

**Transmission of Hepatitis B Virus to Gibbons
by Exposure to Saliva Containing Hepatitis B Surface Antigen**

Principal Investigators : William H. Bancroft, LTC, MC
 Rapin Snitbhan, M.D.
 Robert McNair Scott, LTC, MC
 Markpol Tingpalapong, DVM
 William T. Watson, MAJ, VC

Associate Investigators : Prayot Tanticharoenyos, DVM
 Jerome J. Karwacki, Jr., SP/6
 Suranga Srimarut¹

OBJECTIVE : To determine if human saliva containing Hepatitis B surface Antigen (HB_sAg) contains infectious Hepatitis B Virus (HBV).

BACKGROUND : The presence of hepatitis B surface antigen (HB_sAg) in the whole mouth saliva of antigenemic people has been reported previously (1). The high prevalence of antigen and antibody to HB_sAg in members of the same family, children in institutions, sexual partners, and hepatitis in dental personnel has raised the possibility that saliva may be a vehicle for the transmission of Hepatitis B Virus (HBV) (2). A human bite has been implicated in the transmission of Hepatitis B Virus in one case (3). Since no reports have demonstrated the infectivity of saliva, an attempt was made to transmit HBV by exposing captive gibbons to a pool of human saliva containing HB_sAg.

DESCRIPTION :

Saliva Collection

Seven male and four female Thai adults, who were known to have HB_sAg positive sera, were asked to provide fresh saliva specimens. Since HB_sAg is inconstantly present in saliva (1), samples were collected from the donors on more than one day. Collections were made between 1000-1200 hours, at least two hours after eating any food or brushing the teeth. The mouth was rinsed with clear water before starting the collections. Each collection period lasted one to two hours.

Two types of saliva collections were made. The first involved splitting into sterile containers. These samples were considered to be whole mouth saliva, since they included all oral secretions. No sialagogues were used to obtain these samples. The second type of collection utilized a circular hard plastic cup, 2 cm in diameter with a peripheral suction ring and a central drainage port attached to a flexible polyethylene drainage tube. The cup was positioned so as to completely cover the opening of one parotid duct and attached to the buccal mucosa by gentle suction. This type of collection was considered to consist of unilateral parotid secretions. During the collection of parotid secretions, the donor was sometimes permitted to suck on a hard candy in order to promote secretory activity. For both types of collections, the saliva samples were kept chilled in an ice bath. After removing a 1:0 ml aliquot for testing, the remainder of each sample was promptly frozen at -70°C until pooled. Samples were selected for inclusion in a saliva pool on the basis of the HB_sAg test results. All saliva samples were tested for occult blood using paper strips impregnated with a buffered mixture of organic peroxide and orthotolidine (Labstix; Ames Company,

¹ Virus Research Institute, Dept of Medical Sciences, Ministry of Public Health, Bangkok, Thailand.

Elkhart, Indiana). Multiple serum samples were drawn from each donor in order to determine the geometric mean level of antigenemia during the period of saliva collection.

Preparation of the Saliva Pool

Five people provided samples of whole mouth saliva and parotid secretion; HB_sAg was present in 16/23 (61%) of the former and only 1/8 (12.5%) of the latter (Table 1). The single antigen positive parotid secretion sample had an unusually large volume suggesting the collection cup may have dislodged allowing HB_sAg to enter the sample from the oral cavity. Because of the low antigen yield, parotid secretions were not included in the final saliva pool.

Six of the 11 saliva donors had HB_sAg in one or more samples. Five of the six carried HB_sAg / adr; the other had the adw subtype. In order to maintain uniformity of the antigen subtype, only 18 samples from the adr carriers were used (Table 2). Each sample was thawed quickly, mixed thoroughly together, centrifuged at 10,000 rpm (12,100 X g) for 30 minutes at 4°C, and the supernate used as the saliva pool. The pool appeared visually clear, was positive for occult blood, and gave a reaction for HB_sAg by radioimmune assay only.

