CHAPTER IV

RESULTS

General description of gibbon population

The 101 captive gibbons kept at Krabok Koo Wildlife Breeding Centre, Royal Forest Department in Cha-Cheng-sao, Thailand were included in this study. The gibbons are housed in small monogamous families. The feeding areas inside the center were separated into 3 parts : C, L and R zone. Each area has different cage types and numbers. Demographic data of animals in the center represented 4 gibbon species including *H. lar* (n=72), *H. pileatus* (n=20), *H. agilis* (n=2), hybrid between *H. lar* and *H. pileatus* (n=1), and *N. concolar* (n=6) (Table 6 and Appendix B). White handed gibbon, *H. lar* , is the main population while there is only one couple of black handed gibbons (*H. agilis*) in Krabok Koo Wildlife Breeding Center. *N. concolar* is the species originally found in North Vietnam and Central China [114]. Male and female ratio is approximately 1:1 of each species and animal age is ranging from 1 to 21 years old. Unfortunately, some of the abandoned gibbons had no record of their age. The average age with standard deviation (SD) of each species as shown in Table 6 was calculated from animals that we could estimate their age. General description of each animal including name, microchip code, species, cage number, sex and age are described in Appendix B.

Complete blood count analysis of gibbons

To investigate the health status of captive gibbons in the Krabok Koo Wildlife Breeding Center, fresh blood samples from 40 gibbons were used for complete blood count analysis. The amount of red blood cells is expressed as a percentage of the total whole blood and determined by microhematocrit method [115] . As shown in Table 7, the average hematocrit value is 43.55 % for female and 44.42 % for male animals. In

human, the normal range of the hematocrit is 37 to 47% for women and 42 to 52 % for men.

The white blood cell count (WBC) denotes the number of white blood cells in 1 liter of whole blood. In a normal, healthy individual human, the WBC falls in the range of 4.8 to 10.8 x 10^9/L. The range of WBC in gibbon blood samples was 5.3 –17.1 x 10^9/L. The manual differential WBC count is performed to determine the relative number of each type of WBC present in the blood. In the present study, total white blood cell, percentage of neutrophils, lymphocytes, eosinophils and basophils were counted and compared to the human normal range standard as indicated in Table 7. Percentage of neutrophil and lymphocyte cells in gibbon blood sample (9-91% and 9-90%) showed the wide range if compared to human standard (35-71% and 24-44%). Abnormal range of percent eosinophils and basophils were not detected. At the 95% confidence interval of the difference, chi-square analysis value of percent hematocrit (X^2 = 0.253) and white blood cells (X^2 = 1.688) had no relation to gibbon HBV status (X^2 = 3.84, from table at degree of freedom = 1, α = 0.05).

Table 6 Demographic data of the gibbons in the Krabok Koo Wildlife Breeding Center, Royal Forest Department, Cha-Cheng Sao, Thailand.

Species	Number	M:F	Age ($\bar{X} \pm$ SD range)*
H. lar	73	40:33	12.27 ± 3.937
H. pileatus	20	9:11	12.76 ± 4.265
N. concolor	6	3:3	10.83 ± 1.472
H. agilis	2	1:1	ND

* Average ± SD range of age was calculated from animals which recorded the year of birth only.

Hybrid of *H.lar* and *H. pileatus* , Stevie L0 was included in *H. lar* group.

ND = Not determined

Table 7 Descriptive data and complete blood count analysis of captive gibbons in Krabok Koo Wildlife Breeding Center, Royal Forest Department, Cha-Cheng Sao, Thailand.

No.	Name	Species	Cage	Sex	%Hct	WBC(x10^9)	%N	%L	%E	%B
1	Gift	*H. pileatus*	C23	F	50	10.3	67	31	-	1
2	Daew*	*H. pileatus*	C22	F	28	5.30	37	63	-	-
3	Candy*	*H. pileatus*	C21	F	50	8.15	45	55	-	-
4	Koo	*H. pileatus*	C20	F	40	9.75	9	88	-	3
5	Jock*	*H. pileatus*	C20	M	12	6.95	70	30	-	-
6	Roen	*H. pileatus*	C19	M	55	5.40	56	44	-	-
7	Farouk	*H. pileatus*	C18	M	44	8.70	80	20	-	-
8	Nong Ni	*H. pileatus*	C17	F	47	10.45	66	29	-	5
9	Pilly	*H. pileatus*	C17	M	40	8.25	75	25	-	-
10	Nong Chai*	*H. pileatus*	C16	M	45	9.70	78	22	-	-
11	Saboo*	*H. pileatus*	C15	M	40	5.65	45	53	-	2
12	Ni*	*H. pileatus*	C15	F	50	7.40	62	38	-	-
13	Gomez*	*H. pileatus*	C14	M	-	-	55	45	-	-
14	Chmi*	*H. pileatus*	C14	F	40	9.30	45	55	-	-
15	Saan*	*H. pileatus*	C13	M	47	8.80	40	58	-	2
16	Kristine	*H. pileatus*	C13	F	45	10.30	15	85	-	-
17	Mila	*H. pileatus*	C12	F	45	10.40	27	71	-	2
18	Piercy	*H. pileatus*	C11	F	45	6.90	14	64	-	-
19	Leuis	*H. pileatus*	C12	F	25	12.90	28	72	-	-
20	Thim(Dim)	*H. lar*	C10	F	55	15.40	46	52	-	2
21	Brownie	*H. lar*	C8	M	40	8.95	47	52	-	1
22	Toffee	*H. lar*	C8	F	50	19.75	36	64	-	-
23	Tua	*H. lar*	C7	F	38	10.45	58	42	-	-
24	Phoon	*H. lar*	C7	M	49	17.10	82	17	-	1
25	Gobboly	*H. lar*	C6	M	49	13.45	67	33	-	-
26	Apple	*H. lar*	C6	M	54	5.95	55	45	-	-
27	YingYong	*H. lar*	C5	M	45	12.20	86	13	1	-
28	John	*H. lar*	C4	M	46	7.45	21	79	-	-
29	Jew	*H. lar*	C4	F	45	9.20	73	27	-	-
30	Ted	*H. lar*	C3	M	42	10.50	67	33	-	-

Table 7 Descriptive data and complete blood count analysis of captive gibbons in Krabok Koo Wildlife Breeding Center, Royal Forest Department, Cha-Cheng Sao, Thailand (continued)

No.	Name	Species	Cage	Sex	%Hct	WBC(x10^9)	%N	%L	%E	%B
31	Mek	*H. lar*	C2	F	44	6.85	63	37	-	-
32	Pok*	*H. lar*	C2	M	45	6.15	45	55	-	-
33	Vetan	*H. lar*	R1	F	36	14.70	10	90	-	-
34	Rusty	*H. lar*	R3	M	49	8.50	66	34	-	-
35	Jacko*	*H. lar*	R4	M	47	8.00	76	24	-	-
36	Ivana	*H. lar*	R4	F	48	7.80	59	40	1	-
37	Darkie	*H. lar*	R5	M	47	16.60	91	9	-	-
38	Ice	*H. lar*	R5	F	47	4.50	43	57	-	-
39	Jieb*	*H. lar*	R6	F	43	10.95	62	38	-	-
40	Kong2	*H. lar*	R7	M	48	5.35	-	-	-	-

Normal range of human:

% Hct = Percent hematocrit ; Male 42-52 %, Female 37-47 %

WBC = White blood cell count ; 4.8 to 10.8 x 10^9/L

N = Neutrophil ; 1.5 - 7.4 x 10^9/L, 35-71 %

L = Lymphocyte ; 1.0 - 4.4 x 10^9/L, 24-44 %

E = Eosinophil ; 0.0 - 0.4 x 10^9/L, 0-4 %

B = Basophil ; 0.0 - 0.2 x 10^9/L, 0-2 %

* indicate the gibbon HBV carrier stage

Chi-square analysis value of percent hematocrit (X^2 = 0.253) and white blood cells (X^2 = 1.688) had no relation to gibbon HBV status (X^2 = 3.84, from table at degree of freedom = 1, α = 0.05).

Seroprevalence of HBV in gibbons.

To analyse the prevalence of hepatitis B virus infection in Thai gibbons, 101 gibbon sera were tested for the presence of HBsAg and antibodies to HBs and HBc. Serological testing indicated that 38.61% of the animals were positive for at least one marker of HBV infection (Table 8). Besides HBsAg positivity, also HBV DNA amplification was the standard to determine curculating viruses in gibbons. The HBV *S* gene was amplified from gibbon sera and a 1,038 bps PCR product was detected (Figure12). Nineteen gibbons were chronic carriers as defined by the presence of HBV DNA and HBsAg, in the absence of antibodies to the S protein. Interestingly, four of these animals were negative for anti-HBc even when tested repeatedly using a different ELISA test. Approximately 20% (20/101) of the animals recovered from the infection as evidenced by the presence of antibodies to S and core.

To test whether HBV infection was associated with liver damage, ALT enzyme, albumin, globulin and total protein levels were determined in the sera of 40 gibbons : 12 HBsAg positive, 11 animals recovered from HBV infection and 17 non-infected healthy control animals (Table 9). Independent sample t-test analysis with equal variances was performed to compare the ALT level of both groups. Elevated ALT levels were detected in HBV infected gibbons (68.75 $\pm$ 48.12 U/l) as compared to control animals (33.04 $\pm$ 15.91 U/l, p <0.05). At the 95% confidence interval of the difference, the different ALT level of both groups was 4.76 – 66.67 (Appendix C).

Albumin and globulin level was tested to confirm chronic status of the liver function in each sample. The average with SD of albumin (3.62 $\pm$ 0.33 g/dl) and globulin (2.77 $\pm$ 0.43 g/dl) level of gibbons were a bit higher than human normal range. Total protein levels may be indicative for hygiene and nutrition of gibbons in the center showed normal range (6.40 $\pm$ 0.46 g/dl) as the same with creatinine level (1.17 $\pm$ 0.18 mg/dl) presented the normal kidney function of gibbons.

Table 8 Seroprevalence of gibbon HBV in the Krabok Koo Wildlife Breeding Center, Royal Forest Department, Cha-Cheng Sao, Thailand.

Stage of infection	HBsAg	HBV DNA	Anti HBs	AntiHBc	Number (%)
No infection	-	-	-	-	62 (61.39)
Recovered	-	-	+	+	20 (19.80)
HBV carrier	+	+	-	+	15 (14.85)
	+	+	-	-	4 (3.96)

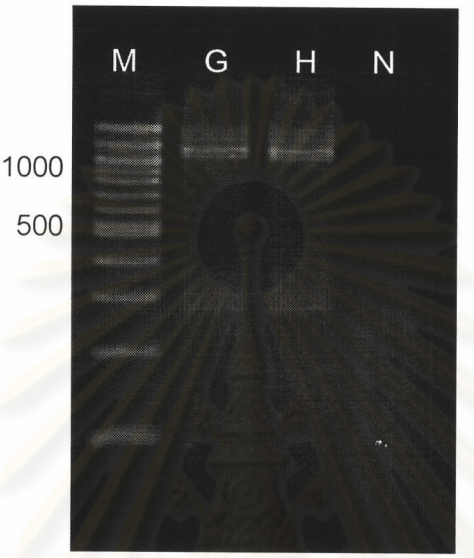


Figure 12 *S* gene amplification of gibbon (G) and human (H) HBV. The expected size of PCR product is 1,038 bps. 500 and 1000 bps DNA marker (M) and negative control (N) are indicated.

Table 9 Blood chemistry analysis of HBV carriers and normal gibbons kept in Krabok Koo Wildlife Breeding Center.

No.	Name	Species	Cage	HBV Stage	ALT	TP	Alb	Glo	Cr
1	Gift	*H. pileatus*	C23	Negative	27	6.1	3.4	2.7	1
2	Daew	*H. pileatus*	C22	Carrier	48	6.4	4	2.4	1.2
3	Candy	*H. pileatus*	C21	Carrier	69	6.6	3.5	3.1	1.2
4	Koo	*H. pileatus*	C20	Recoverd	36	5.6	3.2	2.4	1.3
5	Jock	*H. pileatus*	C20	Carrier	46	6	2.6	3.4	1.3
6	Roen	*H. pileatus*	C19	Recoverd	45	6.8	3.7	3.1	1
7	Farouk	*H. pileatus*	C18	Recoverd	49	6.3	3.5	2.8	1.1
8	Nong Ni	*H. pileatus*	C17	Negative	30	5.8	3.3	2.5	0.9
9	Pilly	*H. pileatus*	C17	Recoverd	47	6.9	3.9	3	1.2
10	Nong Chai	*H. pileatus*	C16	Carrier	50	6	3.5	2.5	1.2
11	Saboo	*H. pileatus*	C15	Carrier	207	6	3.6	2.4	1.2
12	Ni	*H. pileatus*	C15	Carrier	70	5.7	3.7	2	1.1
13	Gomez	*H. pileatus*	C14	Carrier	93	6.7	4	2.7	1.3
14	Chmi	*H. pileatus*	C14	Carrier	87	6.2	3.4	2.8	1.3
15	Saan	*H. pileatus*	C13	Carrier	46	6.3	3.6	2.7	1.2
16	Kristine	*H. pileatus*	C13	Recoverd	42	6.3	3.7	2.6	1.4
17	Mila	*H. pileatus*	C12	Negative	73	6.4	3.6	2.8	0.9
18	Piercy	*H. pileatus*	C11	Recoverd	28	5.7	3.7	2	1
19	Leuis	*H. pileatus*	C12	Recoverd	35	7.5	2.9	4.6	1.1
20	Thim(Dim)	*H. lar*	C10	Negative	20	7	3.9	3.1	1.1
21	Brownie	*H. lar*	C8	Negative	23	6.3	3.6	2.7	1
22	Toffee	*H. lar*	C8	Recoverd	21	7.2	4	3.2	1.4
23	Tua	*H. lar*	C7	Negative	74	6.3	3.7	2.6	1.1
24	Phoon	*H. lar*	C7	Negative	35	6.9	4.1	2.8	1.5
25	Gobboly	*H. lar*	C6	Negative	19	6.3	3.5	2.8	1.5
26	Apple	*H. lar*	C6	Negative	36	7.1	4.3	2.8	1.4
27	YingYong	*H. lar*	C5	Negative	16	6.8	3.6	3.2	1.4
28	John	*H. lar*	C4	Negative	20	5.8	3.4	2.4	1.3
29	Jew	*H. lar*	C4	Negative	18	5.8	3.1	2.7	0.9
30	Ted	*H. lar*	C3	Negative	32	7.3	4	3.3	1.1

