CHAPTER V

CONCLUSION AND DISCUSSION

Captive gibbons in Krabok Koo Wildlife Breeding Center

Wild gibbons (*Hylobates spp.*) can be found throughout the tropical rainforests of South and Southeast Asia, including Thailand, Laos, Cambodia Indonesia and Malaysia. They are at high risk of extinction due to habitat loss and increased illegal pet trade. After categorization as a conserved wild animal in Thailand, hundreds of confiscated and abandoned gibbons have been handed over to the authorities of Thai government. Krabok Koo Wildlife Bleeding Center was established to care the abandoned gibbons before re-establishing and reintroduction program of selected animals to the forest. The hepatitis B virus screening in gibbon population was done to frustrate possible disease spreading of the wild gibbon and humans population.

Two main species of gibbons in Thailand, *H. lar and H. pileatus*, were the main gibbon population in Krabok Koo Wildlife Breeding Center (Table 6). Moreover, the discovery in Thailand of *N. concolar*, the tonkin black crested gibbons normally found in North Vietnam and Central China [114] , suggests that they were illegally hunted and sent through the borderline between Vietnam and Thailand. This event indicated the potency of illegal wild animal trade nowadays.

Most of captive gibbons are housed in small monogamous families and some animals were borne in-house. Due to the lack of historical data of abandoned animals, some analysis were performed with recorded data only. For example, the average age of gibbons in the center was lower than expected because the year of birth was noted mostly for the new born gibbons in the center, but not for wild animals (Table 6).

To investigate the hygiene of captive gibbons, a routine health care program was usually performed. Additionally, blood chemistry analysis was checked to confirm the health status of gibbons (Table 7). Because there is no record of the normal range of gibbon blood components, the human standard range was used as the criteria for investigation. For human red blood cell analysis, the normal values for the hematocrit

will vary with age and sex and the percent hematocrit reading closely parallels the hemoglobin values of the individual. The hematocrit is decreased in anemia and increased in the various forms of polycythemia. In gibbons, the range of percent hematocrit was similar to the normal human criteria which suggests the normal red cells status in most of animals. Chi-squared analysis indicated the significantly unrelated of percent hematocrit and HBV carrier status (Table 7). Anemia or other blood diseases should be checked for some gibbons that had low hematocrit levels.

The WBC is utilized to indicate infection and may also be employed to follow the progress of certain diseases and therapies. In disease stages, a particular white blood cell type may show an absolute increase in number. For example, increasing numbers of neutrophils, eosinophils and lymphocytes may indicate bacterial infection, parasitic infection or viral infection, respectively. Most of the gibbons exhibited WBC in normal range except some cases such as Darkie (R5 cage) presented the over range of WBC and % neutrophils while % lymphocyte was under normal range estimation. Bacterial infections or other blood diseases should be considered in some animals. Statistical analysis showed that number of WBC was not related to HBV carrier status of gibbon (Table 7).

HBV transmission in captive gibbons

Serological analysis of HBV infection in the gibbons kept at the Wildlife Breeding Center showed that 38.61% of animals were infected with HBV; 19 animals were HBsAg and HBV DNA positive carriers and could be a source of viral spread in the gibbon population (Table 8). The prevalence of gibbon HBV carrier was rather higher than the prevalence in Thai human population [59,101]. Similar figures have been reported recently in captive gibbons housed in the center for Gibbon Studies in California, by Lanford *et al.* (2000) [5]. On the other hand, Grethe *et al.* (2000) [14] reported even higher frequencies of HBV infection in animals housed at different zoos. Similarly to the first report on gibbon HBV [3], four of the HBV carrier gibbons were negative for anti-HBc even when tested repeatedly with different ELISAs (Table 8). Undetectable anti-HBc antibody was reported in acute HBV cases as the human HBV-2 type [118]. However,

there is no data of HBV-2 spreading in Thais population. In gibbon cases, the specific immune response against core may be undetectable due to the lack of specific anti gibbon IgG reagents in the commercial test kits.

ALT levels representative for destruction of the liver during gibbon HBV infection, were significantly higher in chronic carriers (68.75 $\pm$ 48.12 U/l) than normal control animals (33.04 $\pm$ 15.91 U/l, p <0.05). Albumin, a protein synthesized by the liver,and globulin levels were detected in the normal range while creatinine levels indicated the normal kidney function in most animals (Table 9).