The pool was divided into aliquots for convenient use and cultured for bacterial growth. Portions reserved for subcutaneous injection were treated with penicillin (1,000 units/ml) and streptomycin (1,000 mcg/ml). Bacterial cultures of the untreated saliva grew a few colonies of alpha streptococcus; the saliva containing antibiotics was bacteriologically sterile. All portions were kept frozen at -70°C until used.

Gibbon Management

Non-breeding white-handed gibbons (*Hylobates lar*) were housed in one wing of a Veterinary Medicine building designed to permit free circulation of air between the animal rooms and the outside area. Temperature and humidity were near ambient. Rooms were double screened to prevent the entry of insect vectors. The gibbons selected for exposure to the saliva were kept in one room apart from the others. Each gibbon was held in an individual, galvanized, metal cage spaced so as to prevent direct contact between animals. Animals were fed a commercial primate diet supplemented with fruits and vegetables. Each had an individual food and water supply. Soiled bedding from the cages was incinerated daily. Only veterinary personnel who had no HB_sAg were permitted to care for the study gibbons. All personnel wore clean protective outer clothing, face masks, and gloves when working with the animals.

Study Gibbons

Ten gibbons with no detectable HB_sAg or anti-HB_s were selected for exposure to the saliva pool. They included five males and five females that ranged in age from 1 to 11 years. Six were born in the SEATO Medical Research Laboratory gibbon colony; the remainder had lived there for 8 to 10 years. One other gibbon (B-66 S) had anti-HB_s and was followed as a control for the methods of antibody detection. No studies of the transmission of viral hepatitis had ever been done in the gibbon colony before.

Exposure to the Saliva Pool

Ten gibbons were exposed to the saliva pool. Two gibbons (Pc-13, Pc-14) received subcutaneous injections of 1.7 ml of saliva every other day for three doses (Table 3). Eight other gibbons were divided into pairs and exposed to 1.0 ml of saliva on each of five successive days by either an aerosol spray of the nose; aerosol spray of the mouth; brushing the teeth with the saliva; or ingesting saliva injected into a banana. Each animal received a total of 5.0 or 5.1 ml of saliva pool over the same five day period. It may be assumed that a large portion of the saliva administered by the oral and nasal routes was swallowed. The antibody positive control gibbon (B-66 S) was not exposed to the saliva.

Method of Follow-up

The first day of exposure to the saliva pool was designated Day 0. On every subsequent day each animal was observed for altered behavior and the rectal temperature was recorded. Once a week, each gibbon was weighed and examined by a veterinarian. Following sedation with a rapidly acting intramuscular anesthetic (phencyclidine hydrochloride or ketamine hydrochloride), a blood sample was drawn for a complete blood count, serum transaminase levels (SGOT, SGPT), and hepatitis B serological tests.

Detection of HB_sAg, Anti-HB_s, and e-Antigen

All serum and saliva samples were tested for HB_sAg by solid phase radioimmune assay (AUSRIA II, Abbott Laboratories, Inc., North Chicago, Illinois) without preliminary treatment. Positive reactions were confirmed by a 50% or more reduction of test serum reactivity after incubation with a human serum containing anti-HB_s. Serum antigen titers were determined by complement fixation. HB_sAg subtypes were detected by immunodiffusion using subtype-specific rabbit antisera. e-antigen was detected in serum by immunodiffusion using serum from a Thai blood donor as antibody.

Anti-HB_s was detected by a solid phase radioimmune assay (AUSAB, Abbott Laboratories, Inc.) interpreted according to the manufacturer's recommendations. Positive reactions were confirmed whenever possible by a 50% or more reduction in reactivity after incubating the test sera with human serum containing HB_sAg/adr. Antibody titers were determined by passive hemagglutination (Electronucleonics Laboratory Inc., Bethesda, Maryland) using erythrocytes coated with HB_sAg/ad.