Table 9 Blood chemistry analysis of HBV carriers and normal gibbons kept in Krabok Koo Wildlife Breeding Center (continued)

No.	Name	Species	Cage	Stage	ALT	TP	Alb	Glo	Cr
31	Mek	*H. lar*	C2	Recoverd	21	5.9	3.6	2.3	1.1
32	Pok	*H. lar*	C2	Carrier	39	6.6	3.9	2.7	1.1
33	Vetan	*H. lar*	R1	Negative	28	6.5	3.6	2.9	0.9
34	Rusty	*H. lar*	R3	Recoverd	55	6.8	3.8	3	1.3
35	Jacko	*H. lar*	R4	Carrier	50	6.4	3.9	2.5	1.2
36	Ivana	*H. lar*	R4	Recoverd	11	6.4	3.8	2.6	0.9
37	Darkie	*H. lar*	R5	Negative	18	6.4	3.7	2.7	1.1
38	Ice	*H. lar*	R5	Negative	25	6.1	3.4	2.7	1
39	Jieb	*H. lar*	R6	Carrier	20	6.2	3.4	2.8	1
40	Kong2	*H. lar*	R7	Negative	41	6.6	4	2.6	1.5

Normal range of human :

ALT = Alanine-aminotransferase : 0-38 U/l

TP = Total protein ; 6.6 - 8.7 g/dl

Alb = Albumin ; 3.4 – 5.5 g/dl

Glo = Globulin ; 2.0-4.0 g/dl

Cr = Creatinine ; 0.5-2.0 mg/dl

Routes of HBV transmission in gibbons

I. Vertical transmission

In order to further analyse gibbon HBV vertical transmission, two carrier families had babies born in the Wildlife Breeding Center (C15 and R6 cages) were analyzed. Tao C15, offspring of Ni and Saboo, was born in 1999 and all family members were found positive for S antigen and HBV DNA. Baby R6 was born in 2000 and his mother, Jieb, is a HBV chronic carrier while his father, Kingkong, had natural immunity against HBV. Both mother gibbons were found HBeAg positive by ELISA test. (Table 10).

HBV isolated from both pairs of mother and baby gibbons were sequenced and submitted to GenBank database (Genbank Accession No. AF477490-91 and AF477493-94, Appendix D). Comparison of *PreS1* sequences, the most divergent part of HBV genome, indicated the close relation of viral strains between mother and baby. After *PreS1* alignment (361 bps), Tao and baby R6 showed only 5 and 2 bases difference from their mother respectively, while 34-41 bases of the HBV sequences from both couples differed as compared to a non related gibbon isolate (Figure 13a). Moreover, the PreS1 gene sequence isolated from Saboo (the C15 father gibbon), showed 8 and 7 bases difference from dam and offspring sequences respectively. The S and core regions of both pairs showed 100% identity among mothers and babies (Figure 13b and 13c) but 23 and 3 bases difference were found when compared to Belle L10 control sequences. The percentage similarity of *PreS1* gene sequences among mother-baby and control strain is shown in Table 11. Thus, taking into account of the relationships of these animals and the sequencing results vertical transmission may seem quite likely to have occurred.

Table 10 HBV serological markers of 2 gibbon HBV carrier families with babies borned in the Krabok Koo Wildlife Breeding Center.

Cage	Name	Species	Sex	Date of Birth	HBV DNA	HBsAg	Anti-S	Anticore	HBeAg
C15	Ni	*H. pileatus*	F	1991	+	+	-	-	+
	Tao*	*H. pileatus*	M	13/05/99	+	+	-	+	+
	Saboo	*H. pileatus*	M	1990	+	+	-	+	ND
R6	Jieb	*H. lar*	F	1982	+	+	-	+	+
	Baby R6*	*H. lar*	M	2000	+	+	-	-	+
	Kingkong	*H. lar*	M	Arrived 1996	-	-	+	+	ND

* baby-borned in house

ND = not determined

(a)

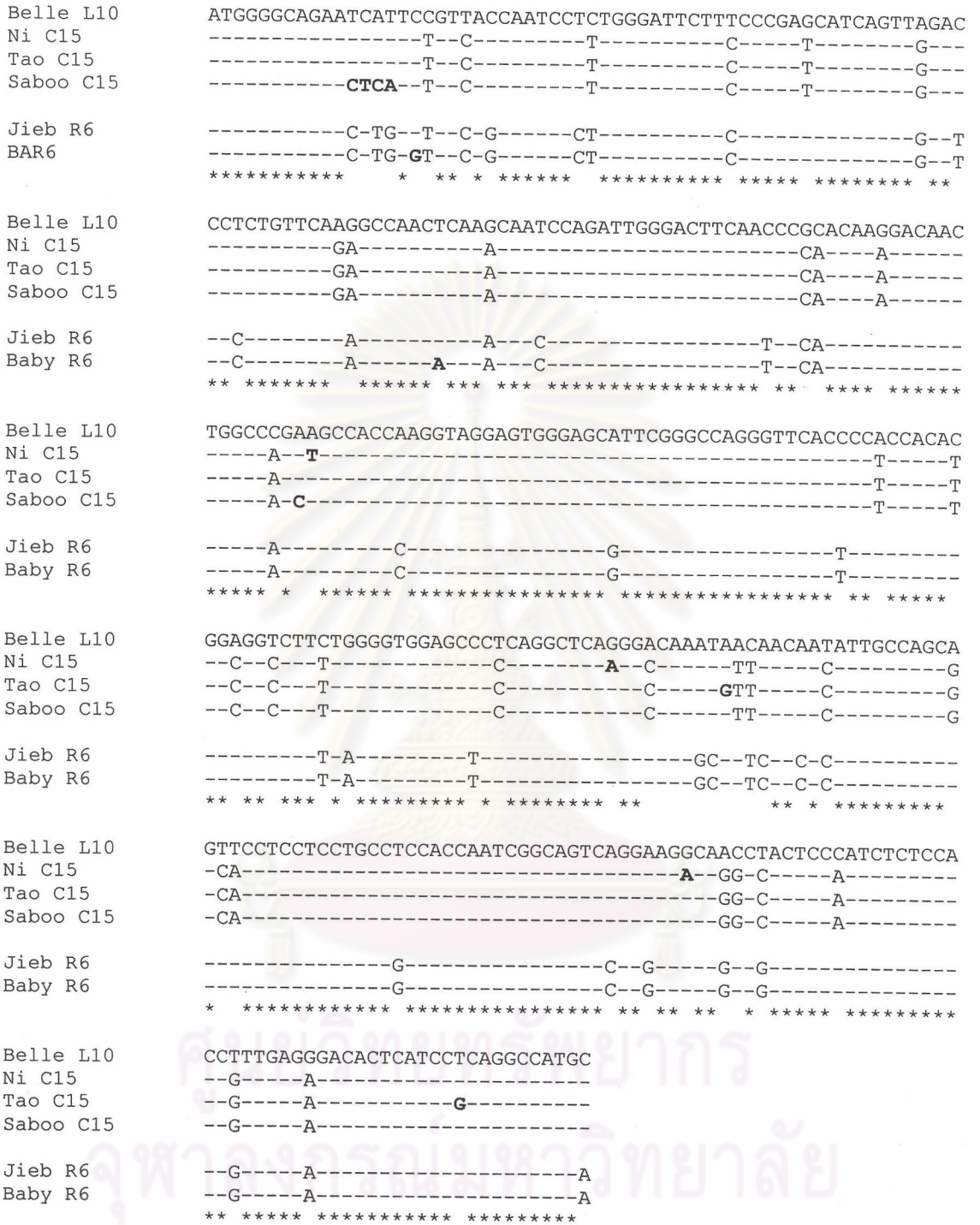
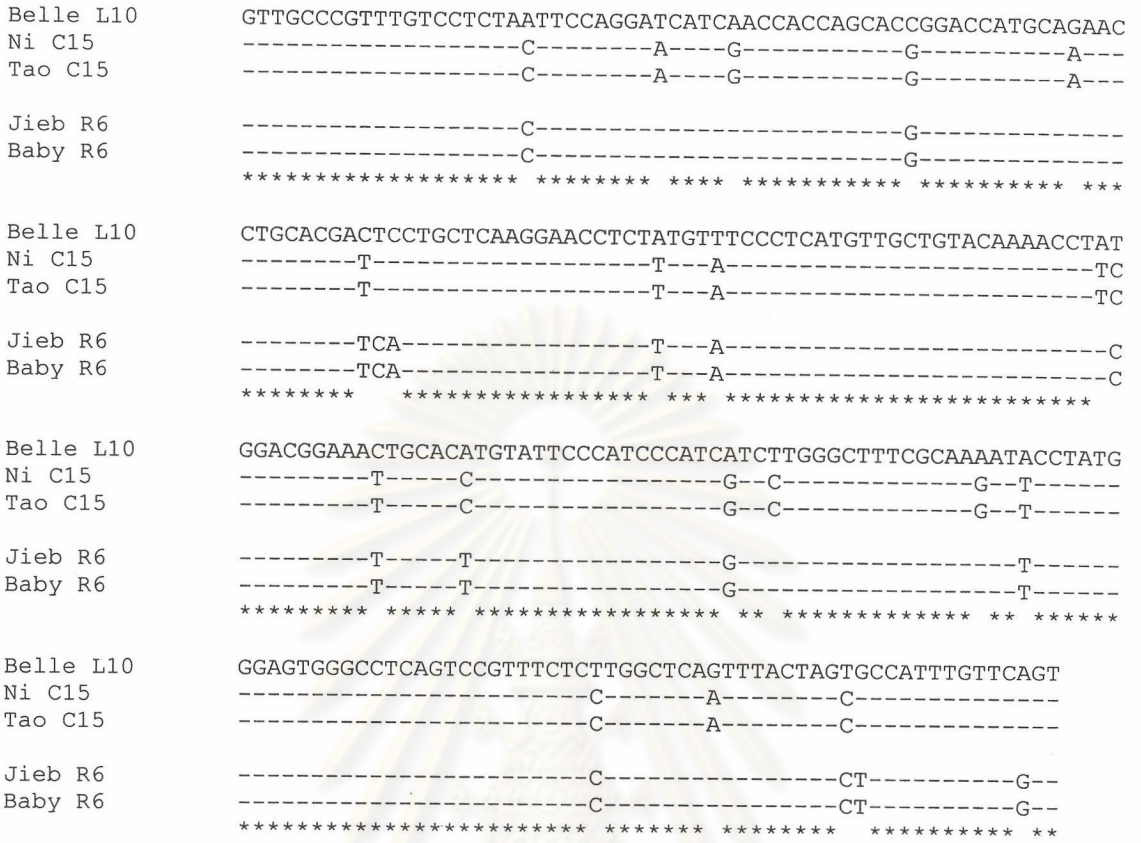


Figure 13 Alignment of HBV sequences from 2 gibbon carrier's families. *PreS1* (a), *S* (b) and *C* (c) sequences were aligned using the Clustal X program. All sequences were compared to Belle L10, accession number AF274497, as a control. Bold letters and dashes indicated different and identical bases residues, respectively.

