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THE BLACKFLY *SIMULIUM BUISSONI* AND INFECTION BY HEPATITIS B VIRUS ON A HOLOENDEMIC ISLAND OF THE MARQUESAS ARCHIPELAGO IN FRENCH POLYNESIA

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Abstract. The hematophagous blackfly *Simulium buissoni* causes skin lesions on an island in the Marquesas archipelago that is holoendemic for hepatitis B virus (HBV). To test the hypothesis of the possible role of this fly in the transmission of hepatitis B, 506 children (age range 2–11 years) were examined for the presence of skin lesions, and attempts were made to detect HBV DNA in and on blackflies using two polymerase chain reaction methods. The mean number of skin lesions showed a positive correlation with the age of these children ($r = 0.12$, $P < 0.05$). Furthermore, it was significantly higher in the rural zone than in the urban zone (mean ± SD 41.02 ± 31.71 versus 17.73 ± 13.43; $P < 0.05$), and showed a correlation with a higher infection rate (73.9% versus 41.3%). Of the 45 pools of 10 insects tested, HBV DNA was not detectable on the inside of the insect, but was detectable on the flies (1–10 particles/insect in three positive pools). Infection by HBV conveyed by the flies is theoretically possible, but their indirect role via the numerous skin lesions caused on children is likely to explain such a high level of transmission.

Hepatitis B virus (HBV) infection represents a major worldwide public health problem because of the ability of this virus to cause a chronic infection in infected individuals, of whom a significant number develop liver disease. Several epidemiologic surveys throughout the Pacific have demonstrated high overall prevalences of HBV infection.[1–4] In French Polynesia, an exceptionally high HBV infection prevalence with a horizontal transmission pattern has been demonstrated in the populations of the Austral archipelago and the Marquesas archipelago.[5, 6] In the latter group of islands, the level and tendency for acquisition of infection were different, depending on the island considered; the infection rate among children was dramatically high on Nuku-Hiva Island (74% in the rural area) while it was moderate on Hiva-Oa Island (19% in rural area). These two islands are very similar in size (330 and 350 km^2, respectively), habitation type (one main population cluster and three or four small villages around the island), total population (2,099 inhabitants on Nuku-Hiva and 1,642 inhabitants on Hiva-Oa), and the populations are almost entirely Polynesian. The ratio of rural versus urban population is approximately 1:2 on both islands. Although the socioeconomic and demographic factors that usually influence the incidence of HBV infection appear identical on the two islands, they cannot explain the differences observed between these two populations.

On Nuku-Hiva Island, the hematophagous blackfly *Simulium buissoni* (discovered by Roubaud in 1906) is a notorious pest of humans. The adult females feed on blood, resulting in painful and allergic reactions in the host. Although this species is not known to transmit disease in humans, its abundance causes considerable skin lesions among children. The swarming of this species, especially in the small, remote villages, poses the question of the association between the presence of this insect and the high seroprevalence of HBV on this island. The hypothesis of the possible role of *S. buissoni* in the transmission of HBV, either directly as a conveyor of the virus or indirectly through the numerous itching lesions produced on the skin, has been proposed because this fly is not present on Hiva-Oa Island. To test this hypothesis, we determined the prevalence of skin lesions and scars in children living on Nuku-Hiva Island

TABLE 1

*Seroprevalence of hepatitis B virus infection on Nuku Hiva Island, according to age and habitation zone**

	Zone	Mothers	Children				
			0–6 months	7–23 months	2–4 years	5–10 years	Total
Seroprevalence of HBsAg, no. positive/total (%)	Urban	14/57 (24.5)	0/8 (0)	0/22 (0)	2/14 (14.2)	5/19 (26.3)	7/63 (11.1)
	Rural	30/55 (54.5)	0/8 (0)	6/23 (26)	5/20 (25)	4/18 (22.2)	15/69 (21.7)
Infection rate, % (anti-HBc), no. positive/total (%)	Urban	44/53 (83)	6/8 (75)†	6/23 (26)	2/13 (15.4)	12/19 (63.2)	26/63 (41.3)
	Rural	53/55 (96.3)	4/7 (57.1)†	13/25 (52)	15/18 (83.3)	19/19 (100)	51/69 (73.9)

* HBsAg = hepatitis B surface antigen; anti-HBc = anti-hepatitis B core antigen.
† Probably maternal antibodies.

and used the polymerase chain reaction (PCR) to detect HBV DNA in and on female blackflies collected in Taipivai, a village that is holoendemic for this virus.