Immune Electron Microscopy

Dane particles were sought in the saliva pool by immune electron microscopy. First, 6.0 ml of the saliva pool were centrifuged at 19,000 rpm (43,500 X g) in a Sorvall RC 2-B centrifuge with an SS-34 rotor for 3 hours at 4°C. As a control for the saliva preparation, human plasma containing HB_sAg/adr was diluted 1:400 in saliva collected from a donor without antigenemia and centrifuged in an identical manner. Second, 5.0 ml of each supernate were centrifuged in a Beckman L 350 ultracentrifuge using an SW 39 L rotor at 35,000 rpm (Approximately 100,000 X g) for 16 hours at 4°C. The top 4.85 ml of supernate was removed with a pipette. The yellow-brown sediment was easily resuspended in the remaining 0.15 ml of supernate to give a 33 X concentration of antigen.

As a third step, each saliva concentrate was divided in two and 0.07 ml mixed with either 0.1 ml of a 1:50 dilution of rabbit anti-HB_s/adr or an equal dilution of serum from the same rabbit obtained before it was immunized. The rabbit antiserum was absorbed to excess with normal human serum. In addition, separate preparations were made of a partially purified HB_sAg/adr in cesium chloride which had previously been shown to contain small particles, filaments and Dane particles. Each sample was incubated at 25°C for one hour, then at 4°C overnight.

The fourth step involved centrifugation of each sample at 17,000 rpm in the Sorvall RC 2-B for 90 minutes (4). The entire supernate was removed with a pipette and each sediment resuspended in three drops of distilled water. One drop of each sample was placed on a collodion-carbon coated copper 200 mesh grid had stained with 2% uranyl acetate before it was examined under a Hitachi HU-IIC electron microscope.

RESULTS :

HBV Infections in Exposed Gibbons

On 3 September 1975, 12 weeks after the first inoculation of the saliva pool, gibbon Pc-13 was positive for HB_sAg (Figure 1). This gibbon had detectable antigenemia for the next two weeks accompanied by a distinct rise in the SGPT values. Anti-HB_s was detected in all subsequent sera.

During the period of antigenemia, Pc-13 had a normal temperature, normal eating behavior, and no change in weight. Jaundice, hepatomegaly, lymphadenopathy, skin rash, and other abnormal physical findings were not found. As soon as antigenemia was detected, this gibbon and the other that was inoculated (Pc-14) were transferred to an empty room to reduce any possibility of inadvertent exposure of the remaining eight study animals.

Gibbon Pc-14 remained well until 10 weeks and 4 days when he escaped from his cage and was bitten severely on the hand by gibbon P-16. Following a surgical repair, during which one finger was amputated, the SGPT value rose to 61 units and the SGOT to 45 units (Figure 2). Both values were at normal levels one week later. At 22 weeks, the serum first contained anti-HB_s by radioimmune assay. The following week the PHA titer of anti-HB_s was 1:32. HB_sAg was not detected in any serum specimen. The incubation period is assumed to be 22 weeks, although it could have been as short as 11 weeks based on the SGPT. It is not possible to be certain whether or not the rise in transaminase was entirely due to the traumatic injury. Since gibbon P-16 showed no evidence of HBV infection, there is no reason to suspect infection of Pc-14 resulted from the bite. None of the gibbons exposed to the saliva by the oral or nasal routes developed HB_sAg or anti-HB_s at any time.

HBV Infections in Unexposed Gibbons

Blood samples collected from gibbons living in the colony between March 1973 and December 1975 permitted tests of 55 animals. While some animals left the colony for a variety of reasons during this period, others were born into it. Two gibbons were found to be chronic carriers of HB_sAg in March 1973; only one of these was still present during the experimental period. Throughout the 34 month observation period, none of the unexposed gibbons developed HB_sAg or anti-HB_s. Gibbon B-66 S, the antibody control, was consistently positive for anti-HB_s.

Immune Electron Microscopy

The partially purified HB_sAg/adr (EHO 17) was found to contain some clusters of particles even without the addition of rabbit anti-HB_s. In the presence of antiserum, particle clusters were more abundant. The mean dimensions of the principal morphological forms were: small spheres, 21.6 ± 3.6 nm diameter; filaments up to 177 nm long; and large spheres, 36.1 nm (Figure 3). Small particles similar to those in EHO 17 were seen in the normal saliva containing HB_sAg/adr serum only after rabbit antiserum was added.