(b)


Belle L10 GTTGCCCGTTTGTCCTCTAATTCCAGGATCATCAACCACCAGCACCGGACCATGCAGAAC
Ni C15 ----------C---------A----G----------G----------A---
Tao C15 ----------C---------A----G----------G----------A---
Jieb R6 ----------C----------G----------
Baby R6 ----------C----------G----------
Belle L10 CTGCACGACTCCTGCTCAAGGAACCTCTATGTTTCCCTCATGTTGCTGTACAAAACCTAT
Ni C15 --------T----------T---A----------TC
Tao C15 --------T----------T---A----------TC
Jieb R6 --------TCA----------T---A----------C
Baby R6 --------TCA----------T---A----------C
Belle L10 GGACGGAAACTGCACATGTATTCCCATCCCATCATCTTGGGCTTTCGCAAAATACCTATG
Ni C15 ---------T-----C----------G--C----------G--T------
Tao C15 ---------T-----C----------G--C----------G--T------
Jieb R6 ---------T-----T----------G----------T------
Baby R6 ---------T-----T----------G----------T------
Belle L10 GGAGTGGGCCTCAGTCCGTTTCTCTTGGCTCAGTTTACTAGTGCCATTTGTTCAGT
Ni C15 ----------C-------A--------C----------
Tao C15 ----------C-------A--------C----------
Jieb R6 ----------C----------CT----------G--
Baby R6 ----------C----------CT----------G--

(c)

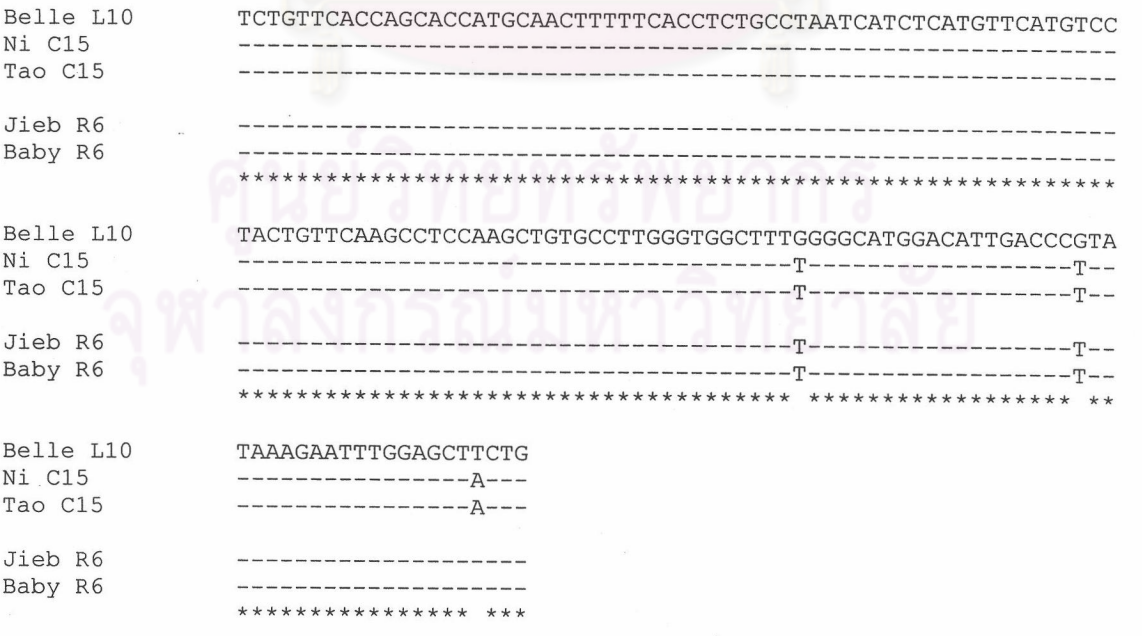

Belle L10 TCTGTTCACCAGCACCATGCAACTTTTTCACCTCTGCCTAATCATCTCATGTTCATGTCC
Ni C15 ----------
Tao C15 ----------
Jieb R6 ----------
Baby R6 ----------
Belle L10 TACTGTTCAAGCCTCCAAGCTGTGCCTTGGGTGGCTTTGGGGCATGGACATTGACCCGTA
Ni C15 ----------T----------T--
Tao C15 ----------T----------T--
Jieb R6 ----------T----------T--
Baby R6 ----------T----------T--
Belle L10 TAAAGAATTTGGAGCTTCTG
Ni C15 ----------A---
Tao C15 ----------A---
Jieb R6 ----------
Baby R6 ----------

Table 11 *PreS1* sequence similarity (%) comparison between gibbon HBV carrier families and control sequences.

Samples	Control	Baby	Mother	Father
Father	C15=89.75	C15=98.06	C15=97.78	C15=100
Mother	C15=90.30 R6=89.17	C15=98.61 R6=99.45	C15=100 R6=100	
Baby	C15=90.58 R6=88.64	C15=100 R6=100		
Control	100			

Control sequences = Belle L10 strain

II. Horizontal transmission

Horizontal transmission of HBV in gibbon population was confirmed in this present study. HBsAg was found in saliva samples kept from HBV carrier gibbons (n=14) while HBV DNA was detected in only 6 samples (Table 12). Both HBV-DNA and HBsAg were not detected in saliva of HBV seronegative (n=6) and HBV recovered gibbons (n=9).

Serological markers of HBV infection in the chronic gibbons are shown in Table 13, including HBV stage of their families. Most of the HBV infected gibbons were of the species *H. pileatus* kept in the C-cage area (11/19). All carriers' partners were HBsAg positive or had been infected with HBV some time earlier.

Gibbon HBV transmission to humans

Due to the high risk of gibbon HBV contact during work, 34 animal keepers of the Krabok Koo Wildlife Breeding Center were screened apart of a pre-vaccination program. Approximately, 50 % of workers already exposed to HBV and 44.1% developed antibodies against viral infection. Only 2 workers were HBV carriers by demonstration of HBsAg, HBV DNA and anti-HBc. The Other 2 presented only HBs antibody but no other markers (Table 14).

Even though the HBV transmission from nonhuman primates was expected in previous study [13], molecular characterization of HBV isolated from 2 carrier keepers must be performed to confirm the original HBV source of workers.

Table 12 HBsAg and HBV DNA detection in sera and saliva of carriers and normal gibbons by ELISA and PCR method.

Samples	Serum				Saliva			
	HBsAg		HBV DNA		HBsAg		HBV DNA	
	+	-	+	-	+	-	+	-
HBV carriers (n= 15)	15	0	14	1	14	1	6	9
Controls (n= 15)	0	15	0	15	0	15	0	15

Table 13 HBV marker detection in serum and saliva of the gibbon HBV carriers and their families in this study

				Serum				Saliva	
Cage	Name	Species	Sex	HBV DNA	HBsAg	Anti-S	Anticore	HBV DNA	HBsAg
C22	Daew	*H. pileatus*	F	+	+	-	+	+	+
C21	Candy	*H. pileatus*	F	+	+	-	-	-	+
C20	Jock	*H. pileatus*	M	+	+	-	+	-	+
	Koo	*H. pileatus*	F	-	-	+	+	-	-
C16	Nong Chai	*H. pileatus*	M	+	+	-	+	-	+
C15	Saboo	*H. pileatus*	M	+	+	-	+	-	+
	Ni	*H. pileatus*	F	+	+	-	-	-	+
	Tao*	*H. pileatus*	M	+	+	-	+	ND	ND
C14	Gomez	*H. pileatus*	M	+	+	-	+	-	+
	Chmi	*H. pileatus*	F	+	+	-	+	+	+
C13	Saan	*H. pileatus*	M	+	+	-	+	+	+
	Kristine	*H. pileatus*	F	-	-	+	+	-	-
C2	Pok	*H. lar*	M	+	+	-	+	+	+
	Mek	*H. lar*	F	-	-	+	+	-	-
R4	Jacko	*H. lar*	M	+	+	-	+	-	+
	Ivana	*H. lar*	F	-	-	+	+	ND	ND

Table 13 HBV marker detection in serum and saliva of the gibbon HBV carriers and their families in this study (continued)

				Serum				Saliva	
Cage	Name	Species	Sex	HBV DNA	HBsAg	Anti-S	Anticore	HBV DNA	HBsAg
R6	Jieb	*H. lar*	F	+	+	-	+	+	+
	Baby R6*	*H. lar*	M	+	+	-	-	ND	ND
	Kingkong	*H. lar*	M	-	-	+	+	ND	ND
R27	Midnight	*H. lar*	M	+	+	-	+	-	+
L14	Nin	*H. lar*	M	+	+	-	+	+	+
	Sang	*H. lar*	F	-	-	+	+	ND	ND
	Baloo	*H. lar*	M	-	-	+	+	ND	ND
L10	Belle	*N. concolar*	F	+	+	-	+	ND	ND
	Caesar	*N. concolar*	M	+	+	-	-	ND	ND
L9	Charlie	*N. concolar*	M	+	+	-	+	ND	ND
	Ozzy	*N. concolar*	M	-	-	+	+	ND	ND

* baby-borned in house

ND = not determined

Table 14 HBV serological study of animal keepers in the Wildlife Breeding Center, Royal Forest Department, Krabok Koo, Cha-Cheng Sao, Thailand.

Stage of infection	HBsAg	HBV DNA	Anti HBs	AntiHBc	No.
No infection	-	-	-	-	15 (44.1)
Recovered	-	-	+	+	10 (29.4)
	-	-	-	+	5 (14.7)
HBV carrier	+	+	-	+	2 (5.9)
Isolated anti-HBs	-	-	+	-	2 (5.9)

Gibbon HBV molecular characterization

Gibbon HBV molecular characterization

I. Electron Microscopy study

Morphology of gibbon HBV was determined under electron microscope by comparison to human viral particles. Comparable size and shape of viral particles from both species are shown in Figure 14.

II. Restriction fragment length polymorphism (RFLP)

HBV DNA, isolated from 19 positive animals was used as the target for RFLP analysis. Two workers (H1 and H2) presented HBV DNA positive and possibly exposed to gibbon viruses, were included in this study. All samples were amplified for *PreS1/ PreS2* region (Figure15a). Subsequently, genotype of gibbon HBV was identified using *Ava* II and *Dpn* II digestion of PreS1/PreS2 amplified PCR products as indicated in Figure 15b and 15c respectively. Samples G1, G2, G3, G4 and G6 represented 5 gibbon viral strains spreading in the Krabok Koo Center.

PreS1 gene amplification from gibbon showed smaller size than human HBV. Patterns of restriction enzyme digestion of gibbon HBV (G1-G6), however, showed different profiles as compared to human HBV (H1-H3). The thirty-three bases deletion after start codon of *PreS1* region caused the different RFLP patterns. Therefore, it cannot be grouped to any human viral genotype including genotype D which was reported to have a *PreS1* deletion in the same region [104] . The RFLP genotypic patterns of carrier gibbons and animal keepers are presented in Table 15. These results were confirmed by partial sequencing and phylogenetic analysis of the *PreS1* gene.

Precore promoter mutations at nucleotide 1762 and 1764 were detected by *Sau3A*I restriction pattern (Figure 16). Four carrier gibbons (G1,G2,G3,G5) showed point mutations in this region while one sample could not be typed (G4). G6 sample presented the normal gene in this region. Noticeably, all mutated samples were positive for HBeAg testing.

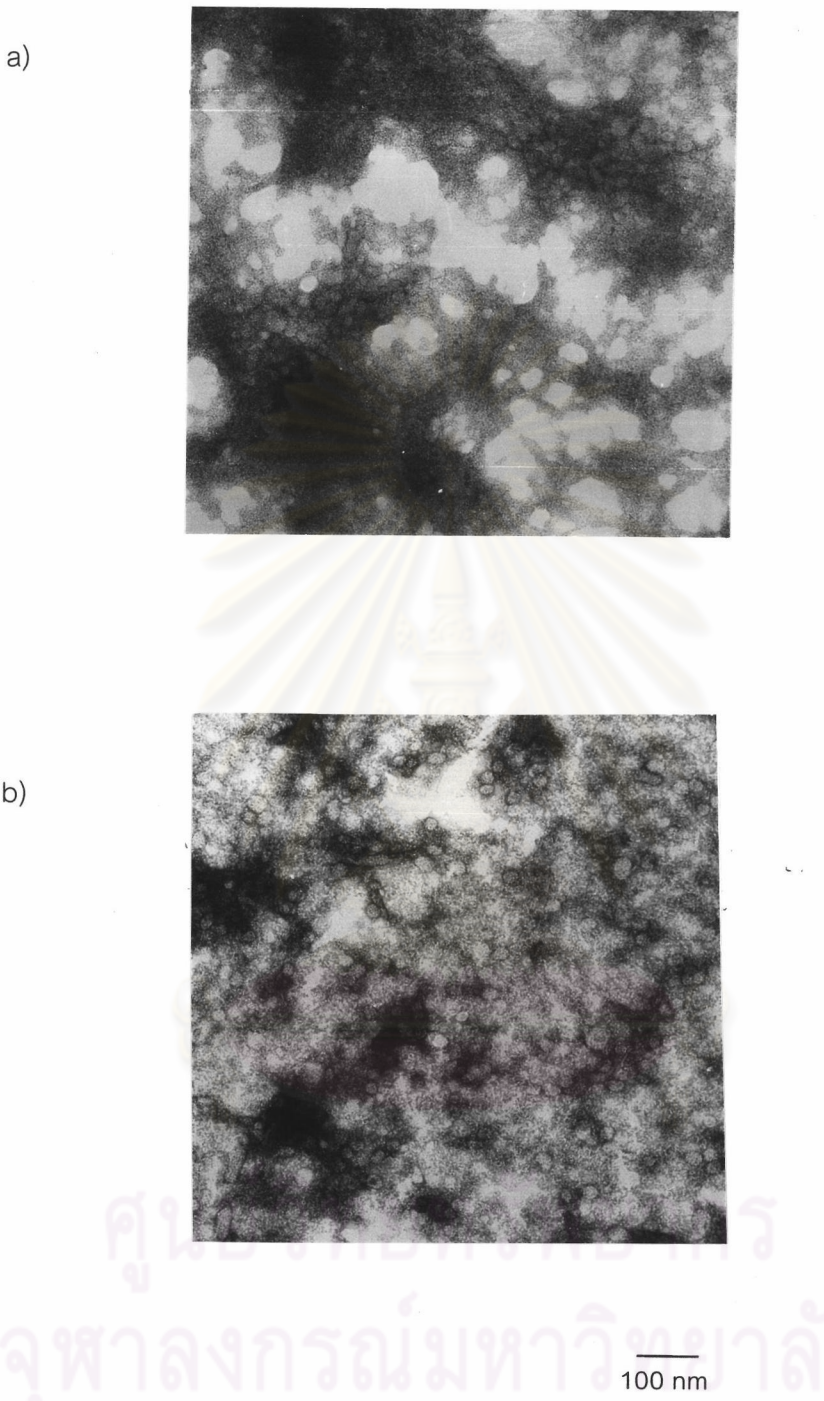
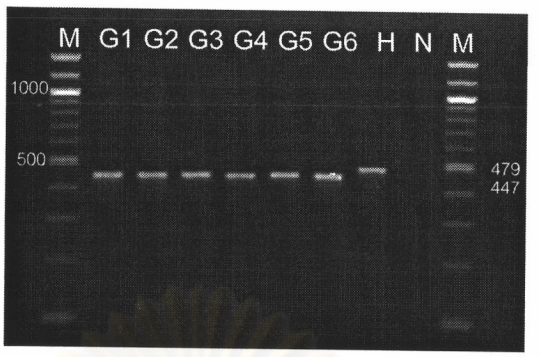
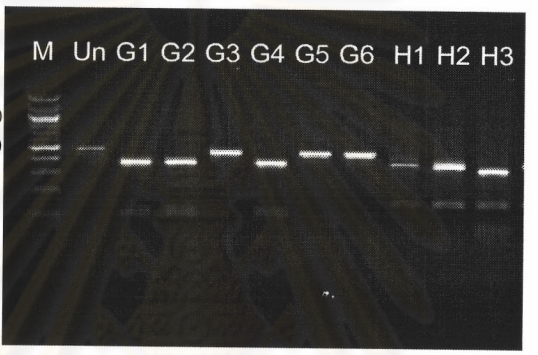