SUBJECTS AND METHODS

Study population

The total population of Nuku-Hiva Island was 2,099 in 1989. It was composed of an urban center (Taiohae) with 1,420 inhabitants and three small rural villages (Taipivai, Akapa, and Hatiheu) with 679 inhabitants. The 132 children included in the HBV seroprevalence study were randomly selected from the entire population of 602 children.

Epidemiology of HBV infection on Nuku-Hiva Island

Seroepidemiologic data regarding HBV infection in 1989 for the 132 children (< 10 years old) and their 112 respective mothers (age range 13–63 years) is shown in Table 1. The infection rate of hepatitis B core antigen was significantly higher in the rural area than in the urban area among both mothers ($\chi^2 = 5.25$, $P < 0.05$) and children ($\chi^2 = 14.4$, $P < 0.001$). The same observation was made with regard to the seroprevalence rate of hepatitis B surface antigen (HBSAg), but the difference was significant only for the mothers ($\chi^2 = 10.5$, $P < 0.01$). The HBsAg rate among children was significantly and surprisingly low (22.2%; $\chi^2 = 5.69$, $P < 0.02$) compared with that of the mothers (54.4%), when one considers that almost all the children are infected by age 10. There is no obvious reason that may explain this discrepancy, except to assume that the situation of the mothers was even worse than that of the the children, since the earlier the infection occurs, the higher the risk of becoming a chronic HBsAg carrier. However, the health and hygiene conditions have improved dramatically in the Marquesas islands during the past 20 years. The current preeminence of the horizontal transmission pattern supports this result.

The theoretical vertical risk of transmission is the expected proportion of HBsAg-positive children at age one. It is determined using the following formula: (% HBsAg positive × % hepatitis B e antigen [HBe] positive among mothers) × 0.90.[7] This coefficient of 0.90 is the generally admitted risk for a one-year-old child of being a carrier when the mother is a double carrier (HBsAg positive and HBe positive). This calculated vertical risk was 1.7% in the urban area and 6.3% in the rural area. They were lower than expected with regard to the HBsAg seroprevalence because of the relatively low HBe seroprevalence among these mothers (7.7% in the urban area and 12.9% in the rural area). The observed vertical risks in these areas are 0% and 11.1%, respectively, which are not significantly different from the theoretical values.

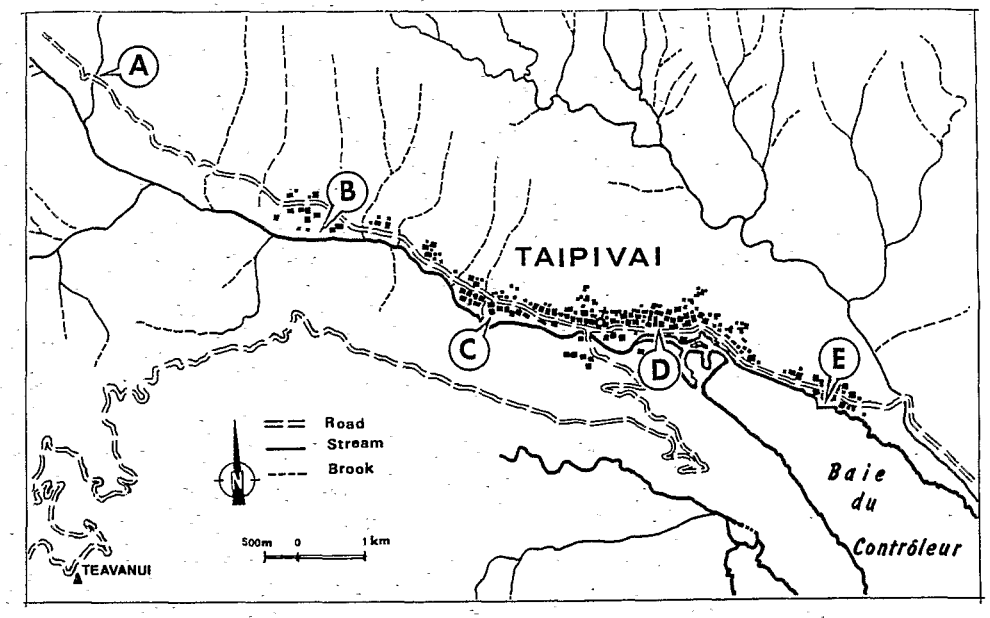

FIGURE 1. Map of Taipivai village on Nuku-Hiva Island in the Marquesas archipelago. The locations of *Simulium buissoni* sampling sites (A, B, C, D, and E) are shown.

