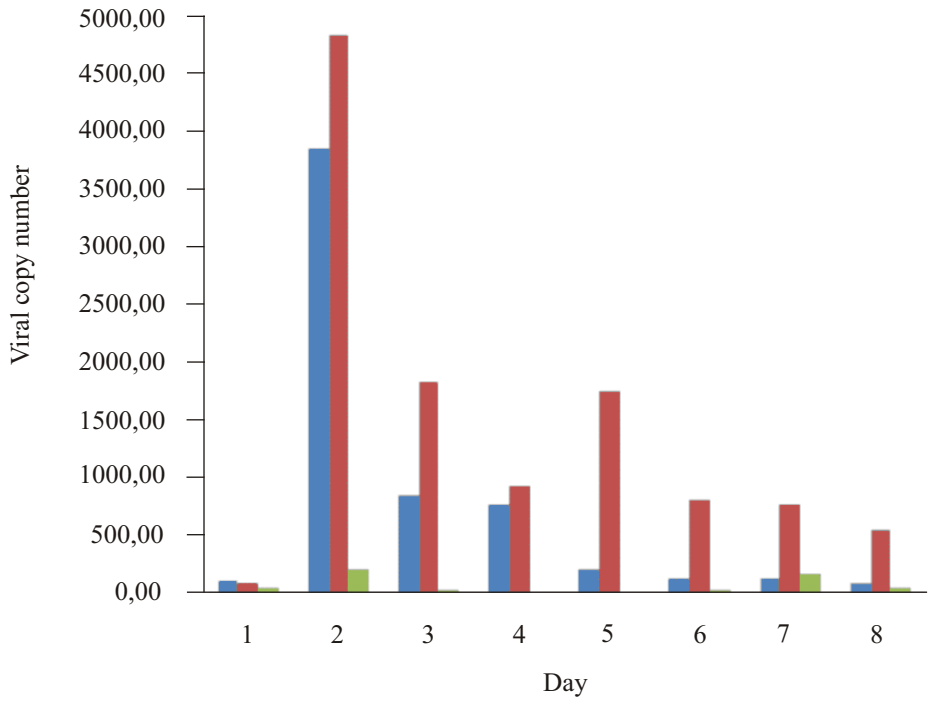


Fig 6 Quantification of GiHBV and OuHBV replication from PTH culture medium using real-time PCR based on viral copy number. GiHBV (■), OuHBV (■), and PTH (■).

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GiHBV wildtype          MGQNHSVTNPLGFFPEHQLDPLFKANTNNPDWDFNPNKDDWPEATKVGVGAFGPGFTPPH 60
GiHBV post-infection    MGQNHSVTNPLGFFPEHQLDPLFKANTNNPDWDFNPNKDDWPEATKVGVGAFGPGFTPPH 60
                        ************************************************************

GiHBV wildtype          GGLLGWSPQAQGITSILPAVPPPASTNRQAGRQPTPISPPLRDTHPQAMRWNSTVFHQTL 120
GiHBV post-infection    GGLLGWSPQAQGITSILPAVPPPASTNRQAGRQPTPISPPLRDTHPQAMRWNSTVFHQTL 120
                        ************************************************************

GiHBV wildtype          QDPRVRGLYYPAGGSSSGTV 140
GiHBV post-infection    QDPRVRGLYYPAGGSSSETV 140
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A

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OuHBV wildtype          MGQNLSVTNPLGFFPEHQLDPLFRANTNNPDWDFNPNKDTWPDATKVGVGAFGPGFTPPH 60
OuHBV post-infection    MGQNLSVTNPLGFFPEHQLDPLFRANTNNPDWDFNPNKDNWPEATKVGVGAFGPGFTPPH 60
                        ************************************.**:****************

OuHBV wildtype          GGLLGWSPQAQGVTTILPAVPPPASTNRQSGRRPTPISPPLRDTHPQAMQWNSTVFHQAL 120
OuHBV post-infection    GGLLGWSPQAQGVTSILPAVPPPASTNRQAGRRPTPISPPLRDTHPQAMQWNSTVFHQTL 120
                        **************:**************:***************************:*

OuHBV wildtype          QDPRVRGLYFPAGGSSSGTV 140
OuHBV post-infection    QDPRVRGLYYPAGGSSSGTV 140
                        *********:**********
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B

Fig 7 Alignment of partial S amino acid sequences between wildtype and post-infection apes HBV onto PTH culture: (A) GiHBV, (B) OuHBV. Note : conserved sequences (*), conservative mutation (:), semi-conservative mutation (.), non-conservative mutation ( ).

between wildtype and post-infection virus.