Figure 14 Morphology of probable HBV particles purified from sera of gibbon (a) and human (b) samples. Negative staining with 2% uranyl acetate was done and photographed under transmission electron microscope. Total magnification x 82,000.

a)



b)



c)

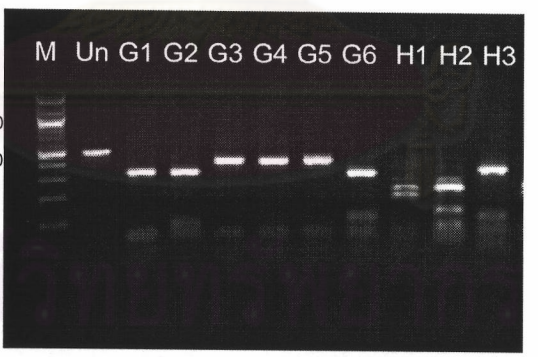


Figure 15 HBV genotype characterization by RFLP analysis. Gel electrophoresis of *PreS1* DNA isolated from gibbons and humans, before (a) and after digested with *Ava* II (b) and *Dpn* II (c). Six samples isolated from gibbons (G1-G6) and three samples from human (H1-H3) were analyzed. 500 and 1000 bps DNA marker (M) and the uncut sample (Un) are indicated.

Table 15 RFLP genotype characterization by action of *Ava* II and *Dpn* II on the segment of *PreS1/PreS2* region of gibbon and human (animal keepers) HBV.

PCR product size (bps)	group	*Ava* II digestion* fragment size (bps)	*Dpn* II digestion* fragment size (bps)	Samples
447	I	no digestion	306, 84, 57	Baby R6 Jieb R6 Pok C2 Saan C13 Midnight R27 Jacko R4 Nin L14
447	II	390, 57	318, 84, 45	Candy C21 Daew C22 Gomez C14 Chmi C14 Jock C20 Nongchai C16 Ni C15 Tao C15 Saboo C15
447	III	390, 57	363, 84	Caesar L10
447	IV	no digestion	363, 84	Charlie L9
450	V	no digestion	369, 81	Belle L10
479	C7**	358,121	214, 178, 52,35	H1 (human)
479	C1**	358,121	249,109, 69, 52	H2 (human)

* all cutting sites were confirmed by DNA sequencing

** human HBV genotype characterization followed the REA profile of human HBV [104]

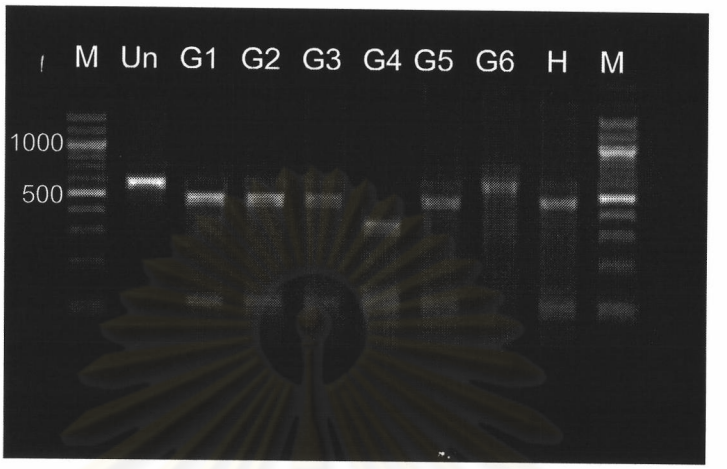


Figure 16 Precore promoter 1762/1764 analysis by RFLP . Gel electrophoresis of *X* region isolated from gibbon and human, digested with *Sau3A* I are presented. Six samples isolated from gibbons (G1-G6) and one sample from human (H) were analyzed. The expected size was 596 bps for wild type while mutant sample presented 465 and 131 bps. 500 and 1000 bps DNA marker (M) and the uncut sample (Un) are indicated.

III. Gibbon HBV DNA sequencing and phylogenetic tree analysis

In order to confirm infection with a gibbon HBV, the complete genome of two representative viruses was sequenced (G25 and G26). Comparing these sequences to other *Hepadnaviridae* demonstrated that these viruses form a cluster seperated from the six known human HBV genotype clades and from the chimpanzee, gorilla and woolly monkey hepadnaviruses (Figure17). The deletion of the 33 basepairs after preS1 start codon of gibbon HBV was confirmed.

Grouping of gibbon HBV was performed by phylogenetic tree analysis; preS1 phylogram analysis including human genotype A-G, orangutan, chimpanzee and woolly monkey revealed the separate clustering of these gibbon HBV (Figure 18). Most animals from the three different cages cluster into the three separate groups observed. HBV from two chronically infected mothers (Jieb and Tao) was more closely related to their infant, with a higher internal edge value at the node of phylogram, than sire strains. Surprisingly, animals in the closed cage such as area C were infected with a closely related strain of HBV since these viruses shared the root of the phylogenetic tree.

Amino acid alignment of *PreS1/PreS2* region from some gibbon isolates including various HBV genotype of human and other primate HBV sequences was shown in Figure 19. The 11 amino acid deletion at 5' site of *PreS1/PreS2* gene was found in nonhuman primate group and human HBV genotype D while 1 amino acid deletion was found in genotype E sequences. Woolly monkey HBV sequences represented the distinct group of nonhuman HBV.

To determine the subtype of gibbon HBV isolates, amino acid sequences of S protein from primate species were aligned at position 110-165 (Figure 20). Following the human HBV serotype characterization by amino acid sequences of S gene as previous report (Table 1)[56], gibbon HBV showed the various serotype including *adw2*, *adr* and *ayw3*.

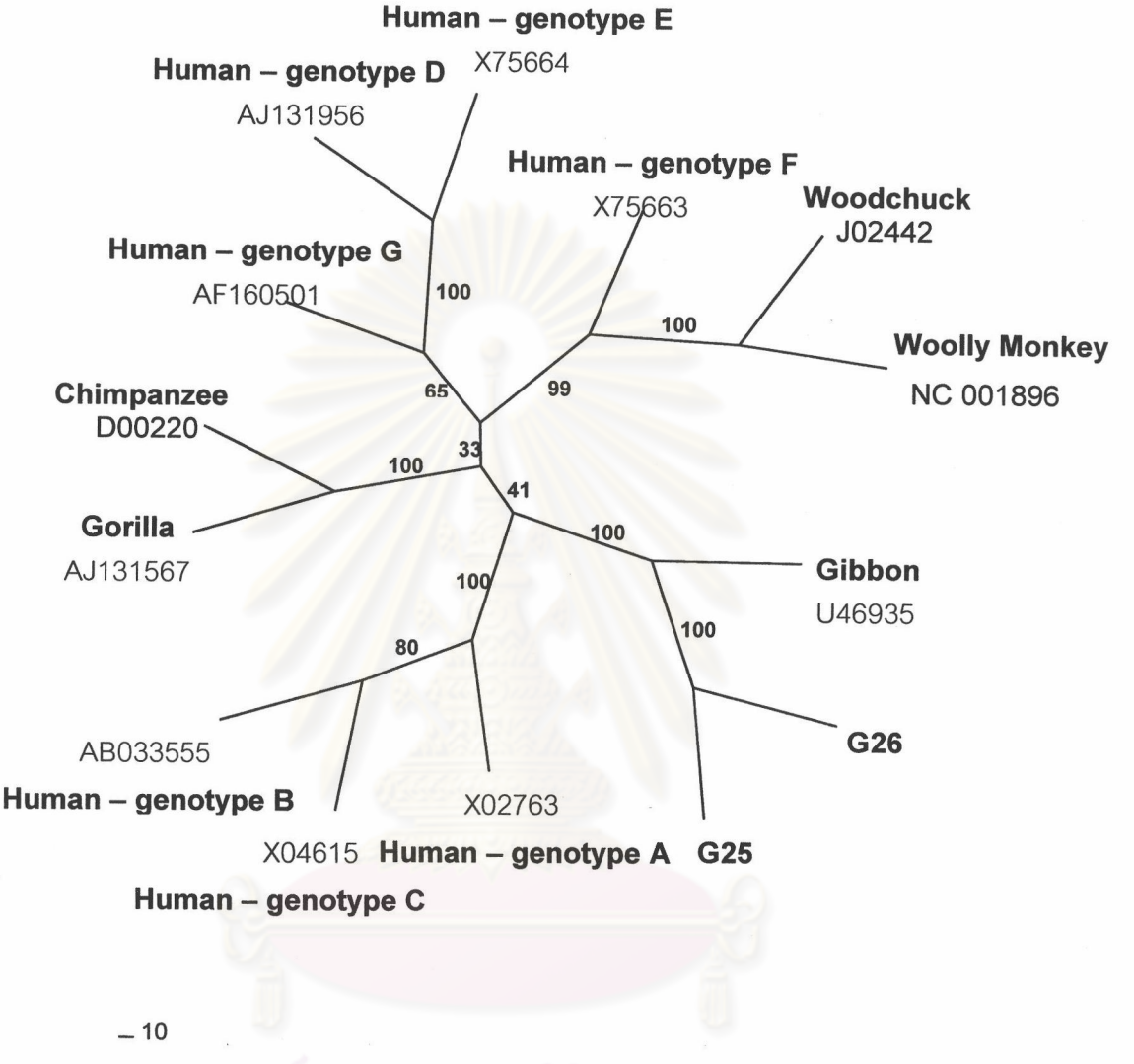


Figure 17 Phylogenetic tree representing an unrooted consensus tree obtained by the neighbor-joining method based on the whole genome sequence of HBV from human and apes. Bootstrap analysis was applied using 100 values. The human and nonhuman isolates are identified by their Genbank accession numbers. Whole genome sequence data of G25 (Belle L10) and G26 (Caesar L10) gibbon HBV were submitted in Genbank data libraries accession number AY077735 and AY077736, respectively.

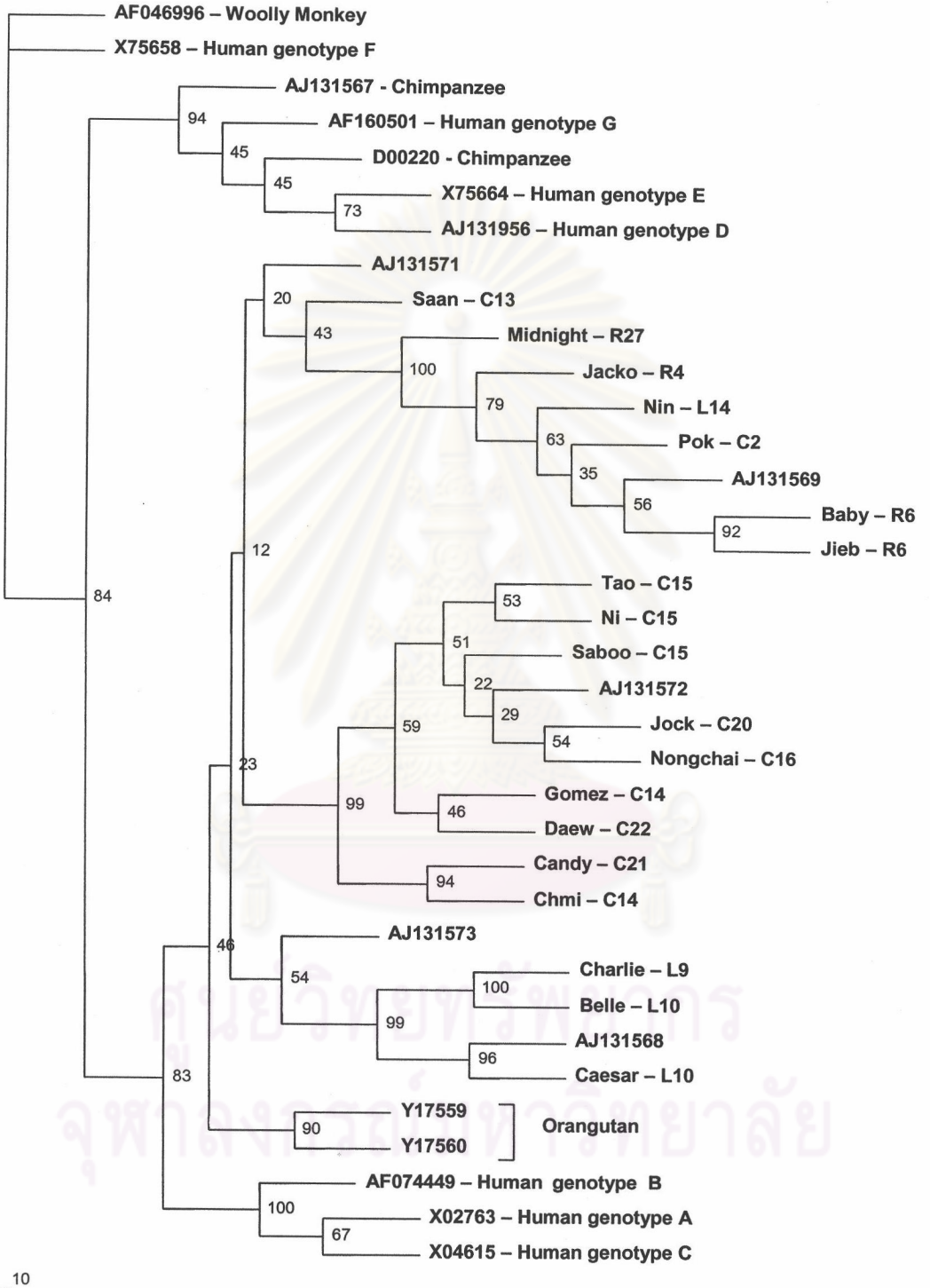

AF046996 – Woolly Monkey
X75658 – Human genotype F
AJ131567 - Chimpanzee
AF160501 – Human genotype G
D00220 - Chimpanzee
X75664 – Human genotype E
AJ131956 – Human genotype D
AJ131571
Saan – C13
Midnight – R27
Jacko – R4
Nin – L14
Pok – C2
AJ131569
Baby – R6
Jieb – R6
Tao – C15
Ni – C15
Saboo – C15
AJ131572
Jock – C20
Nongchai – C16
Gomez – C14
Daew – C22
Candy – C21
Chmi – C14
AJ131573
Charlie – L9
Belle – L10
AJ131568
Caesar – L10
Y17559
Y17560
Orangutan
AF074449 – Human genotype B
X02763 – Human genotype A
X04615 – Human genotype C
94
45
45
73
20
43
100
79
63
35
56
92
12
84
53
51
22
29
59
54
23
99
46
94
46
54
100
99
96
83
90
100
67
_10