HBV is transmitted by sexual contact and by parenteral exposure, although it is thought that mother-to-child perinatal transmission is responsible for high rate endemically in several regions of the world. More than 25-50 % of the chronic HBV carriers results through vertical transmission or horizontal transmission by nosocomial exposure in early childhood. Most of infants and young children were infected by vertical transmission from carrier mothers [119,46]. In gibbons, vertical transmission was strongly supported by > 98% identity of *PreS1* gene of HBV isolated from mother and baby gibbons (Figure 13, Table11). Hence, base sequence changing may have occurred through mutations in time; baby R6 younger than Tao at the date of blood sampling showed higher sequence similarity. Offspring from chronic mother may be exposed to the infected blood and bodies fluid during birth or contacted to milk or saliva since the early stage of life and most of them will become HBV carriers [120] . Noticeably, all animals which share cages with gibbon carriers were infected by HBV and anti-core antibody was detected (Table 13). These data suggests that sexual contact or horizontal HBV transmission of family members could be a source of viral spreading in this gibbon population.

Similar to observations made in human HBV, HBsAg could be detected in saliva of all HBV carriers [102] . Until now, few cases have been reported on HBV infection from saliva by a human bite [121,122] but the high prevalence of HBV among dentist personnel and family members suggests that HBV might spread by saliva [123,124]. However, currently no report of HBV transmission from captive animals to human exists. In the present study, HBV DNA was detected in saliva of six gibbons, representing circulating HBV infectious particles in body fluids (Table 12). HBV-positive human saliva

administered intradermaly, but not orally, to gibbons caused HBV transmission [17]. Thus, HBV DNA detected in gibbon saliva may indicate a potential risk for horizontal transmission especially if the animals are injured after fighting or accidental injury of animal keepers. Two workers (5.9%) of Kabok Koo Center were found positive for HBsAg and HBV DNA, which corresponds to seroprevalence of HBV in general adult Thai population [101] (Table 14). Other two samples presented only anti-S antibody without any other markers suggested the vaccinated character. However, their historical data has no record.

Although the electron microscope study of gibbon and human HBV demonstrated the similar in size and shape of both viral particles (Figure 14), molecular characterization by RFLP and phylogenetic analysis confirmed the separate clustering of gibbon HBV compared to human and nonhuman primate HBV. Phylogenetically, the three different clusters within the gibbon viruses observed could relate to genomic variants as described by Grethe *et al.* (2000) [14]. Interestingly, animals in the C and L cage areas shared the same branches of each group that suggested the possibility of brother and sister relationship of these animals or saliva contamination during fight or feeding in the early age. *PreS1* amplified products from human and gibbon HBV showed different size indicated that 33 bps deletion could detected only in gibbons and circulated viruses in two workers had an origin from human HBV source (Figure 15a). DNA sequencing confirmed that 33 bps deletion in *PreS1* region was found only in gibbon, but not in human samples. HBV RFLP analysis of the two human HBV isolated from animal care workers indicated that they were infected by human HBV genotype C (Figure 15b and 15c). Using the same procedure, gibbon HBV represented four different groups distinct from human HBV RFLP profile (Table 15).

Precore promoter mutation AGG $\rightarrow$ TGA at nucleotide 1762/1764 previously linked to a relatively moderate down-regulation of the synthesis of HBeAg [125,126] but also observed in the HBeAg-positive stage [103], have been associated with more-severe liver damage [127] and with increased response to interferon [128]. In gibbon HBV carriers, this mutation was detected in four HBeAg positive animals including Pok-C2, Midnight-R27, Nin-L14, all are anti-HBc positive, and Caesar-L10 with anti-HBc negative (Figure 16). One sample (G4) showed the untypical pattern after digested with *Sau3A* I. This sample

probably has the variant sequences in core region and it was identical to *Sau3A* I cutting site. Unfortunately, core gene sequencing and ALT status in these gibbons could not be analyzed in this study due to the limitation of samples.

The recent finding that chimpanzees as well as orangutans can be infected by HBV in the wild makes any proposed spread of HBV from the New World extremely unlikely [99,129,15]. In contrast, all data support the idea that these viruses are indigenous to the different nonhuman primate populations. Moreover, the close relation between the human genotype C viruses, the orangutan HBV and gibbon HBV, all found in South East Asia, is not consistent with the presumption that there was a single transmission between humans and apes in the Old World. However, this event involves a common ancestor of human genotypes A to E and the ape viruses, which rather spread through cross-species transmission in South East Asia [130]. Gibbon and chimpanzee HBV are more closely related to each other than to the human genotypes. More sequence data on different isolates are needed as well as possible evidence for transmission of nonhuman primate HBV to humans.