Examination of children for scars and lesions

A total of 506 (98%) of 516 children (263 boys and 243 girls, age range 2–11 years) were examined. Three hundred forty-eight were from the urban center and 158 were from the rural area of the island. There was no difference in the age distribution of urban and rural children. For each child, the total number of scars and lesions on the arms and legs were scored. Statistical analysis was done using the PCSM statistical package (Programme Conversationnel de Statistiques pour les Sciences et le Marketing, Delta Soft, Meylan, France).

Collection of S. buissoni *blackflies on Nuku-Hiva Island*

Blood-seeking female blackflies were collected during the day by human bait catches. The catchers were free of HBV infection as determined by serologic and PCR tests. The flies were sampled in the village of Taipivai (380 inhabitants), where the highest infection rate has been observed.[6] The village was divided into five sampling sites (A, B, C, D, and E) (Figure 1). Negative control blackflies were collected in an uninhabited area (Terre Deserte). After collection, the insects were frozen in liquid nitrogen, sent by air to Tahiti, and stored at −80°C until tested.

Preparation of DNA samples from S. buissoni

For each sampling site, nine pools of 10 insects each were extracted (a total of 45 pools for the five sampling sites). Each pool of insects was soaked for 30 min in a microtube containing 200 μl of TE buffer (10 mM Tris HCl, 1 mM EDTA,

pH 8) plus 1% NP40. This supernatant should contain the HBV particles washed from the external surface of the blackflies (legs, wings, and mandibles). Subsequently, the insects were removed, blotted on filter paper, and transferred to a new microtube where they were homogenized in 200 µl of TE buffer plus 1% NP40 with a glass rod. This homogenate should contain the HBV particles extracted from internal regions of the flies (intestinal contents, ovaries, and salivary glands). The DNA in the supernatants and homogenates was purified by incubating them for 1 hr at 70°C in 200 µl of lysing solution (10 mM Tris HCl, 10 mM EDTA, 10 mM NaCl, pH 8, 5% sodium dodecyl sulfate, 50 µg/ml of proteinase K), and extracting twice with phenol and twice with chloroform/isoamyl alcohol. The homogenates were dialyzed overnight at 4°C against 0.1× TE buffer (membrane type VS; Millipore, Bedford, MA). The DNA was precipitated with ethanol, and the pellet was resuspended in 100 µl of water. Ten microliters of each extract was used as template DNA to detect HBV using each of the two PCR systems.

Polymerase chain reactions for HBV

Two PCR assays (S gene and X gene amplifications) were used to detect HBV DNA. The sequences of the primers were selected for the conserved regions of the viral genome determined from subtypes adw, adr, and ayw (C. Brechot, Institut Pasteur, Paris, France). The MD03/MD06 primers directed the amplification of a 128-basepair (bp) DNA fragment in the S gene that contained 112 specific nucleotides. The MD24/MD26 primers directed the amplification of a 243-bp DNA fragment in the X gene that contained 234 specific nucleotides.[8]

Amplification of HBV DNA was performed in a 50-µl reaction mixture containing 10 ul of the DNA template and 40 ul of a mixture containing two units of *Taq* polymerase (Perkin-Elmer-Cetus, Norwalk, CT), 1× PCR buffer (10 mM Tris HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin), 0.2 mM of each primer, and 100 mM of each dNTP.[9] The reaction mixture was overlayed with 50 µl of mineral oil. The DNA was denatured at 94°C for 5 min, and 35 amplification cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min were performed, followed by an final extension of at 72°C for 5 min in an automatic thermo-cycler (Hybaid; Ceralabo, Auber Villiers, France). After amplification, 10 µl of the PCR product was subjected to electrophoresis on a horizontal 1.5% agarose gel in 1× Tris-borate-EDTA buffer (100 mM Tris-boric acid, 2 mM EDTA). The gel was stained with ethidium bromide and DNA bands were visualized with an ultraviolet transilluminator.