Figure 18 Phylogram based on nucleotide sequences of *PreS1* gene using the Kimura two-parameter matrix and neighbor-joining method. The number of each node indicated the percentage of bootstrap replicates (of 100 total). The distance along the horizontal axis among isolates is proportional to genetic divergence. Human and nonhuman primate HBV were compared to gibbon HBV isolates identified in this study. The Genbank accession number of reported sequences were indicated in phylogram. *PreS1* isolates from gibbon HBV presented in this tree were indicated by name and cage of animals as shown in Table 13 . All sequences were submitted to GenBank database: accession number AF477482-AF477494 and AF274495 (Candy-C21), AF274496 (Daew-C22), AF274497 (Belle-L10), AF274498 (Caesar-L10), AF274499 (Charlie-L9) and AF275378 (Nin-L14).

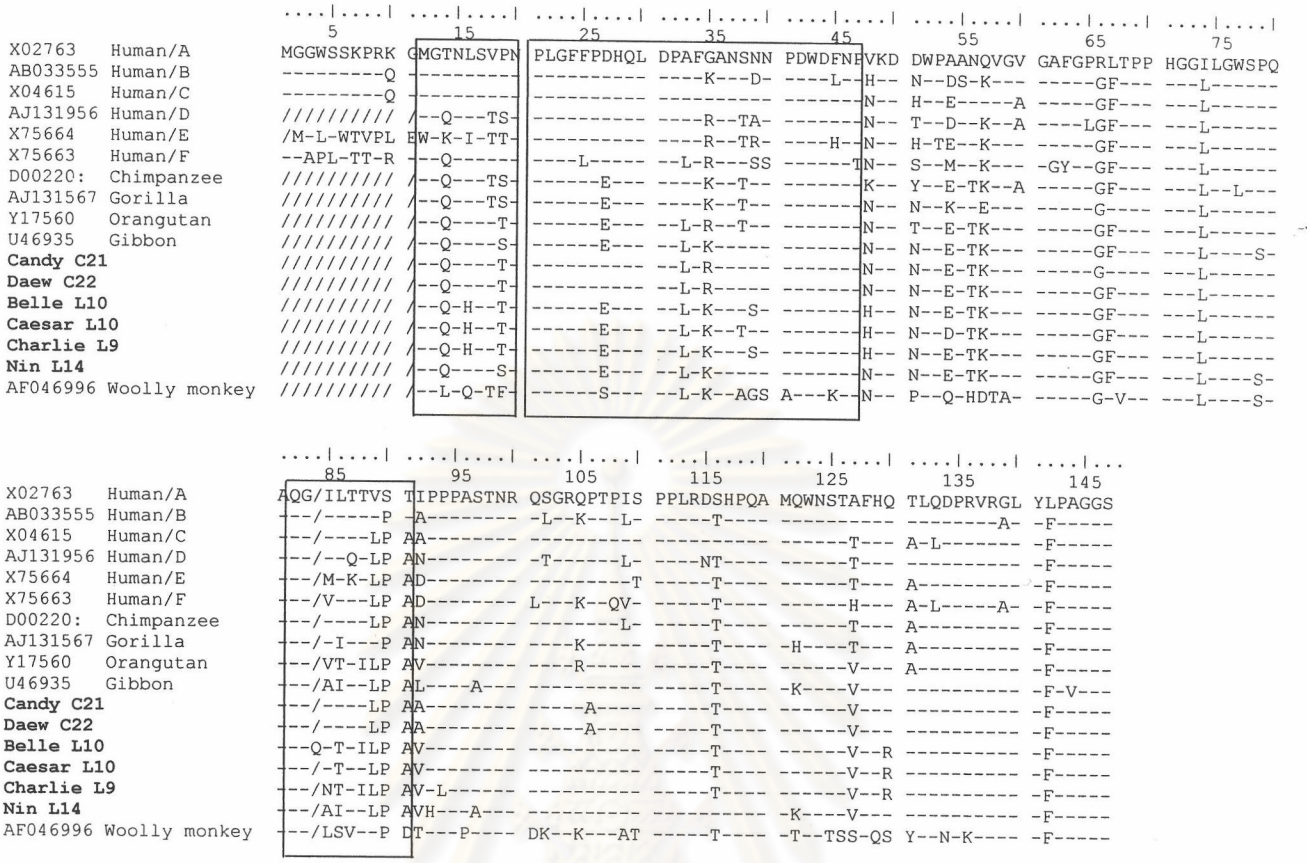


Figure 19 Alignment of PreS1/Pres2 amino acid across six genotypes of human HBV and other nonhuman primate hepadnaviruses. The respective primates and genotype were indicated each sequence. All isolates in present study noticed by bold letter. Slash (/) presents insertion or deletion while dash (-) indicates identical amino acid to master sequence (Human HBV genotype A ; X02763) . Boxes region represent the reported residues of HBV binding receptors.

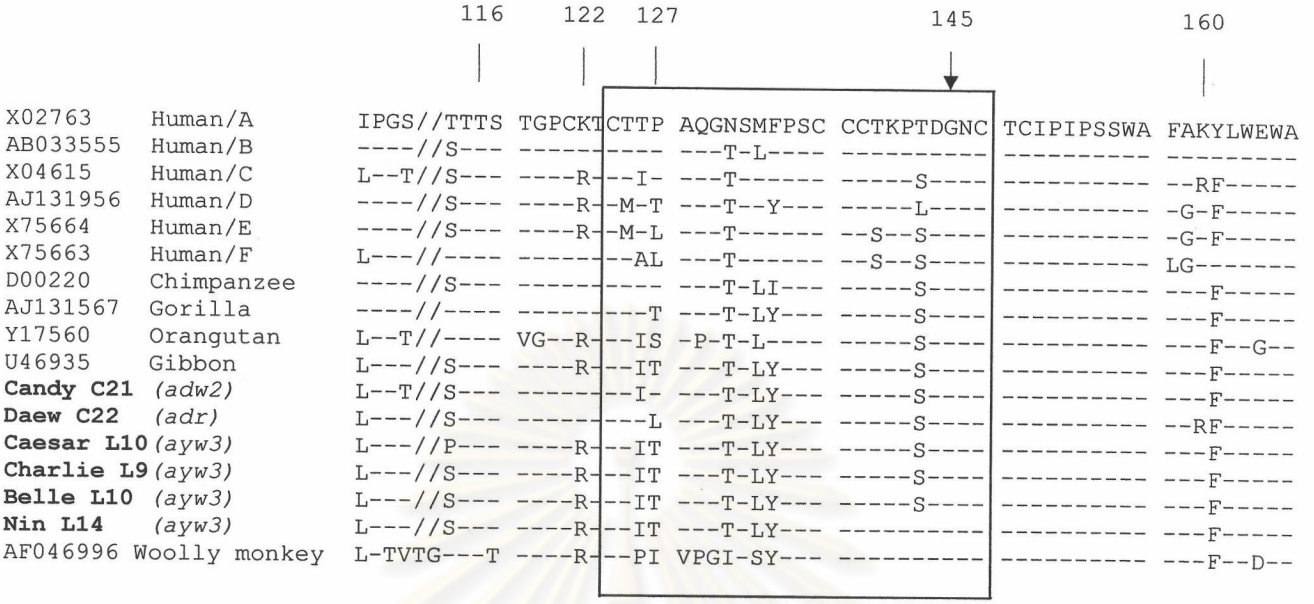


Figure 20 Alignment of S protein among six genotypes of human HBV and other nonhuman primate hepadnaviruses. The respective primates and genotype were indicated each sequence. All isolates in present study noticed by bold letter. Slash (/) presents insertion or deletion while dash (-) indicates identical amino acid to master sequence (Human HBV genotype A ; X02763) . Boxes represent the "*a*" determinant region of HBV which reacted to protective immunity. Arrow indicates the specific glycine (G) of this region. Position of amino acid refered to human HBV sequences. Gibbon HBV serotype identified from amino acid sequences was indicated in parenthesis.

Surface antigen mapping of gibbon HBV

To test the conformational structure of gibbon HBV surface protein compared to human viruses, a panel of antibodies against surface antigen of human HBV was used. According to a previous study, F4-7b antibody was reported with a high efficiency binding to human HBV [116] . Standard curve of ELISA assay plate coated by F4-7b and detected by F4-7b-biotin conjugate, followed with streptavidine/HRP and TMB substrate colur development, is shown in Figure 21.

HBsAg positive sera isolated from 5 gibbons (Belle, Candy, Nongchai, Saboo and Midnight) and 2 HBV chronic patients from Thailand (PS and SOM) were included in the present study. Gibbon HBV samples were selected from different groups referring to *PreS1* gene phylogenetic analysis (Figure 18). Amount of HBsAg of each samples was estimated using a standard curve of the F4-7b ELISA assay (Table 16). Different dilutions of each sample estimated by the $OD_{450/620}$ around 1.00 detecting by F4-7b as coated and captured antibody of HBsAg ELISA plate assay.

Surface antigen mapping of gibbon HBV was determined by a panel of antibodies to HBV surface protein. Estimation of antibody binding region is shown in Figure 22. Efficiency of antibodies binding to gibbon and human HBsAg was determined by ELISA (Table 17) and recalculated into the correlation factor number comparison to the standard plate detected by F4-7b anti-S (Table 18). The F9H9, 9A and 1Ff4 mAbs showed a similar binding to human and gibbon HBV, compared to binding factor of F4-7b reference assay (Table 18). Polyclonal anti-S from sheep gave a high binding capacity while anti-127 mAb could not bind to both antigens. Interestingly, a sample from gibbon, Midnight exhibited the highest binding factor of all antibodies. On the other hand, anti-PreS2 mAb adhered to gibbon viruses with higher reactivity than human samples.

Anova analysis to test correlation of antibodies bound to gibbon and human HBV indicated that the error variance of antibodies binding is equal across groups ($F = 2.379$, $p = 0.029$).

To test HBsAg binding by nonlabelled biotin-conjugated antibodies, ELISA assay plate was coated with anti-PreS1 and anti-PreS2 as solid phase before sample

incubation. F4-7b labeled biotin was added in the capture step following with streptavidine/HRP conjugate. Anti-PreS2 non-labeled biotin was used as a positive control for ELISA system. As shown in Table 19, Anti-PreS1 monoclonal antibody bound to only one human sample, but not for all gibbon viruses.

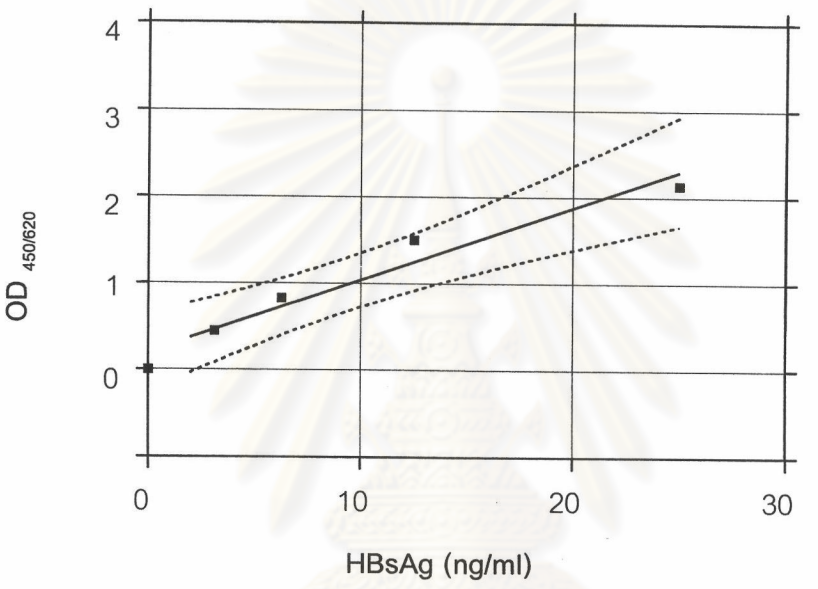


Figure 21 Standard curve of HBsAg detected by F4-7b ELISA assay plate.