The high prevalence of hepatitis B virus infection in captive gibbons of the Krabok Koo Wildlife Breeding Center shows that HBV is an important infectious agent that should be aware in captive gibbons. Since chronic carrier gibbons are the main source of HBV in the center, separating carriers and early vaccination of newborn babies should be done to reduce the number of infected animals. In additional, vaccination to non-immune gibbons for preventing the HBV infection in Karbok Koo Wild Life Breeding center should be evaluated for further recommendation of new arriving gibbons. Although transmission of HBV between nonhuman primates and humans, similar to zoonotic HIV transmission [131], could not be confirmed, these studies could provide more insight in the molecular evolution and transmission routes of HBV and facilitate our understanding of the origin of HBV and the viral pathogenesis.

Surface antigen mapping of gibbon HBV

Since the first isolation of gibbon HBV was reported by Mimms *et al.* (1993) [3], this unique variant HBV was indicated by non-reactivity to a monoclonal anti-PreS2 (aa

116-134) and an anti-PreS1 mAb which binds the PreS1 hepatocyte receptor region (aa 27-35). However, this event was explained by amino acid changing of the gibbon HBV genome.

Genome sequencing of gibbon viruses indicated the 33 bps deletion after the start codon of *PreS1* gene [97] and might cause the different conformational structure of surface protein of gibbon HBV. Similarity of gibbon and human HBV particle was investigated by electron microscope in the present study. The HBsAg specific monoclonal antibodies F9H9, 9A and 1Ff4 showed positive binding to viral particles of both gibbon and human (Table 17,18). Polyclonal anti-S from sheep which bound to several region of HBsAg, gave high binding capacity to all samples while anti-127 mAb could not bind to both HBV groups due to the fact that this antibody was developed for detecting recombinant HBsAg derived from yeast (personal contact to Dr. R.A. Heijtink). Statistical analysis presented the equal variance of antibodies binding to human and gibbon HBV and indicated the similarity of surface HBV binding sites of both species to anti-HBV antibodies.

Midnight, a gibbon sample showed the higher binding capacity to all antibodies compared to others gibbon and human antigens. Interestingly, midnight did not show the special group different from others by *PreS1* sequencing or RFLP analysis. However, the sufficient binding factor in serum of midnight may cause the false positive of ELISA assay. Unfortunately, serotype identification and *S* gene sequencing of midnight did not perform due to the limitation of samples.

Although the binding epitope of anti-PreS2 antibody was not identified, we assume that it should bind to the non-variance of PreS2 region because the anti-PreS2 mAb adhered to both human and gibbon viruses with strong reactivity (Table 17,18). The Pre-S2 region is also reported as hepatocyte receptor [69]. An alternative explanation for the poor reactivity of this antibody to human samples may be the presence of factors in the sera that bind to the specific receptor region and block the anti-PreS2 antibody binding region.

Anti-PreS1 mAb bound to only one human sample, but not for all gibbon viruses supported the lack of binding site or conformational changing of PreS1 protein due to the 33 bps deletion of gibbon HBV (Table 19).

Inhibition binding of gibbon HBsAg by immune anti-HBs

Hepatitis B vaccine is widely used to prevent HBV infection, usually for children of HBsAg-positive mother, haemodialysis patient and medical personnel. Several reports described the physical, chemical and immunological characteristics of recombinant DNA vaccine. The anti-HBs antibodies induced after immunization are mainly reactive with "*a*" determinant (aa 120-147) since this region was reported as a specific antibodies against HBV, especially in the second loop (aa 139-147) [81].

The inhibition in solution assay was performed with solid phase human monoclonal F4-7b for capturing human and gibbon HBV. Antibodies from vaccination studies with Engerix-B, GenHevac-B and Hepagene-B were compared in titrated amounts with reference monoclonal antibodies (F4-7b) and HBIg (Hepatect). Blocking binding of gibbon HBV by human anti-S after vaccination seems less efficient than human HBV (Figure 23, Table 20). Interestingly, immune antibodies induced from Engerix B vaccine showed the minimum binding capacity to HBV, especially to gibbon viruses. This supported the previous data of HBsAg vaccine that induced lower immune response than vaccine containing *PreS2/S* and *PreS1/PreS2/S*, such as GenHevac B and Hepagene B, respectively [87].