Southern blot analysis with digoxigenin-labeled DNA probes

For the Southern blots, the DNAs were transferred to nylon membranes (Hybond N; Amersham, Arlington Heights, IL) using 20× SSC (0.3 M sodium citrate, 3 M NaCl) and irradiated with ultraviolet light. The DNA probes used (MDO9 and MD29) are specific to the S and X amplified fractions and they do not overlap in sequence with the primers. These probes were 3′-end labeled with digoxigenin-dUTP (no. 1362372; Boehringer, Mannheim, Germany) according to the manufacturer's instructions.[10] The filters were prehybridized, then hybridized with the labeled probes under stringent conditions for 1 hr at 65°C. Digoxigenin-labeled DNA was detected immunologically by an antibody-enzyme conjugate anti-digoxigenin-alkaline phosphatase (no. 1363514; Boehringer), and a positive reaction was documented on x-ray film by the dephosphorylation of a chemiluminescent substrate ((3-(2′-spiroadamantine)-4-methoxy-4-3″-phosphoryloxy)-phenyl-1,2-dioxetane [AMPPD]).

Detection limits of the PCR assays

To test the sensitivity of the two PCR methods, negative control blackfly homogenates and supernatants were spiked with a range of 10-fold concentrations of serially diluted HBV DNA standard (Abbott Laboratories, North Chicago, IL). The amount of HBV particles ranged from 3×10^6/ml to 30/ml.

Controls in the PCR assays

To prevent cross-contamination, which would generate false-positive results, the technical precautions described by Gerken and others were used to ascertain the validity of our results.[10] For each DNA extraction series, a negative control (TE buffer) was included. For each PCR experiment, five negative controls consisting of no DNA plus reagents were run. To check the specificity of the reactions, five pools of negative controls blackflies were tested.

TABLE 2

Number of scars and lesions in 2–11-year-old children on Nuku Hiva Island

Zone	No. of children	Mean ± SD no. of. lesions (range)
Urban		
Taiohae	348	17.73 ± 13.83* (1–91)
Rural		
Taipivai	76	46.12 ± 38.53 (2–200)
Hakapa	36	41.31 ± 22.72 (8–101)
Hatiheu	46	32.37 ± 22.62 (8–94)
Total	156	41.02 ± 31.71* (2–200)
Total	506	25.00 ± 23.68 (1–200)

* $P < 0.05$ versus total urban and total rural children.

RESULTS

Scars and lesions among children

The results, according to the habitation zone, are shown Table 2. The overall mean ± SD number of lesions was significantly higher ($P < 10^{-4}$, by Student's *t*-test) in the rural area (41.02 ± 31.71) than in the urban zone (17.73 ± 13.83). This was found to be positively correlated with age (all children analyzed); Pearson's coefficient $r = 0.12$ with the regression line $Y = 16.2897 + 1.2595\ X$, $P < 0.05$. There was no significant difference in the number of lesions between both sexes (26.63 ± 25.26 for boys and 23.24 ± 23.68 for girls).

Detection of HBV by PCR

Specificity. All supernatants and homogenates from the five pools of negative control flies caught in Terre Deserte, an uninhabited area of Nuku Hiva Island, were negative for HBV DNA in either of the two PCR systems.

Detection limits. In the supernatants, the detection limit was 10–30 HBV particles ($3–9 \times 10^{-5}$ pg of DNA) in one PCR reaction using the S gene PCR, while it was 1–3 HBV particles ($3–9 \times 10^{-6}$ pg of DNA) in one PCR reaction using the X gene PCR. In the homogenates, the detection limit was 100–300 HBV particles ($3–9 \times 10^{-4}$ pg of DNA) in one PCR reaction using the S gene PCR, while it was 10–30 HBV particles ($3–9 \times 10^{-5}$ pg of DNA) in one PCR reaction using the X gene PCR. The X gene PCR was 10-fold more sensitive than the S gene PCR, and both PCR systems were 10-fold more sensitive for the supernatant than for the homogenate.

TABLE 3

Results of the two polymerase chain reactions (PCR) for hepatitis B virus on supernatants of Simulium buissoni *blackflies**

	Samping sites					Negative control†
	A	B	C	D	E	
X gene PCR	0/9	1‡/9	1/9	1/9	0/9	0/5
S gene PCR	0/9	1‡/9	0/9	0/9	0/9	0/5

* Values are the no. of positive pools/total no. tested.
† Blackflies from an uninhabited area.
‡ Same pool positive using both PCR assays.

