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Stercorarial Shedding and Transtadial Transmission of Hepatitis B Virus by Common Bed Bugs (Hemiptera: Cimicidae)

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ABSTRACT Transtadial persistence and stercorarial shedding of hepatitis B virus (HBV) in common bed bugs, *Cimex lectularius* L., was studied by using experimental infectious blood feedings, infectious intrathoracic inoculations, and virus detection by polymerase chain reaction and Southern hybridization. Results showed that HBV persisted after an infectious blood meal in bed bug bodies for up to 35 d after the infectious blood meal. It was passed transtadially through one molt regardless of instar, was shed in fecal droplets for up to 35 d after the infectious blood meal, but was not passed transovarially. In bugs inoculated intrathoracically, HBV was detected for 21 d postinoculation. Previous studies detected the hepatitis B surface antigen found on both infectious and noninfectious particles in bed bugs. In this study, the presence of nucleic acids amplified from a conserved core region of the viral genome in bodies and feces of *C. lectularius* suggests that the HBV virus may be mechanically transmitted in feces or when bugs are crushed, during feeding.

KEY WORDS bed bugs, *Cimex lectularius,* hepatitis B virus, polymerase chain reaction

HEPATITIS B VIRUS (HBV) infection is an important cause of acute and chronic hepatitis, cirrhosis, and primary hepatocellular carcinoma (PHC) worldwide (Hamilton 1994). A high incidence of HBV infections has been noted in underdeveloped countries as compared with developing countries (Shafritz 1990). Although the primary modes of transmission are thought to be parenteral, perinatal, and sexual exposure, up to 40% of people infected with HBV are not associated with these risk factors (Alter and Mat 1994). Detection of hepatitis B virus surface antigen (HBsAg) in unengorged nymphal and adult bed bugs from huts in Africa (Wills et al. 1977, Jupp et al. 1978, Ogston et al. 1979) provided evidence that bed bugs acquire virus from humans, and might be capable of transmitting HBV either mechanically or biologically. Laboratory experiments showed that HBsAg could be detected in bed-bug bodies for 6–7 wk (Jupp and McElligott 1979) and in the feces for 5–6 wk (Taylor and Morrison 1980, Jupp et al. 1983) after infected feeding. Transtadial transmission of HBsAg occurred for one, but not two, molts in bed bugs, and HBsAg was detected for 52 d after a single molt (Jupp and McElligott 1979). Neither Jupp et al. (1979) nor Taylor and Morrison (1980) found evidence of transovarial viral transmission, using HBsAg as the biological marker. Jupp et al. (1980, 1983) demonstrated through experimental passage that HBV did not replicate in bed bugs. Due to the host specificity of the virus, few transmission studies have been conducted. Jupp et al. (1991) found no evidence of oral or biological transmission by *C. lectularius* to chimpanzees; however, mechanical transmission by interrupted feeding or fecal contamination was not examined.

Despite the body of literature on the subject, there are still several gaps in our knowledge of the interactions between HBV and hematophagous hemiptera. Only one study (Silverman et al. 2001) has attempted to measure the persistence, fate, and shedding of HBV in bed bugs by molecular tools (polymerase chain reaction and Southern detection) to detect HBV nucleic acids, instead of the HBsAg. All previous studies used immunological assays to detect HBsAg, a protein on the surface of all three particles produced by the HBV. Only one of these three particles contains viral DNA and is infectious (Blumberg et al. 1965). The concentration of noninfectious particles associated HBsAg exceeds the concentration of infectious particles by 10^4 or greater. Consequently, studies that rely entirely on detection of HBsAg cannot indicate if infectious particles are present, nor the concentration of infectious particles. Detecting HBV nucleic acid would provide evidence that infectious particles could be present. Additionally, there have been no studies that examined the fate of HBV when the gut is circumvented by intrathoracic inoculation in insects.

Studies with laboratory-reared *Cimex lectularius* infected by feeding on HBV-positive patients were conducted under a human use protocol approved by the University Committee on Research Involving Human or Animal Use (IRB# 96–039).