The saliva pool contained no clusters of particles in the absence of antiserum. The addition of specific antibody, however, precipitated many clusters which included small particles with a mean diameter of 23.3 ± 2.7 nm and large spheres averaging 41.8 nm in diameter (Figure 3). Filamentous forms were not found in the saliva pool.

DISCUSSION : It is certain gibbons, Pc-13 and Pc-14 were infected with Hepatitis B virus following an exposure to the pool of human saliva. Because of the precautions taken to avoid other exposures to HBV, it is highly unlikely they were infected in any other way. During the study period, one other gibbon and four animal technicians were known to carry HB_sAg. All five antigen carriers were kept separated from the study gibbons. Throughout 34 months of observations, none of the other gibbons developed serologic evidence of infection, whether or not they were included in this experiment. The detection of large particles in the saliva pool, similar in size to Dane particles (5), adds additional weight to the probability that the saliva was the source of the infection.

Why didn't the eight gibbons exposed by the oral and nasal routes become infected? It is probable the concentration of infectious material was too low. It can be assumed that each animal swallowed

some of the saliva. Krugman (6) demonstrated that MS-2 serum was highly infectious by mouth. The MS-2 serum contained a concentration of HB_sAg detectable by immunodiffusion. The saliva pool used in this study had antigen detectable only by radioimmune assay. The relative insensitivity of immunodiffusion compared to radioimmune assay suggests the MS-2 serum contained a much higher titer of HB_sAg than the saliva and probably more infectious particles as well.

The incubation periods of 12 and 22 weeks observed for the gibbons were similar to the ranges reported for chimpanzees, 2 to 15 weeks; rhesus monkeys, 12 to 15 weeks; and human children, 4 to 15 weeks.

The presence of detectable HB_sAg in saliva correlated in general with high serum titers of antigen and the concentration of occult blood. Exceptions to this generalization were observed, however. Perhaps the presence of 3-antigen in the donor's blood is also important for selecting infectious saliva donors.

SUMMARY : A pool of whole mouth saliva collected from five human carriers of HB_sAg/adr, was found to contain antigen particles with mean diameters of 23.3 and 41.8 nm by immune electron microscopy. Two gibbons received subcutaneous injections of the pool and developed serological and, in at least one animal, biochemical evidence of Hepatitis B virus infection at 12 and 22 weeks, respectively. Although none of eight other gibbons that were exposed by the nasal or oral routes were infected, the experiment demonstrated that human saliva can serve as a vehicle for the transmission of hepatitis B virus.

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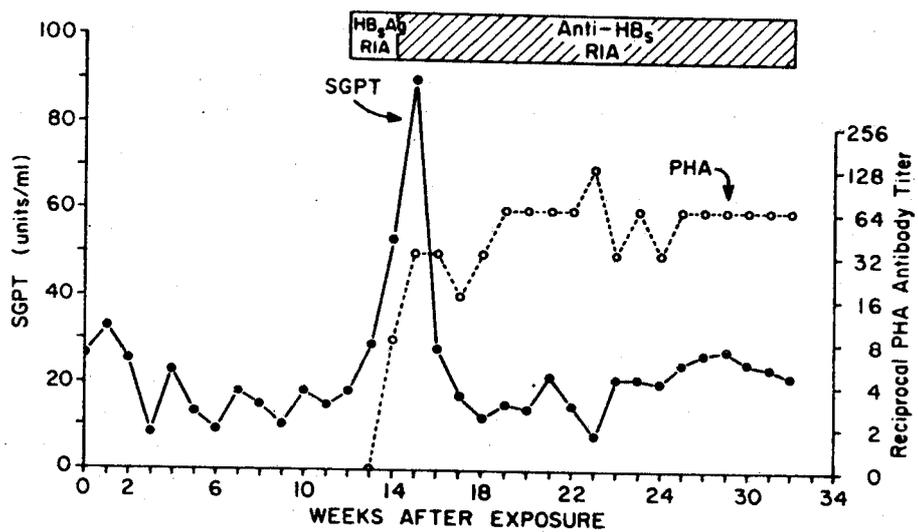