Detection of HBV DNA in *S. buissoni* blackflies from Taipivai village. All 45 homogenates tested were negative for HBV DNA using either the X gene or S gene PCR. Consequently, based on the above detection limits in the homogenate and the amount of template DNA used in one PCR reaction, the number of viral particles extracted from one fly may be assumed to less than 10 HBV particles.

The PCR results for the presence of HBV DNA in the supernatants are shown in Table 3. Of the 45 supernatants tested, only three were positive for HBV DNA. The HBV-positive insects were found at sampling sites B, C, and D (one of nine pools was HBV positive at each site) using the X gene PCR, while only site B was HBV positive (one of nine pools was positive) using the S gene PCR. Sites A and E were negative using both PCR assays.

The results image obtained by Southern blotting (Figures 2 and 3) showed that small amounts of HBV DNA were contained in the HBV-positive supernatants, except at sampling site B, which was positive using both PCR assays. Although the PCR is not a very accurate quantitative method, one may estimate the number of HBV particles using the calibrated positive controls. The number of HBV particles was approximatively one particle for the supernatants from sites C and D (i.e., one particle per insect), and 10 particles for the supernatant from site B (i.e., 10 particles per insect). The same results were obtained when the tests were repeated on another aliquot of DNA extracts. A higher molecular weight band (approximately 450 bp) was seen in the supernatants from sites A, B, and C when the S gene was amplified (Figure 2).

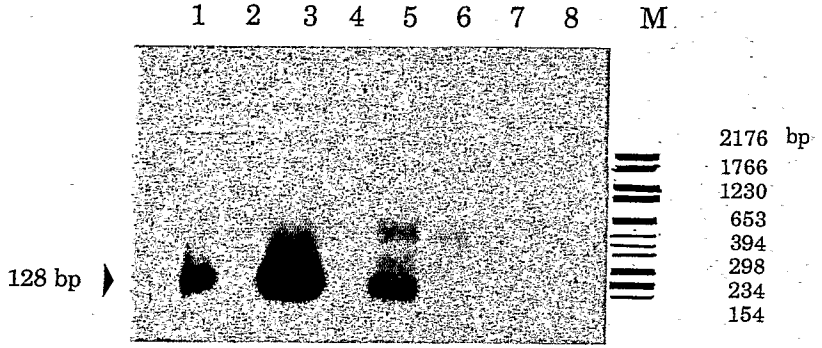

FIGURE 2. Southern blot of amplified S gene products of hepatitis B virus (HBV) (128 basepairs [bp], primers MD03/MD06) with specific digoxigenin-labeled DNA probe MD09. Lane 1, positive control containing three HBV particles; lane 2, no DNA (control); lane 3, positive control containing 30 HBV particles; lanes 4–8, supernatants from sampling sites A, B, C, D, and E, respectively. M = DNA molecular weight markers.

DISCUSSION

The first important result of this study was the overall high mean ± SD number of lesions found among children living on Nuku-Hiva Island (25.0 ± 23.68). As many as 200 lesions were counted in one child in the rural part of the island, where the mean number of lesions was significantly higher than in the urban area ($P < 0.05$). This result is consistent with the observation that the blackfly is more abundant in the three rural villages than in the Taiohae urban zone. Regardless of the zone, the number of lesions was positively correlated with age, but no difference was found between the sexes. The fact that the hematophagous blackflies bite males and females equally and that the number of lesions and scars accumulate as the children grow up are likely to explain this finding. The higher mean number of lesions in the rural zone compared with the urban zone (41 versus 17.7) is consistent with the higher HBsAg prevalence (21.7% versus 11.1%) and the higher HBV infection rate (73.9% versus 41.3%) among children < 10 years old in the urban zone.