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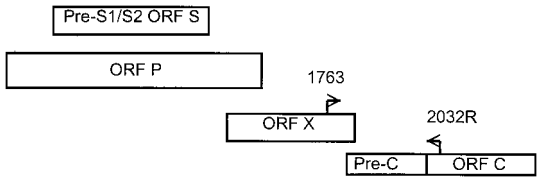

Fig. 1. Diagram of primer location in HBV genome, showing the conserved region of the genome encoding the core structural protein, producing a 270-bp size fragment with PCR. Four overlapping open reading frames (ORF) are present. The ORF C region encodes a core, structural protein of the nucleocapsid and the ORF S/pre-S encodes viral surface glycoproteins. Primers (see text) amplified a conserved region encoding the core structural protein, producing a 270-bp size fragment. ORF, open reading frame. C, core. Pre-C, precore. S, surface.

Our objective was to redress these issues by studying HBV and bed bugs.

Materials and Methods

Hepatitis B Virus-Infected Serum Collection. Sera were obtained from HBV-positive patients and from HBV-negative volunteers as controls and frozen (-70°C) until used. The Abbott HBV DNA assay (Abbott Laboratories, Abbott Park, IL) was used to quantify hepatitis B viral levels (picogram of HBV DNA per milliliter of serum).

Experimental Feedings and Manipulations. Approximately 100 *C. lectularius* individuals, drawn from a laboratory colony originally collected from Fort Dix, NJ, in 1973 (Bartley and Harlan 1974), were collected and placed into each of two clean jars with fresh filter paper as a substrate 1 wk before the experimental feeding. The filter paper was replaced on the morning of that feeding. One jar of bugs was fed on the forearm of an HBV-positive volunteer (viral titer, 1,500 pg/ml) (exposed cohort) and the other jar on an HBV-negative volunteer (unexposed cohort). Engorged bugs were sorted into exposed and unexposed adult and immature cohorts, respectively. Each cohort was placed into a clean jar with fresh filter paper. Exposed cohorts were held in a glove box at 25°C and unexposed cohorts were held in the insectary at 25°C until collection described later. All bugs were held in their respective cohort jars throughout the experiment until collected at one of the designated intervals. Unexposed cohorts' samples were always handled before the HBV-exposed samples during manipulations and collections to avoid contamination.

To determine whether HBV remained in individual bed bugs transtadially, engorged immature *C. lectularius* were held for 14 d at 25°C to allow for molting. After a molt, they were sorted individually into 1.5-ml labeled eppendorf tubes and frozen (−70°C). To determine how long HBV was detectable in adult bed bugs, whether it was passed transovarially, and whether it was shed stercorarially, the following manipulations were performed. Usually, five adult *C. lectularius* were collected at designated intervals after-feeding (on days 1, 3, 7, 14, 21, 28, or 35), their legs were removed with a sterile scalpel and placed in a 1.5-ml eppendorf tube, and the remainder of their bodies were placed in another 1.5-ml eppendorf tube. Then, the filter paper substrate in the jar was examined for eggs and a portion of filter paper to which eggs had been attached was placed in a clean jar to hatch. Newly hatched nymphs were placed in one 1.5-ml eppendorf tube. The remaining filter paper was placed in a labeled petri dish and frozen (−70°C) for later analysis of fecal spots. Each time a sample was taken, fresh filter paper was placed in each cohort jar. All samples were then frozen (−70°C) until nucleic acids were extracted for PCR and Southern hybridization detection of HBV (see below).

Some *C. lectularius* were inoculated intrathoracically with 0.5 μl of an HBV-positive serum suspension (equivalent to ≈45 viral particles), or a control suspension, according to methods described elsewhere (Rosen and Gubler 1974). The HBV-positive suspension consisted of 1:5 mixture of HBV serum (>2000 pg/ml HBV-DNA) and E-199 cell culture broth mixture. The control suspension consisted of 1:5 mixture of HBV-negative serum and E-199. Infected cohorts were kept in a sealed glove box at 25°C with wet cotton pads for humidity.