* OD of each value was calculated from duplicate test

Dot lines represent the upper and lower range of standard curve

Table 16 HBsAg concentration of gibbon and human sample present in this study. Dilution factor and OD $_{450/620}$ of each sample were indicated. Concentration of HBsAg was calculated from standard curve of F4-7b (Figure21)

Sample	Dilution	OD450/620	HBsAg conc. (μg/ml)
Belle	1:8000	0.946	71.240
Candy	1:8000	0.99	75.536
Nongchai	1:2000	1.136	22.356
Saboo	1:4000	1.34	54.452
Midnight	1:10	0.384	0.022
PS	1:1000	0.87	8.004
SOM	1:400	1.129	4.438

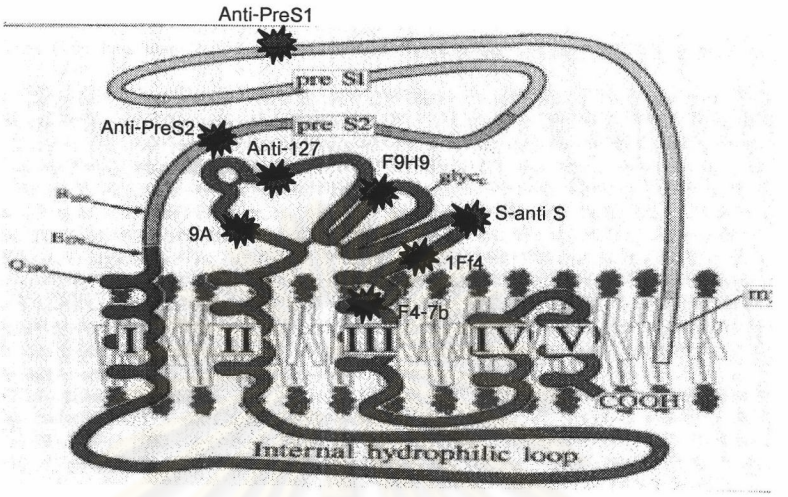


Figure 22 Image of HBV surface protein binding sites by a panel of antibodies in present study. HBV surface protein structure was modified from Kann *et al.* (1997) [117].

Table 17 Antibodies binding to surface antigen of gibbon and human samples determined by F4-7b ELISA assay plate at $OD_{450/620}$

Samples	Antibodies ($OD_{450/620}$)*						
(dilution)	F4-7b	sheep anti-S	F9H9	9A	1Ff4	anti-PreS2	anti-127
Belle (1:8000)	1.3470	2.0185	0.2505	0.5215	0.0110	0.5989	-0.0025
Candy (1:8000)	1.7540	2.3505	0.2960	0.4455	0.0140	1.1261	0.0045
Nongchai (1:8000)	0.4995	1.1370	0.0500	0.0845	0.0025	0.2561	-0.0025
Saboo (1:6000)	1.4085	2.2880	0.2105	0.3555	0.0110	0.9045	0.0020
Midnight (1:5)	1.0980	2.7250	0.9920	1.5655	0.8215	1.7299	-0.0230
PS (1:800)	1.9780	2.4310	0.3475	0.6255	0.0095	0.5783	-0.0007
SOM (1:600)	0.8955	1.2895	0.0930	0.1845	-0.0060	0.1572	-0.0060

* OD of each value was calculated from duplicate test.

Sample dilution was indicated in parenthesis.

Table 18 OD correlation of antibodies binding to human and gibbon HBV surface protein determined by ELISA assay. Correlation factor number of each sample was compared to the F4-7b ELISA standard plate at $OD_{450/620}$.

Samples (dilution)	Antibodies (Cor. Fac.)						
	F4-7b	sheep anti-S	F9H9	9A	1Ff4	anti-PreS2	anti-127
Belle (1:8000)	1.0000	1.4895	0.1860	0.3871	0.0080	0.4444	0
Candy (1:8000)	1.0000	1.3401	0.1687	0.2540	0.0080	0.6420	0
Nongchai (1:8000)	1.0000	2.2763	0.1001	0.1692	0.0050	0.5128	0
Saboo (1:6000)	1.0000	1.6244	0.1494	0.2524	0.0078	0.6422	0
Midnight (1:5)	1.0980	2.4818	0.9034	1.4258	0.7481	1.5755	0
PS (1:800)	1.0000	1.2290	0.1757	0.3162	0.0048	0.2924	0
SOM (1:600)	1.0000	1.4400	0.1038	0.2060	0	0.1756	0

Anova analysis indicated that the error variance of antibodies binding to both viruses is significantly equal across groups ($F = 2.379$, $p = 0.029$).

Table 19 Antibodies binding to surface antigen of gibbon and human HBV determined by anti-PreS1 and anti-PreS2 antibodies coated ELISA plate. Assay plate was captured by F4-7b monoclonal antibody.

Samples	Antibodies ($OD_{450/620}$)	
(dilution)	anti- PreS1	anti-PreS2
Belle (1:300)	0.0285	2.5215
Candy (1:300)	0.0490	2.5455
Nongchai (1:50)	0.0145	2.5615
Saboo (1:200)	0.0125	2.5665
Midnight (1:5)	0.0010	0.3250
PS (1:100)	0.3970	2.4645
SOM (1:100)	0.0515	2.1405

* OD of each value was calculated from double test

Concentration of coating antibodies

Anti-PreS1 = 15 ug/ml

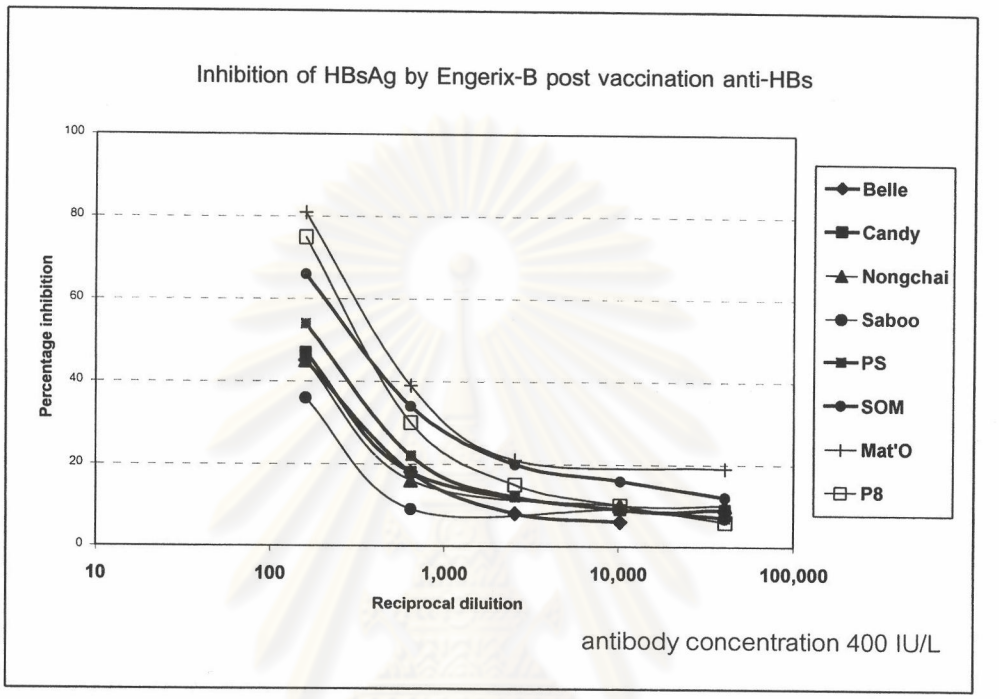
Anti-PreS2 = 5 ug/ml

Inhibition binding of gibbon and human HBV by immune anti-HBS

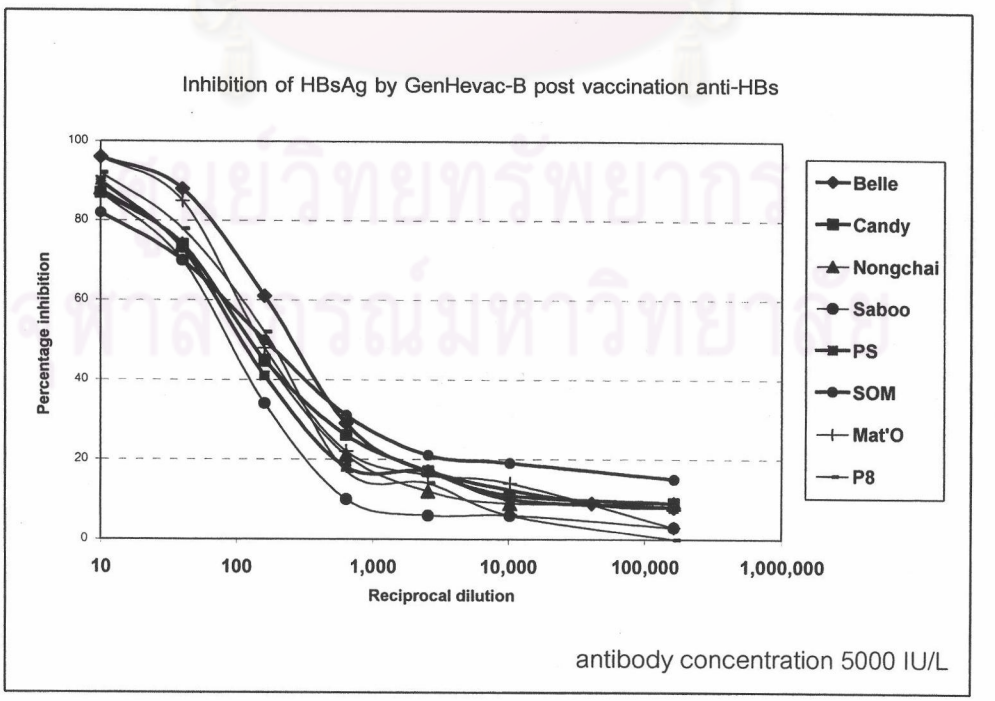
To investigate the neutralizing efficiency of immune anti-S produced from the vaccinated human, HBsAg positive sera isolated from 4 gibbons (Belle, Candy, Nongchai and Saboo), 2 patients from Thailand (PS and SOM) and 2 human HBV subtypes, adw2 (Mat'O) and adr (P8), were tested for the inhibition binding assay[105]. Antibodies from vaccination studies with Engerix-B, GenHevac-B and HepageneB were compared in titration range with reference monoclonal antibodies (F4-7b) and HBIg (Hepatect). The anti-HBs from vaccinees showed the same capacity of binding blocking to human as gibbon HBV (Figure 23). Thus, binding of immune anti-HBs from Engerix-B vaccination seems higher specific to human HBV than gibbon viruses (Figure 23a). The percentage of inhibition binding of gibbon and human HBsAg studied at the fixed antibody concentration (about 50 IU/L) is shown in Table 20. The results showed the similar capacity of human immunoglobulin (Hepatect) and F4-7b antibody to inhibit binding of gibbon and human HBV. Interestingly, anti-S from plasma of vaccinated persons presented the blocking binding of both virus strains, except anti-S produced from Engerix B vaccination. The low binding blocking of gibbon viruses (43.25 ±4.92) was detected comparison to human HBV (69.00±11.74).

Figure 23 Inhibition binding assay of immune anti-S including Engerix-B (a), GenHevac-B (b) and Hepagene-B (c), human immunoglobulin : Hepatect (d) and human monoclonal antibody, F4-7b (e) to gibbon and human HBV in a HBsAg ELISA assay.

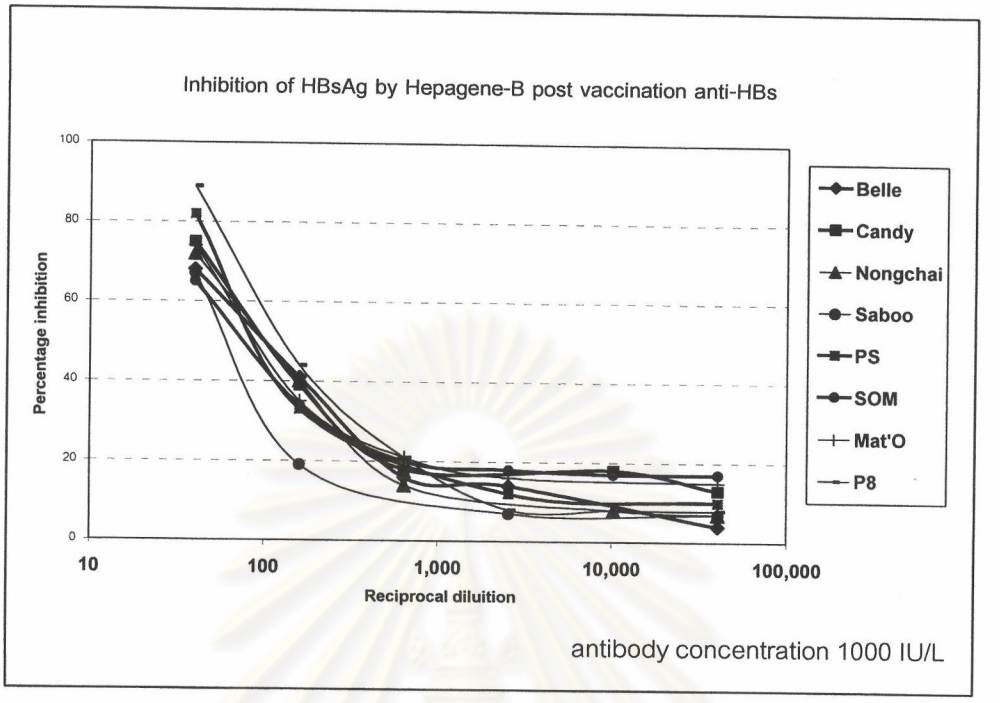
(a)