Figure 1. Response of gibbon Pc-13 to the subcutaneous injection of a pool of human saliva containing HB_sAg. Antigenemia (HB_sAg) was followed sequentially by hypertransaminasemia and the appearance of antibody detected by passive hemagglutination (PHA) and radioimmune assay (RIA)

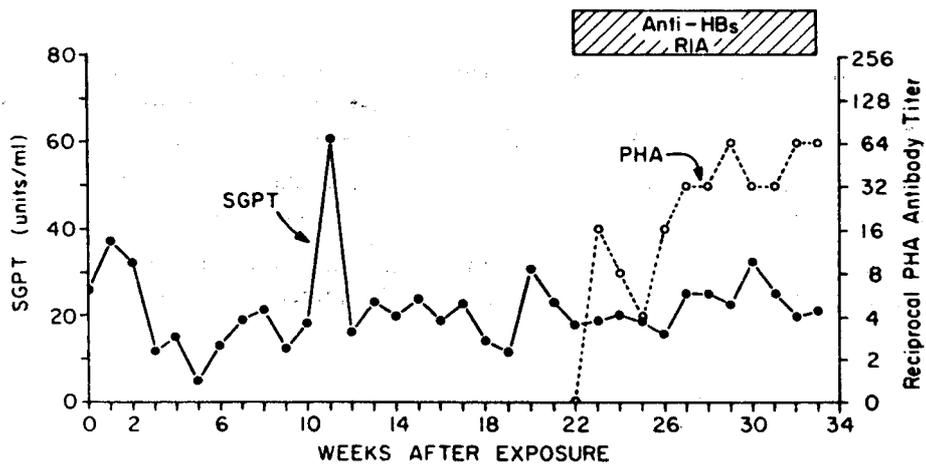


Figure 2 Response of gibbon Pc-14 to subcutaneous inoculation of a pool of human saliva containing HB_sAg at 0 weeks. Hypertransaminasemia at 11 weeks immediately followed a surgical procedure on one hand. Anti-HBs was first detected by radioimmune assay (RIA) at 22 weeks. HB_sAg was not detected