Support by this study, particles of gibbon and human HBV were caught by anti-S from serum of vaccinees measured by inhibition binding assay. The immunogenicity of the human HBV vaccine is possibly related to the protective antibodies against S gene within "*a*" determinant region [81]. Due to the amino acid of gibbon S protein, especially in "*a*" determinant region, presented the similar codons comparison to human viruses (Figure 20), this may cause the cross-reaction of antibodies produce from HBV vaccinated persons to gibbon HBV particles. Although the 11 amino acids deletion was detected in PreS1 protein of gibbon viruses, but protective antibodies binding sites were not included in this region. All data supported that human HBV immunization showed the protective activity against gibbon HBV, whether less efficiency than human viruses was suggested. Animal cares worker or people which close contact to gibbons should be vaccinated to avoid the accidental viral transmission by animal bite.

Expression of HBV surface protein and binding to human hepatocyte cells

Due to the restriction of gibbon serum collection, the purified HBV particles isolated from gibbon sera for binding and infection assay was not possible. Referring to the previous data [63], the binding and infectivity test of human HBV were performed by molecular techniques. The cloning of HBV surface gene into expression vector was the alternative way to study the biological characteristic and property of gibbon HBV.

Binding of HBV surface protein to the receptors on host cells was the first step and the most important for HBV infection [7]. The adherence of surface protein to specific receptors of different host species was unidentified. According to the data of human HBV, several proteins on hepatocyte cells have been identified as the receptor binding sites for HBV infection (Figure 19). However, many reports indicated that PreS1 is the most specific region for HBV binding to species specific host cells. It is still a doubt whether the 33 bases deletion after start codon of gibbon *PreS1* gene interfered to the binding of gibbon HBV particles to human host cells.

In the present study, the surface protein of gibbon virus was expressed in the similar structure as natural expectation. Due to the recombinant protein expression in bacteria and yeast cells lacked the glycosylation of protein modification process [83], mammalian cells were used for expression of the gibbon surface protein to avoid the conformational changing.

In the first experiment of cloning and expression HBV surface protein from gibbon and human, complete *PreS1/PreS2/S* gene was cloned into pCR2.1 vector before subcloned into VR1012 Neo^{+} mammalian cell expression vector. Both recombinant envelope proteins were expressed in CHO cells and qualitative protein detection was confirmed by immunostaining with F4-7b mAbs (Figure 24) and anti-PreS2 antibodies. Unfortunately, the secreted protein from clones could not be detected neither transient transfection cells nor stable cell lines by ELISA HBsAg assay (Table 21). Noticeably, surface protein was produced and located in cytoplasm of CHO cells as determined by F4-7b antibody staining. This event indicated that the intracellular protein expression system from mammalian cells was a success, but only the protein

transportation system may require more specific factor such as the inducible protein or enhancer binding peptide to complete this process.

In 1998, Seyec *et al.* described the plasmid construction of HBV envelope protein within the downstream HBV sequence beyond to the polyadenylation signal which played a role to enhance the recombinant protein secreting [106]. The second experiment of surface protein expression was set up following this previous report and indeed, the human and gibbon enveloped protein became detectable using the same procedure (Table 22). Using F4-7b and anti-PreS2 antibodies, immunostaining assay indicated the binding of these antibodies to the correct form of S and PreS2 proteins (Figure 25). CHO stable lines expressing HBV surface protein were selected by geneticin G418 (neomycin) resistant phenotype. After 3 weeks of neomycin selection, the unstable cells without recombinant DNA integration were discard. Daughter cells grown from single mother clone and formed the cluster of cells. Although the very low frequency of recombinant DNA integrated into genome of host cells was reported, stable line selection was usually performed by drug resistant pressure [109]. In present study, the recombinant protein produced from LMS/VR1012/Neo$^+$ plasmid becomes detectable by ELISA assay. Expressed protein seems localize in some organelles of host cells that suggested as golgi aparatus. This result may indicate the possibility of protein transport out of the cells [132] . Even though the integrated HBV surface gene was not detected in CHO stable lines, but secreted protein was continuously detected in supernatant. Due to the LMS/VR1012/Neo+ plasmid contains the complete HBV *X, S* and *PreS* genes, it may enhance the process of HBV gene integration into host cells since the previous data indicted that these regions play a role on HBV gene integration step and induce the hepatocellular carcinoma progression [133,134] . However, CHO stable cell lines expressing gibbon HBV surface protein could not be selected in this study even after triple repeated experiments were done. The recombinant gibbon HBV secreted proteins may somehow interfere in human host cells and reduce the neomycin drug resistance feature of mammalian host cells.