DISCUSSION

Compared to what was described by Glebe *et al*. (2003), the growth of *T. javanica* hepatoyctes in this study were slower; the proliferation of hepatocytes reached to its 80% confluence after 14 d. Animals condition might be one of the factors causing this difference. Animals used in the study of Glebe *et al*. (2003) were captive bred with known age, meanwhile the animals used in this study were wild caught, in

which the age of the animals were determined by estimation. The number of hepatocytes was influenced by the liver size and weight, so it depends on the age of the animal. To obtain hepatocytes, Glebe *et al.* (2003) described the use of pump to control the speed and pressure of the solution to minimize cells damage caused by high pressure during perfusion stages. In our study, perfusion was performed manually using syringe thus more damaged cells might have occurred which lead to the decreasing of cells number.

Despite the difference of the perfusion methods, in this study *T. Javanica* hepatocytes were successfully isolated and cultured showing different morphologic appearance during cell proliferation on day 1, day 4, day 7, and day 14. On day 1, hepatocytes appeared as individual elongated-shape cells, while on day 4 it formed colony of polygonal-shape cells, and on day 14 the surface of the substrate covered by hepatocytes with 80% confluence. The polygonal-shape of the PTHs showed in this study was similar to the morphology of the hepatocytes from the study published by Glebe *et al*. (2003).

Similar to the study conducted by Yan *et al.* (2012) on *T. belangeri*, in this study we demonstrated by immunofluorescence assay that *T. javanica* hepatocytes also carry NTCP as cell surface receptor to facilitate the entry of HBV into the cell. In humans, the hepatocytes have a surface protein NTCP specifically interacts with the large surface protein of HBV, thereby functioning as a viral entry receptor. The expression of NTCP correlates with the susceptibility of the target cells. Experimental reduction of NTCP expression markedly inhibited HBV and HDV infection on known susceptible cells (Iwamoto *et al.* 2014; Yan *et al.* 2014).

Both ape hepatitis viruses (GiHBV and OuHBV) infected the PTH up to eight days post infection, as demonstrated by the amplification of HBV S gene from supernatant of infected cells. This results were also supported by the viral copy number that can be detected up to the eighth day using quantification PCR method.

HBV binds to its receptor (NTCP) and enters the cell through endocytosis and fuses to the endosome, thus releasing its nucleocapsid. A comprehensive illustration of replication of the hepadnaviral genome was described by Beck and Nassal (2007). The existence of NTCP, and the ability to amplify its gene indicated the capability of *T. javanica* hepatocytes in supporting the replication of non human primate HBV without causing cythopathic effect. This is in accordance to the study conducted by Glebe *et al.* (2003). Decreased ability to support the replication of cells after the eighth day probably caused by cell death or reduced cell proliferation ability.

Declining of viral copy number after day 2 probably was caused by the reduction of cells number. Primary cell cultures are cultures of cells obtained directly from normal tissue or organ, and have not been subcultured through passages. Cells in primary culture have a limited lifespan and at one stage cells will not multiply and die. Primary cell cultures are still carrying cell genotype origin, and primary hepatocyte culture can lose their phenotype and further cellular mechanisms of the cell cannot support further viral replication (Lin *et al.* 2007). Generally, the replication graphic trend was decreasing two days post-infection, the fact that there was an increase of viral copy number of OuHBV on day 5 was still unknown.

Sequences of GiHBV partial S gene from wildtype and post-infection were aligned and showed one nucleotide change from G to A at position 78 (G78A, identity 99.76%) and caused amino acid changes, from glycine to glutamate (Gly138Glu) with identity 99.29% (Fig 7A). Meanwhile, in OuHBV there were 14 nucleotides changes in partial S gene sequences from wildtype and post-infection to the cells (identity 96.67%): T2875C, A2918C, A2967C, A2977C, T3022A, T3071A, T3077C, A3112G, G3116T, A7G, G10T, A20G, A28G, and A44T. Nucleotides changes resulted in the changes of six amino acids (identity 95.7%): Thr40Asn, Asp43Glu, Thr75Ser, Ser90Ala, Ala119Thr, and Phe130Tyr (Fig 7B). These changes might have occurred due to the fact that *Tupaia* hepatocytes are not the original host for apes HBV, so the changes occurred was thought as adaptation process for the HBV to replicate in a new host.

The primary *T. javanica* hepatocytes culture developed in this study has similar characteristic to *T. belangeri* hepatocytes that has been described previously with polygonal-shape morphology. The PTH in this study was able to proliferate *in vitro* and reach 80% confluence in growth medium after 14 d of incubation. The *T. javanica* primary hepatocytes also showed evidence for the presence of NTCP receptors, as captured by immunofluorescence assay. There were amino acid sequences mutations of partial S geneafter the infection of apes HBV onto PTH culture. Considering the above mentioned findings, we conclude that *T. javanica* hepatocytes can be utilize as an alternate *in vitro* system for the replication of apes HBV (GiHBV and OuHBV). To this date, this report provides the first

evidence for the existence of a novel *in vitro* model of apes HBV infection on Indonesian primary Tupaia hepatocytes culture, which can be explored further for the potential study of immunotherapeutic approaches against viral hepatitis B.

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