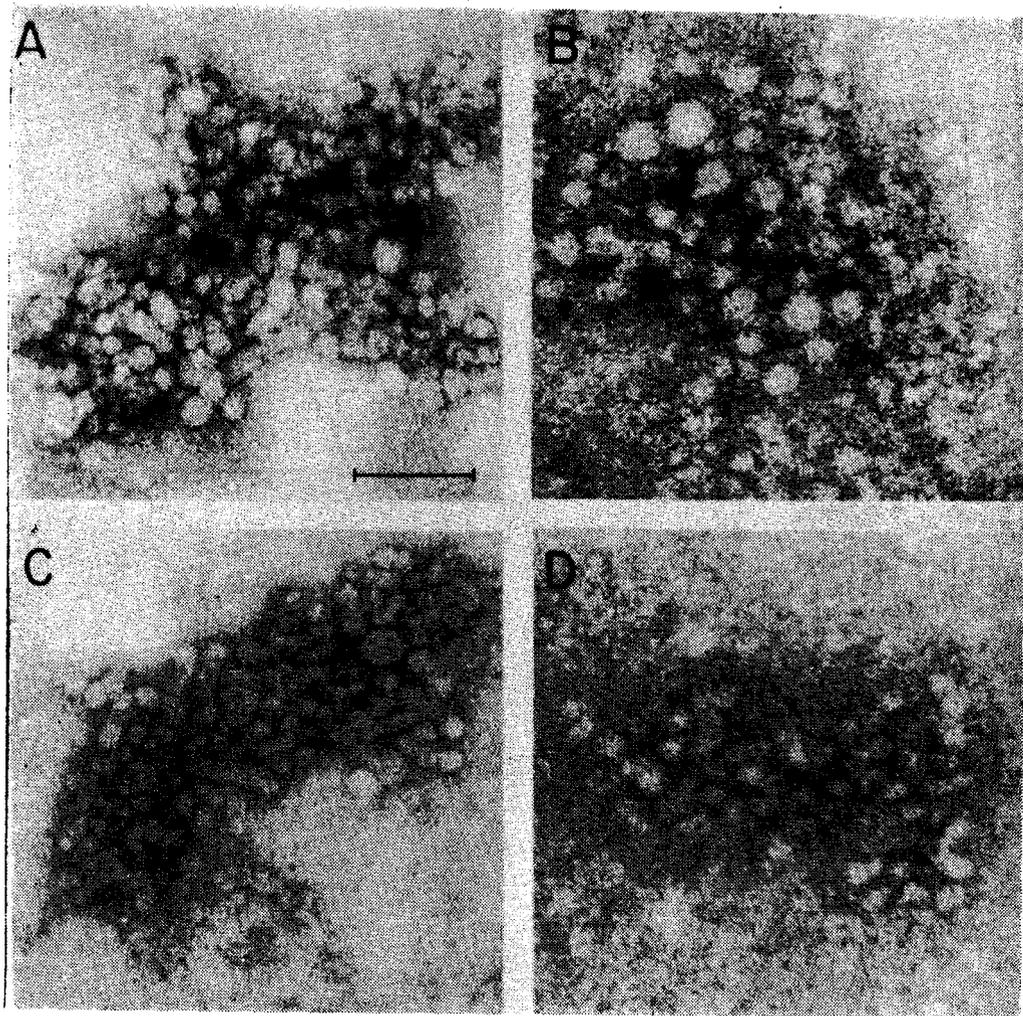


Figure 3 Electron micrographs of clumps of HB_sAg particles derived from human plasma and saliva. A; partially purified HB_sAg/adr (EH017) with normal rabbit serum C; EH017 with rabbit anti-HB_s. B, D; similar small and large particles from the human saliva pool precipitated with rabbit anti-HB_s. Filamentous forms were not found in the saliva. The bar represents 100 nm (X 192,000)

Table 1. Frequency of HB_sAg in Two Types of Saliva Preparations

Donor No.	Whole Mouth Saliva				Parotid Secretion			
	No. Spec.	Occult Blood +	HB _s Ag + (RIA)	Mean Vol. (ml)	No. Spec.	Occult Blood +	HB _s Ag + (RIA)	Mean Vol. (ml)
1	2	2	2	12.5	1	1	1	4.5
2	7	7	6	11.3	2	2	0	10.5
3	2	2	0	14.5	2	1	0	7.5
4	6	6	4	4.3	1	1	0	7.5
5	6	6	4	6.4	2	2	0	3.8
Total	23	23	16		8	7	1	

Table 2. Whole Mouth Saliva Samples from Carriers of HB_sAg/adr Used in the Saliva Pool

Donor	Serum		Saliva Samples			
	HB _s Ag* Titer	e Antigen	Occult Blood Reaction	No. Tested	HB _s Ag +	Volume added to saliva pool (ml)
1. Male 36 years	1:128	+	4+	2	2	62.6
2. Male 36 years	1:32	-	4+	2	2	75.0
			3+	2	2	
			2+	2	1	
			1+	1	1	
3. Female 35 years	1:28	-	4+	1	1	20.0
			3+	2	2	
			2+	3	2	
4. Male 33 years	1:11	N.T.**	4+	1	1	12.2
			Trace	1	1	
5. Female 28 years	1:11	+	4+	1	1	1.5
						171.3

* Geometric mean complement fixation titer

** Not tested

Table 3. Method of Exposure of Gibbons to Human Saliva Pool

Saliva Pool		Gibbon		
Route of Exposure*	Total Dose (ml)	No.	Age (yr)	Sex
Subcutaneous	5.1	Pc - 13	2	F
		Pc - 14	2	M
Nasal Aerosol	5.0	Pc - 20	2	M
		S - 81	11	F
Oral Aerosol	5.0	Pc - 21	1	F
		S - 83	8	M
Toothbrush	5.0	Pc - 16	1	F
		P - 16	9	M
Banana	5.0	P - 24	1	F
		B - 40	9	M

* The subcutaneous doses were given on Days 0, 2 and 4. All other methods of exposure were used on Days 0 to 4 inclusive.