Taipivai is a rural village where blackflies swarm and where the highest HBV infection rate was observed (100% of 5–9-year-old children).[6] An interesting finding of our study was the presence of HBV DNA in three of 45 supernatants (representing the outside of flies) but in none of 45 extracts (representing the internal organs and hemolymph of flies). Hepatitis B virus DNA was

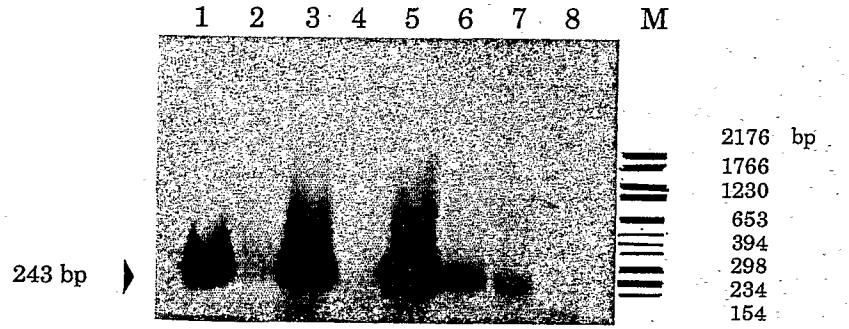

FIGURE 3. Southern blot of amplified X gene products of hepatitis B virus (HBV) (243 basepairs [bp], primers MD24/MD26) with specific digoxigenin-labeled DNA probe MD29. Lane 1, positive control containing three HBV particles; lane 2, no DNA (control); lane 3, positive control containing 30 HBV particles; lanes 4–8, supernatants from sampling sites A, B, C, D, and E, respectively. M = DNA molecular weight markers.

detectable in the supernatants of one of nine pools of insects caught at each of three sites (B, C, and D) in Taipivai village. The number of viral particles was very low and estimated to be one particle per insect at sampling sites C and D and 10 particles per insect at sampling site B.

Although the PCR is a very sensitive test, false-positive results due to contamination are a possibility. However, this was minimized by the negative results of the no DNA controls in each series of DNA extractions and the PCR experiments, and by the use of negative control blackflies caught in an uninhabited area. Furthermore, the high specificity of the results was ensured by performing the amplification in two regions of the HBV genome (S gene and X gene) using very specific primers with a high GC content and highly stringent hybridization conditions.[8] Despite these modifications, we observed on Southern blots of the S gene a nonspecific, higher molecular weight band in several supernatants. We have no explanation for the presence of this band.

Under these conditions, the detection limits of the two PCR assays for the supernatants were similar to those recently published by others.[10–12] The X gene PCR, which is approximately 10 times more sensitive than the S gene PCR, was able to detect 1–3 HBV particles ($1–3 \times 10^2$ HBV/ml of supernatant). In the case of homogenates, both PCR assays were 10 times less sensitive when compared with the supernatants. The presence of nontarget insect DNA or insect inhibitors of the DNA polymerase may explain this finding, as reported previously.[13, 14] Based on this detection limit, one may assume that there were less than 10 HBV particles per insect. The number of viral particles absorbed during the blood meal from highly viremic patients (10^7 HBV/ml) would be 10^4 particles, assuming a blood meal volume of 1 μl, which is much greater than the PCR detection limit. Since the feeding periodicity is approximately six days, the virions are probably degraded by insect enzymes between two blood meals.

Experimental studies on chimpanzees have demonstrated that the infectious dose of HBV by injection was as low as 100 particles.[15] Thus, HBV infection due to viral particles conveyed by the flies is theoretically possible because 1–10 particles per insect could be detected and the biting index measured in the village of Taipivai is 95/hr/person (unpublished data). In practical terms, however, this hypothesis is unlikely because 1) only three of 45 pools of insects tested were HBV positive, 2) the probability that a new bite occurs on a lesion rather than on intact skin is low, and 3) the viral particles detected are not necessarily infective because the PCR may also detect defective HBV genomes or virions already neutralized or degraded between two blood meals.

In conclusion, although small amounts of HBV DNA were found on blackflies, our results do not support the hypothesis of direct transmission of HBV by the blackfly *S. buissoni* as an explanation for the the high infection rate on Nuku-Hiva Island. On the other hand, the indirect role of this fly seems beyond doubt because of the scratching of lesions it causes on children. Contamination by close contact between children and/or by means of contaminated hands and fingernails is a more likely explanation for this situation. An HBV vaccination program that includes all newborn children, an important goal in the control of HBV infection, was implemented in 1992. Concurrently, to promote tourism and agricultural development, an *S. buissoni* eradication program on Nuku-Hiva Island will be implemented.

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