Secreted envelope proteins of gibbon and human HBV were concentrated by PEG-8000 for reducing the excess solution in samples before precipitation by centrifugation. The concentrated proteins were tested for HBV surface protein specificity

by immunoblot detection with F4-7b antibody (Table 23 and Figure 26). The results confirm that the concentration process did not disturb the conformation of both proteins. However, the presence of the PreS1 region of recombinant protein from both viruses could not be confirmed because of the lack of effective anti-PreS1 antibody binding.

In the binding assay between concentrated recombinant HBV envelope protein and human hepatocyte cell line (HepG2 cells), purified human viral particles were used as positive control while unbound HepG2 cells were defined as negative control of FACs analysis. The positive binding signal of FITC-streptavidine conjugated to F4-7b was detected only in positive control reaction, but no signal finding for the others (Figure 27). From the present data, the binding of viral particles to host cell receptors was not require only the specific binding proteins, but also the precise conformational structure to fit into recognition sites. Unbinding of recombinant envelope HBV protein may result from several reasons. For example, the large surface protein (L protein) which is translated from *PreS1/PreS2/S* gene may be less expressed compared to M and S protein because of the weak promoter of this region [8]. Purified human viral particles might have higher protein binding sites than concentrated surface protein and this may effect to protein adherence precess. M protein was reported previously with the specificity to host cell surface binding [65], complex protein undetectable by anti-PreS2 may cause by the fact that HepG2 receptor protein was competitive attachment to M protein. Additional, the concentration process did not get rid of the crude proteins in cell supernatant such as FBS, host cell (CHO cells) secreted protein and chemical substance which may interfere to the binding specificity. The last factor, which remained to explain the unsuccessful experiment, was the abnormal recognition sites of both surface protein subjects. The expressed L, M and S recombinant proteins of human and gibbon viruses may form the incorrect structure and HepG2 cells already loss their specific receptors on cell surface already due to immortalizing properties. Anyway, through improvement of some factors in this study, including the purification of recombinant proteins before binding to HepG2 cells, the use of primary hepatocyte cells and optimization of binding procedure and detecting system, should result in the more insight in the viral-host cell interactions.

In vitro HBV infection

Virus attachment is one of the important initial steps that determine the host and organ tropism of viruses. In this study, an attempt to perform the binding between gibbon HBV envelope protein and host cell receptors had been done, however the results were not determined. Another trying was processed on infectivity study by detection the HBV replicative product (cccDNA).

Viral infection and replication are functions of the viral delivery system and viral load. The elementary structure of a virus is designed to fulfill these two fundamental functions. The delivery system consists of components forming external structures of viral particles (surface components), and the payload carried inside a viral particle and containing the genome and associated component which protect the genome from degradation and supply functions for initiating steps of replication. These components may aid in translating or transcribing the genome, integrating it into the host DNA, or keeping it quiescent [135].

Components of the delivery system initiate the replication cycle by attachment to "receptors" on the cell surface and initiating penetration into the cytoplasm. Cytoplasmic penetration is usually associated with a loss of external components (uncoating). Viruses that replicate within the nucleus have additional delivery functions that permit viral components to be introduced into the nucleus.

Since the human primary hepatocyte cells were supposed to variably susceptible to human HBV infection due to the specific receptor expression on their cell surface and already enrich with the initiated materials for viral replication [11,136], these cells were usually used for *in vitro* HBV infection experiment. However, the limitation of primary cell culture was the restriction of normal liver source, optimum isolation method to prevent the cell injury and the contamination of white blood cells during the isolation step. The most severe problem of these cells was a short period of cell life after culturing and also the optimum medium components for maintain the culture. However, the infectivity of gibbon and human HBV positive sera were tested in human primary hepatocyte cells, but replication was not detected thus far.

The specific binding receptors in liver cells are believed to express on the plasma membrane of human primary hepatocyte cells. Thus, it is benefit to maintain these cells in culture as long as possible. One research group has been constructed the immortalizing hepatocyte cell line which exhibits characteristics similar to primary human hepatocyte cells [112] . NKNT-3 cell lines were generated by retroviral transfer in normal primary adult human hepatocytes of an immortalizing gene that can be subsequently and completely excised by Cre-Lox site specific recombination. After induced the Cre recombinant expression plasmid, NKNT-3 was switched to be mortal and showed the similar morphology and protein expression as human hepatocyte cells. This beneficial cell line can be used for the temporary metabolic support of patients awaiting liver transplantation or spontaneous reversion of their liver disease. In this experiment, NKNT-3 reversed cells were applied for gibbon and human HBV positive sera infection due to their similar properties to human hepatocyte cells.