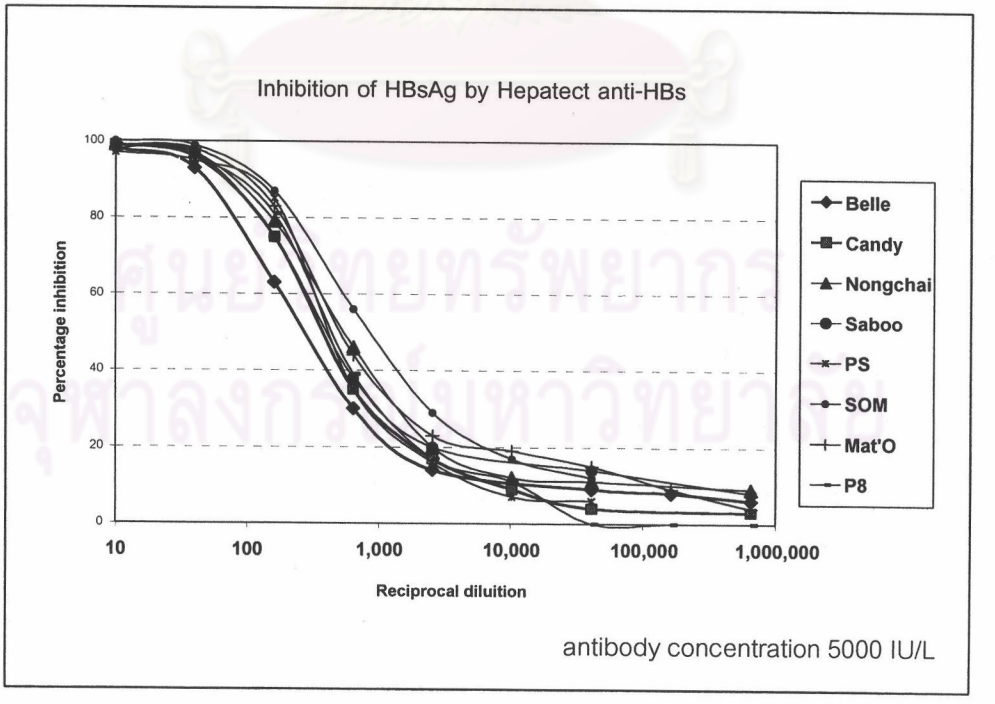
(b)



(c)


Inhibition of HBsAg by Hepagene-B post vaccination anti-HBs
Percentage inhibition
100
80
60
40
20
0
10
100
1,000
10,000
100,000
Reciprocal diluition
Belle
Candy
Nongchai
Saboo
PS
SOM
Mat'O
P8
antibody concentration 1000 IU/L

(d)


Inhibition of HBsAg by Hepatect anti-HBs
Percentage inhibition
100
80
60
40
20
0
10
100
1,000
10,000
100,000
1,000,000
Reciprocal diluition
Belle
Candy
Nongchai
Saboo
PS
SOM
Mat'O
P8
antibody concentration 5000 IU/L

(e)

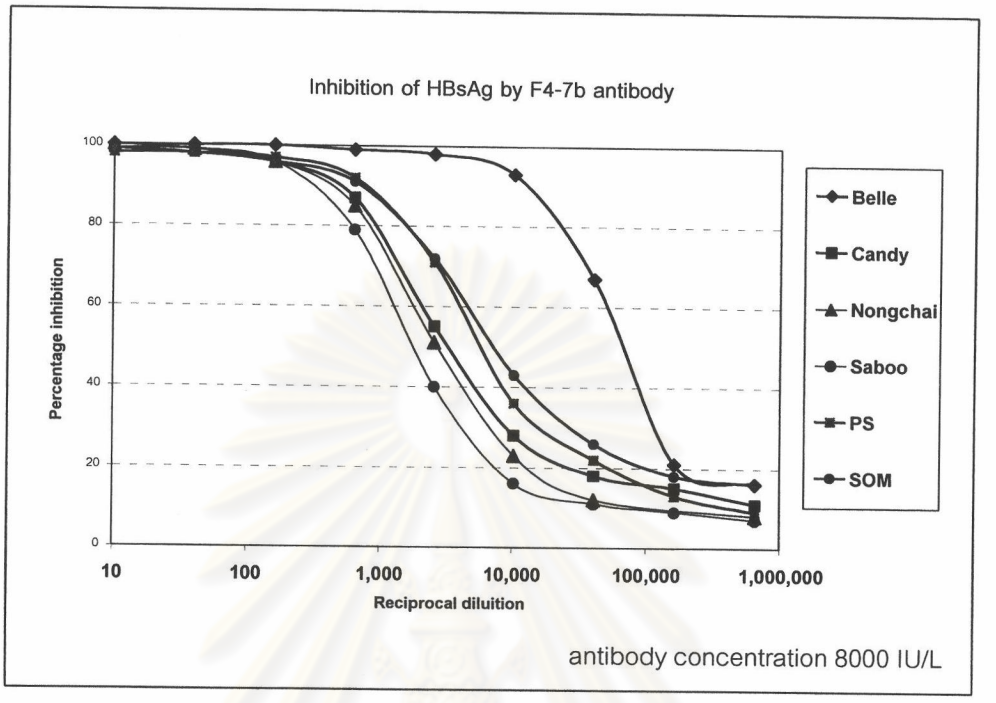


Table 20 Percentage of inhibition binding of immune anti-S generated from Engerix B, GenHevac B, Hepagene B vaccination to gibbon and human HBsAg comparison to Hepatect HBIg and F4-7b mAb. The fix antibodies concentration (50 IU/L, at the reciprocal dilution 1:160) was selected to present in table.

Samples	Anti-HBs (%Inhibition binding)				
	Engerix B	GenHevac B	Hepagene B	Hepatect	F4-7b
Belle	45	61	41	63	100
Candy	47	45	39	75	96
Nongchai	45	45	40	79	96
Saboo	36	34	19	75	96
X±SD	43.25±4.92	46.25±11.12	34.75±10.53	73.00±6.93	97.00±2.00
PS	54	41	33	85	97
SOM	66	50	34	87	96
M'O	81	48	35	83	ND
P8	75	52	44	81	ND
X±SD	69.00±11.74	47.75± 4.79	36.50± 5.07	84.00± 2.58	96.50± 0.71

ND = not determine

Cloning and expression of HBV surface protein (L/M/S protein)

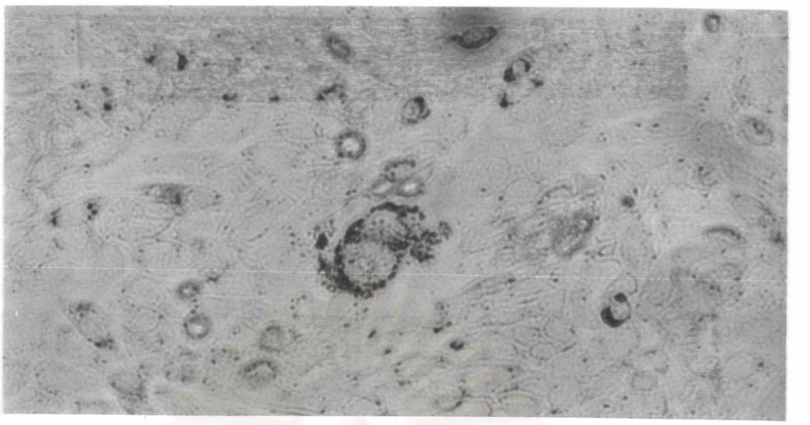
Due to the limitation of viral particles purified from gibbon serum, the binding study of HBV particles to human host cells is not possible. To support the binding study, gibbon and human HBV surface genes were cloned and expressed in mammalian CHO cells. A first set of cloning experiment, sLMS/ VR1012/Neo$^+$ plasmids were constructed with 1489 and 1522 bps of gibbon and human HBV insertion, respectively. These plasmids carried only the gibbon and human *PreS1/PreS2/S* genes encoding for envelope protein. The viral surface proteins synthesized in transfected cells was detected by F4-7b mAb (Figure 24) and anti-PreS2 antibodies (data not shown) immunostaining. Unfortunately, the secreting surface proteins produced from both plasmids expressed in transient transfected cells and stable cell lines was too weak to be detectable by ELISA assay. (Table 21)

The second set of plasmids, LMS/VR1012/Neo$^+$, expressed surface protein of gibbon and human HBV were constructed and transfected into CHO cells. They contained the genes coding for the envelope proteins and also had the downstream HBV sequences beyond to the polyadenylation signal. This additional viral sequence contained the X gene/enhancer II region, which is known to increase the level of surface gene transcription [106]. In particular, the present of the posttranscriptional regulatory element facilitates cytoplasmic accumulation of transcript protein. The surface protein of both clones were detected by immunostaining using anti-PreS2 (Figure 25) and F4-7b antibodies (data not shown). Interestingly, the HBV surface secreating protein of both clones became detectable in supernatant of transient transfected cells, and stable cells of gibbon and human expressed plasmid, respectively (Table 22). Due to the lacking of CHO stable cell lines expressed gibbon HBV protein, supernatant from transient transfection of this sample was used to perform the next experiment.

HBV surface protein detected in supernatant of stable lines was concentrated with PEG-8000 to reduce the volume of sample. The concentrated protein was confirmed their conformation and amount of protein by ELISA and immunoblot (dot blot) analysis (Table 23 and Figure 26).

According to the previous report, HBV surface proteins, especially *PreS1* region, showed the specific binding to the host cell receptors [9]. To test the quality of viral surface protein expressed from recombinant plasmid and secreted in supernatant of stable cell lines, concentrated protein was directly incubated with the human hepatocyte HepG2 cells. HBV purified particles isolated from human positive sera was used as positive control. As shown in Figure 27, only binding between purified human viral particles and HepG2 cells was detected by F4-7b antibody staining and FACs analysis.

(a)



(b)

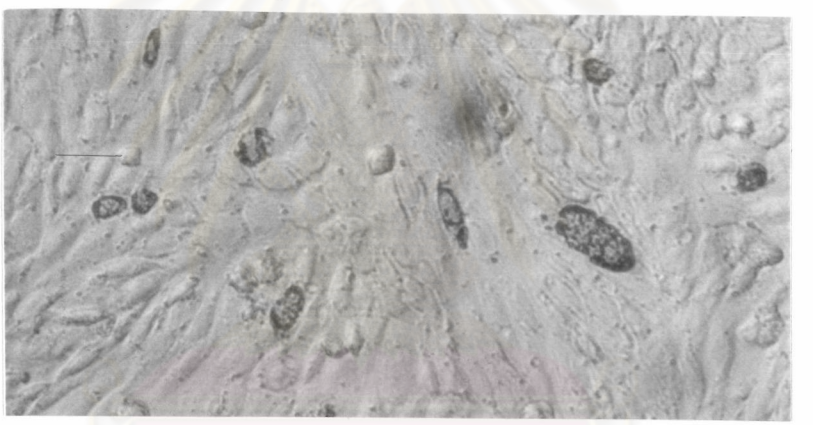


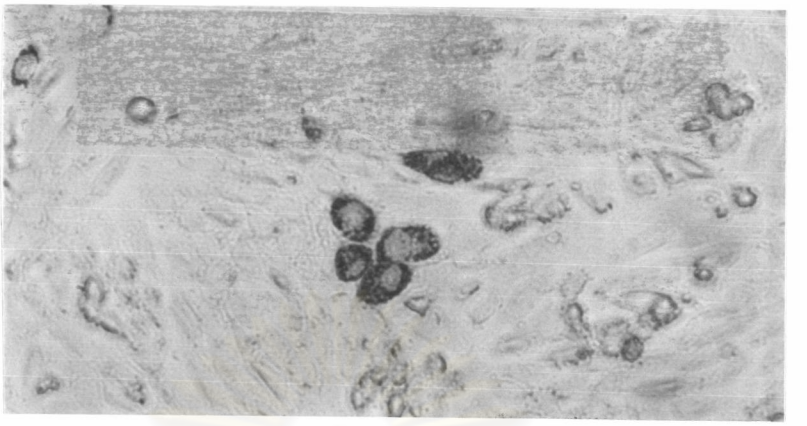
Figure 24 F4-7b mAb staining of surface protein of gibbon (a) and human (b) HBV expressed by sLMS/ VR1012/Neo^{+} plasmids in CHO cells. Magnification x200.

Table 21 ELISA assay of secreted HBV surface protein expressed from gibbon and human sLMS/ VR1012/Neo$^+$ plasmids in supernatant of CHO culture.

Cell lines	$OD_{450/620}$*	
	Transient transfected cells	Stable cell lines
CHO	0.0280	0.0307
CHO/ VR1012/Neo$^+$	0.0310	0.0337
CHO/ gibbon sLMS/ VR1012/Neo$^+$	0.0360	0.0360
CHO/ human sLMS/ VR1012/Neo$^+$	0.0350	0.0347

* average $OD_{450/620}$ was calculated from triplicate results.