Sensitivity of PCR and Southern Hybridization. The sensitivity of PCR and Southern blot to detect HBV nucleotide target sequence was assessed by analysis of 10-fold serial dilutions of HBV serum used in the experimental feedings (1,500 pg HBV DNA per ml). One picogram of HBV DNA is equal to $\approx 2.8 \times 10^5$ genomic equivalents (Hollinger 1996). The serum was diluted beyond the theoretical end point of one virus particle. A second set of serial dilutions was made and a whole *C. lectularius* was added to each tube to determine the level of detection in the bugs. A third set of serial dilutions was prepared and dispensed onto a piece of filter paper to determine the level of detection from filter paper. Nucleic acids were then extracted from these samples as described below.

Nucleic Acid Extraction, and PCR and Southern Hybridization Procedures. Bed bug legs or bodies were ground with tight-fitting pestels in 1.5-ml eppendorf tubes in the presence of DNAzol and Polyacryl Carrier (Molecular Research Center, Cincinnati, OH). The optimal volume and ratio of DNAzol and Polyacryl Carrier for different sample constituents was determined in preliminary studies (Blow 1998). Consequently, bed bug bodies and legs were ground with 250 μl DNAzol and 5 μl of Polyacryl Carrier. Fecal spots (4–5 < 1 mm diameter) from filter papers were cut out, placed in an 1.5 ml eppendorf tube, and nucleic acids extracted with 750 μl of DNAzol and 15 μl of Polyacryl Carrier. Filter paper extractions were performed overnight at 4°C. Genomic DNA was precipitated from the lysate with cold ethanol, washed with ethanol and dried, reconstituted in 10 μl of water, concentrated by centrifugation, and frozen (−20°C) until subjected to PCR.

Oligonucleotide primers were used to amplify a 270 bp fragment from the conserved precore and core