Unfortunately, the NKNT-3 reversion system using Cre recombinase enzyme produced from recombinant plasmid was not successful in our study. The SV 40 gene, which expected to remove out after recombinase enzyme active, was still detectable. This problem may effect the susceptibility of host cells to both human and gibbon viral particles.

Although much information about the molecular biology of HBV has been gained, to date it is still unclear about the mechanism of attachment and penetration of HBV into human hepatocyte, the natural host cells of HBV. Since the binding of the HBV particle to the plasma membrane of human hepatocytes is considered as an important initial step in viral entry and replication of HBV, HBsAg is thought to play a crucial role in the interaction with hepatocyte plasma membrane.

The recombinant SHBsAg binds specifically to the Annexin V (AnV), Ca2+-dependent phospholipid binding protein present on plasma membrane of human hepatocytes, as reported by Hertogs *et al.*(1993) [77] . Enhancing AnV expression in human heptocyte cell lines (HepG2) was expected to increase the infectivity of HBV *in vitro*. Human AnV expression vector was constructed and transfected to HepG2 cells. The protein expression was detected by immunostaining with anti-AnV either transient

transfected cells or stable cell lines (Figure 28). Unluckily, the HBV infection into AnV expressed CHO stable lines was not a success.

It was commonly believed that the binding receptors should be presented on plasma membrane specific to each species. This could explain the restriction of HBV infection only to host and tissue tropism. Anyway, more than one receptor may involve in the binding and infection process and cause the unsuccessful infection experiments.

Paran *et al* . (2001) [78] reported the HBV infection system of cell culture by using the DMSO treatment to increase the HBV susceptibility of human hepatocyte cell lines (HepG2). In the present study, 2% DMSO was added into HepG2 culture medium 7 days before HBV positive sera infection. The morphology of treated cells changed and was easily detected under the light microscope. Increased cell size with more nuclei and slow growing features were found. Every 7 days after infection, replication markers of gibbon HBV, cccDNA replication form and M protein (*PreS2* gene), were analyzed in the host cells by PCR and immunostaining, respectively (Figure 29, 30). The chemical treatment of host cells was needed to express most of the proteins, including HBV receptor which less producing after become to immortalized cell, and enhanced the binding between viruses and host cells [12] . Even though the infection process using DMSO treated cells may not be similar to binding between gibbon viruses and human host cells in nature, but the fact that gibbon HBV can replicate in human hepatocyte cells was confirmed. On contrastingly, positive human HBV serum did not show the infectivity in human host cells. This event probably caused by the low amount of HBsAg in human serum (Table 16). The purified viral particles before infection may increase the viral infectivity of both species.

In conclusion, our study showed seroprevalence and molecular characterization of gibbon HBV compared to human viruses. HBV isolated from both species have the similar structure as confirmed by EM study. Phylogenetic relationship of human and nonhuman HBV strains showed the close ancestor even though these viruses are indigenous to the different population. Similarity between human and gibbon S antigen was confirmed by ELISA binding assay of a panel of antibodies to HBV envelope protein and binding of both antigens were inhibited by immune antibodies prepared from immunized persons. Cloning and expression of surface antigen of gibbon and human

HBV were performed to study the specific binding region of HBV to the human host cells. Although the natural entry step of gibbon HBV into hepatocyte cell line was not successed, the viral replication in human hepatocyte cells was strongly demonstrated. The finding of gibbon HBV replication in human cell never be reported elsewhere. From these results, the possibility of cross contamination between gibbon and human HBV might occur. In addition to other nonhuman primates HBV, the cross transmission may not be excluded.

Although this study can show the replication of gibbon viruses in human cells *in vitro*, the HBV and cells interaction between both species *in vivo* should perform. Chimeric mice generated human hepatocyte xenograft may be an alternative way to study the possibility of HBV cross-species transmission. The correlation of specific receptors on human hepatocyte cells and specific binding region on viral surface protein should be studied in the future for explanation the narrow host range of HBV infection. Efficiency of human HBV vaccine to induce the protective immune response against gibbon HBV should be investigated in gibbons. Moreover, the species specific HBV vaccine produces from antigenic region of each species should induce the best protective immune response of these species. For example, the complete surface gibbon HBV produce from LMS/VR1012/Neo^{+} plasmid of gibbon HBV may use as gibbon HBV vaccine that can induce the stronger protective antibodies than using human HBV vaccine.