(a)



(b)

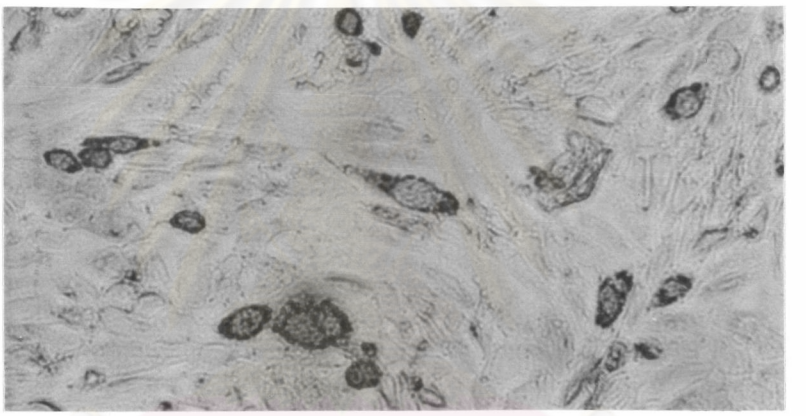


Figure 25 anti-PreS2 mAb staining of surface protein of gibbon (a) and human (b) HBV expressed by LMS/ VR1012/Neo^{+} plasmids in CHO cells. Magnification x200.

Table 22 ELISA assay of secreted HBV surface protein expressed from gibbon and human LMS/ VR1012/Neo$^+$ plasmids in supernatant of CHO culture.

Cell lines	$OD_{450/620}$*	
	Transient transfected cells	Stable cell lines
CHO	0.0250	0.0202
CHO/ VR1012/Neo$^+$	0.0255	0.0243
CHO/ gibbon LMS/ VR1012/Neo$^+$	0.0500	0.0410
CHO/ human LMS/ VR1012/Neo$^+$	0.0237	0.2280

* average $OD_{450/620}$ was calculated from triplicate results.

Table 23 Comparison of secreted HBV surface protein of gibbon and human LMS/ VR1012/Neo$^+$ plasmids expression in CHO stable cells. HBsAg in CHO supernatant before and after concentration with PEG-8000 were determined by ELISA assay.

Cell lines	$OD_{450/620}$*	
	Supernatant before concentrated	Pellet after concentrated
CHO	0.0180	0.0240
CHO/ VR1012/Neo$^+$	0.0224	0.0452
CHO/ gibbon LMS/ VR1012/Neo^{+**}	0.0280	0.0760
CHO/ human LMS/ VR1012/Neo$^+$	0.2390	0.5310

* average $OD_{450/620}$ was calculated from triplicate results.

** supernatant was collected from transient transfected cells

Figure 26 Immunobloting of gibbon and human HBV surface protein expressed in CHO cells. Supernatant before and after concentration with PEG-8000 of human (a and d) and gibbon (b and e) surface protein were tested. Supernatant from CHO cell (c) used AS negative control, while concentrated supernate of HepG2.2.2.15 lines (f) and serial dilution of HBsAg positive serum (dilution 1:10 to 1:10,000, G-J) were used as positive control, respectively.

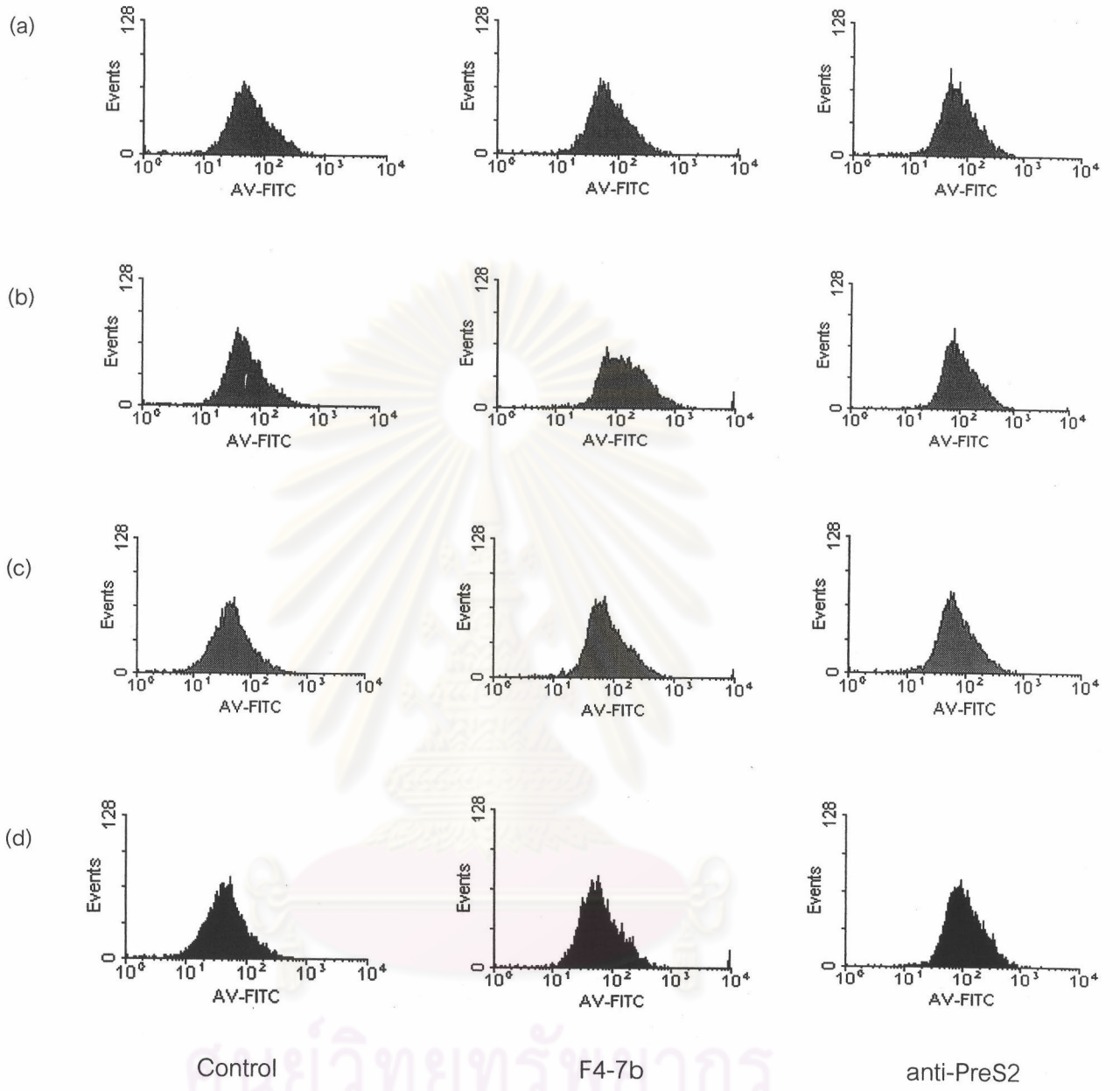


Figure 27 Histogram of FACs analysis for HBV protein binding to human hepatocytes cells. The concentrated surface proteins of gibbon (c) and human (d) HBV expressed in CHO stable cell lines were incubated with HepG2 cells and detected the protein complex by F4-7b and anti-PreS2 FITC conjugated antibodies. Normal HepG2 cells (a) and HepG2 bound to purified viral particles (b) were used as negative and positive control, respectively.

In vitro HBV infection in human hepatocyte cells

Hepatitis B virus infection in cell culture is the ideal model for study the mechanism of viral binding, penetration and replication in human cells. Additional, this model is expected to demonstrate the HBV cross-transmission among different animal species and might be useful for the specific receptors binding study. Until now, there are still no stable cell lines that support HBV infection. In this present study, several cell lines were tested for susceptibility to HBV infection.

Primary hepatocyte cells were isolated from normal human liver and cultured in enrich medium before infected with HBV positive serum. The cccDNA form of HBV which detected only in replicating infected cells was not found. The same result was seen in reversed NKNT-3 cell lines.

The specific receptor, Annexin V protein, was reported as the binding protein to human HBV particle in a previous study [77]. HepG2 cells transiently expressing Annexin V were used as target cell for the HBV infection study. The expression of Annexin V was detected by anti-AnV antibody as shown in Figure 28. Unfortunately, the infected cells were not detected due to lack of the cccDNA form in cell culture.

Since the first report on the possiblity to infect human hepatocytes, some chemical reagents have been used to increase the susceptibility of the hepatocytes. The DMSO treated human hepatocyte HepG2 cells were used in this study and gibbon HBV serum infection was done. The rc and cccDNA of HBV were detected in infected cells by PCR (Figure 29). As shown in Figure 30, HBV surface protein was detected inside infected cells by anti-PreS2 antibody immunostaining .

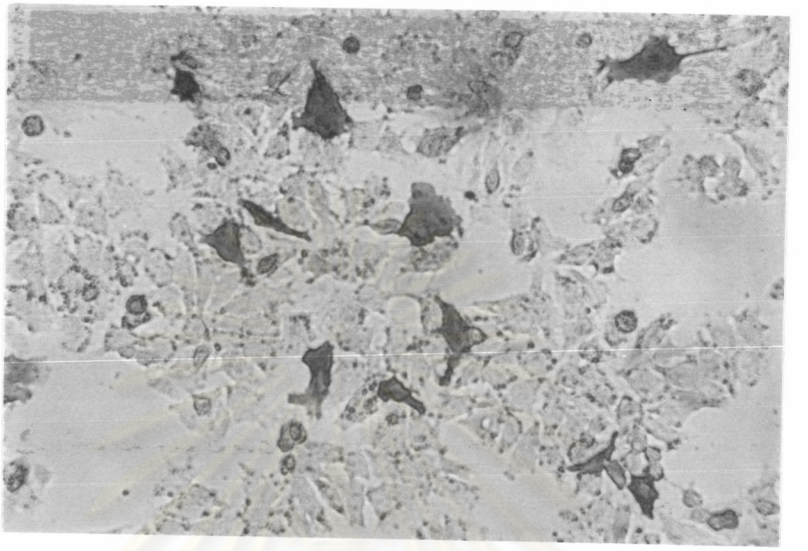


Figure 28 Immunostaining of Annexin V expressed in HepG2 cells. The target protein was detected by anti-Annexin V antibodies. Magnification x200

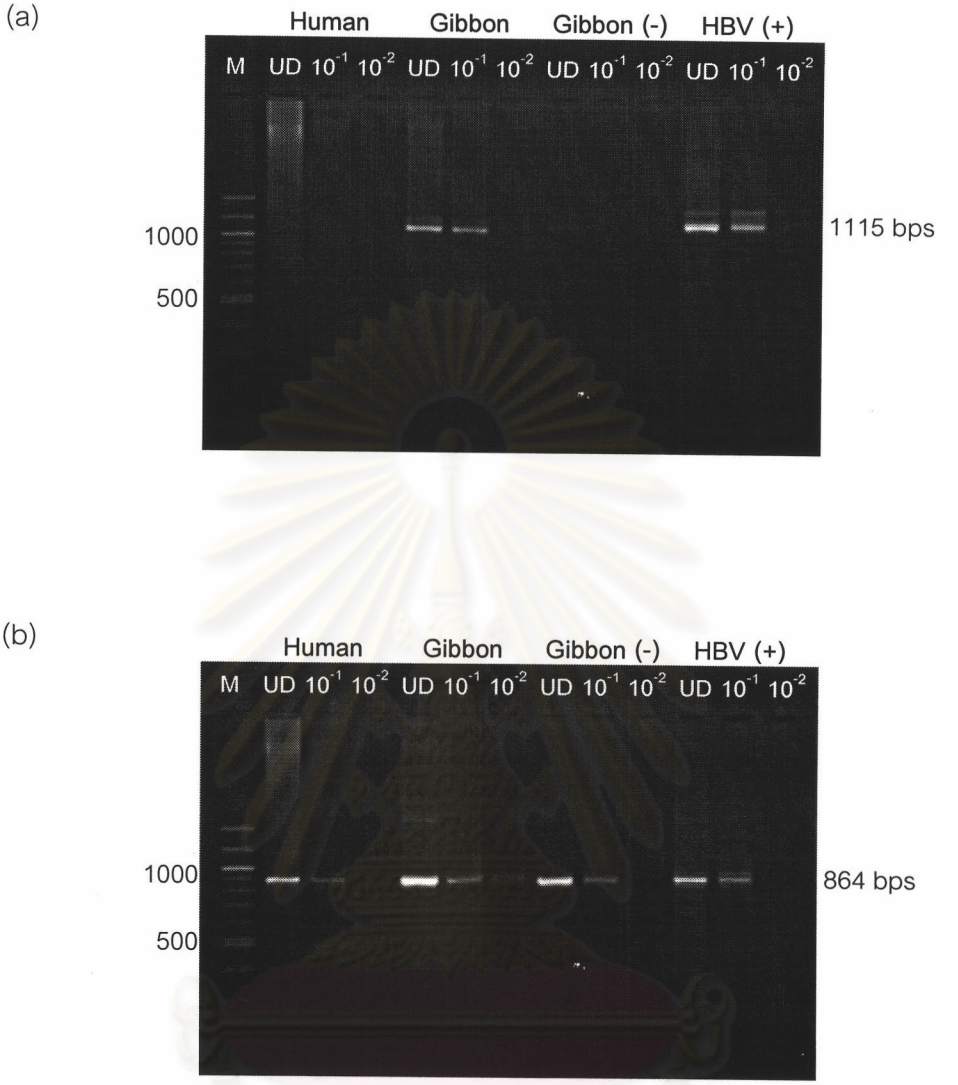


Figure 29 cccDNA (a) and rcDNA (b) detection in DMSO treated human hepatocyte (HepG2) cells at 14 days after infection. DNA template was extracted from cells infected with human and gibbon HBV positive sera. Gibbon HBV positive serum (Gibbon-) and DNA extract from liver biopsy of HBV patient (HBV+) were used as negative and positive control respectively. Each template was tested within serial dilution including undiluted DNA (UD), 10 times dilution (10^{-1}) and 100 times dilution (10^{-2}). Expected size of amplified products, 500 and 1000 bps DNA marker (M) are indicated.

(a)

(b)

Figure 30 Anti-PreS2 antibody staining of gibbon HBV infected HepG2 cells (a) and normal HepG2 (b). Magnification x200.