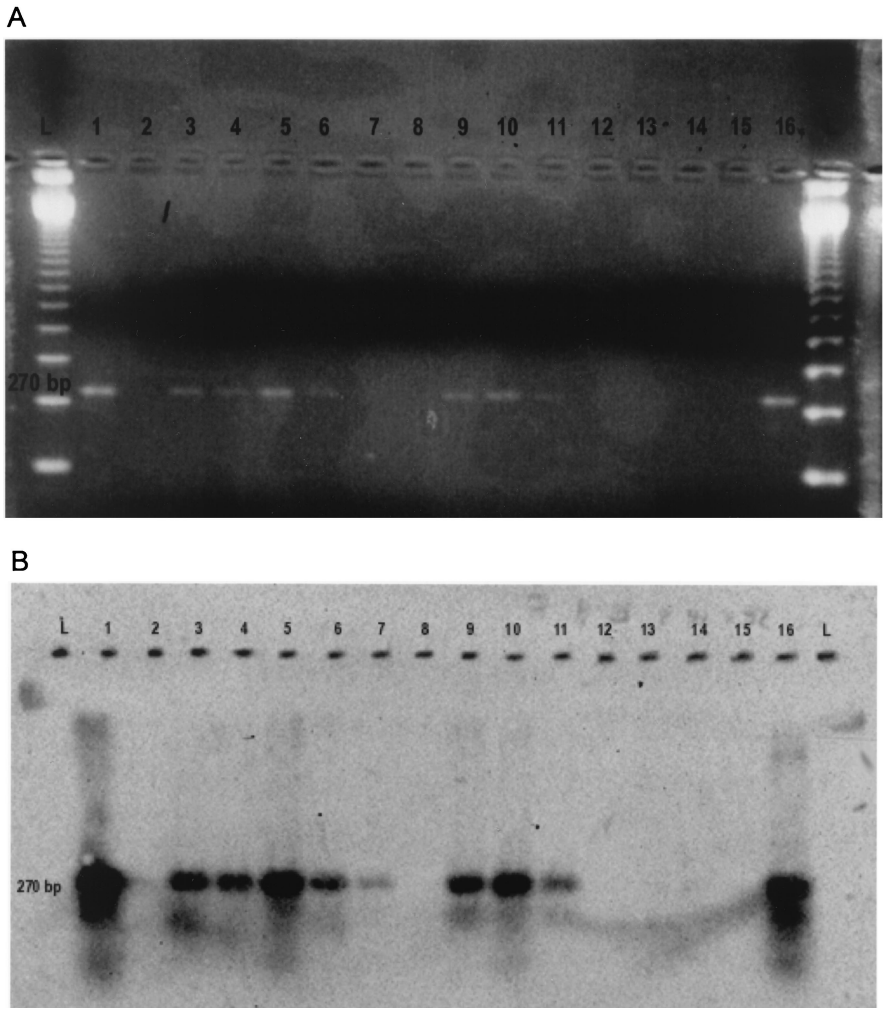

Fig. 2. Determination of the effect of bed-bug tissue on the sensitivity of HBV detection. (A) Agarose gel of PCR products; bands at the 270-bp position indicate positive samples. (B) Southern hybridization. Lanes: 1-positive control; 2-negative control, 3-straight serum (400 virions), 5–1:100 (40 virions), 6–1:1,000 (4 virions), 7–1:10,000 (0.4 virions), 8–1:100,000 (0.04 virions), 9-serum plus bug (400 virions), 10-1:10 serum plus bug (400 virions), 11-1:100 serum plus bug (40 virions), 12-1:1,000 serum plus bug (4 virons), 13-1:10,000 serum plus bug (0.4 virons), 14 1–100,000 serum plus bug (0.04 virions), 15- negative control, 16 positive control, L-123 bp lambda ladder.

region of the HBV DNA genome, corresponding to mapped nucleotides 1891–1920 (Lauder et al. 1993) (Fig. 1). The sense primer, beginning at nucleotide map position 1763, was 5′-GCTTTGGGGCATGGACATTGACCCGTATAA-3′ and the antisense primer, beginning at map position 2032, was 5′-CTGACTACTAATTCCCTGGATGCTGGGTCT-3′ (Fujiyama et al. 1983). A 2.5-μl aliquot of sample DNA was subjected to PCR (Perkin Elmer GeneAmp PCR, Wellesley, MA) in a 50-μl reaction volume containing 2.5 U of *Taq* DNA polymerase (Perkin Elmer), 200 μmol/liter each of four bases (100 mM pH7, dNTPs Li-salt; Boehringer Mannheim, Mannheim, Germany), 1 μmol/liter of the primer pair, 50 mmol/liter Tris-HCl (pH 8.3) (Perkin Elmer 10 × PCR Buffer II), 1.5 mmol/liter $MgCl_2$, and 0.01% (wt:vol) gelatin (25 mM $MgCl_2$, Perkin Elmer) (Kaneko et al. 1989). Reaction conditions were as follows: 1 min at 94°C, followed by 30 cycles of 94°C for 1.5 min, 42°C for 1.5 min, 72°C for 3 min, and then a final extension reaction for 7 min at 72°C. Nested PCR was not done due to a high rate of false positives in preliminary studies (Blow 1998).

PCR products were visualized as follows. Aliquots (8 μl) from the PCR reactions were separated by electrophoresis on 2% agarose gels containing 0.5 μg/ml ethidium bromide and 1.0 × Tris-borate (TBE). After electrophoresis, gels were exposed to UV illumination to visualize band location and separate PCR products. The PCR products were transferred from gels to nylon membranes for Southern hybridization. An oligonucleotide internal to the PCR oligonucleotides was used to probe PCR products. The sequence was 5′-TGTTCACCTCA CCATACAGC-3′, and began at map position 1913 of the HBV genome. This probe was labeled at the 3′ end with digoxigenin-ddUTP,

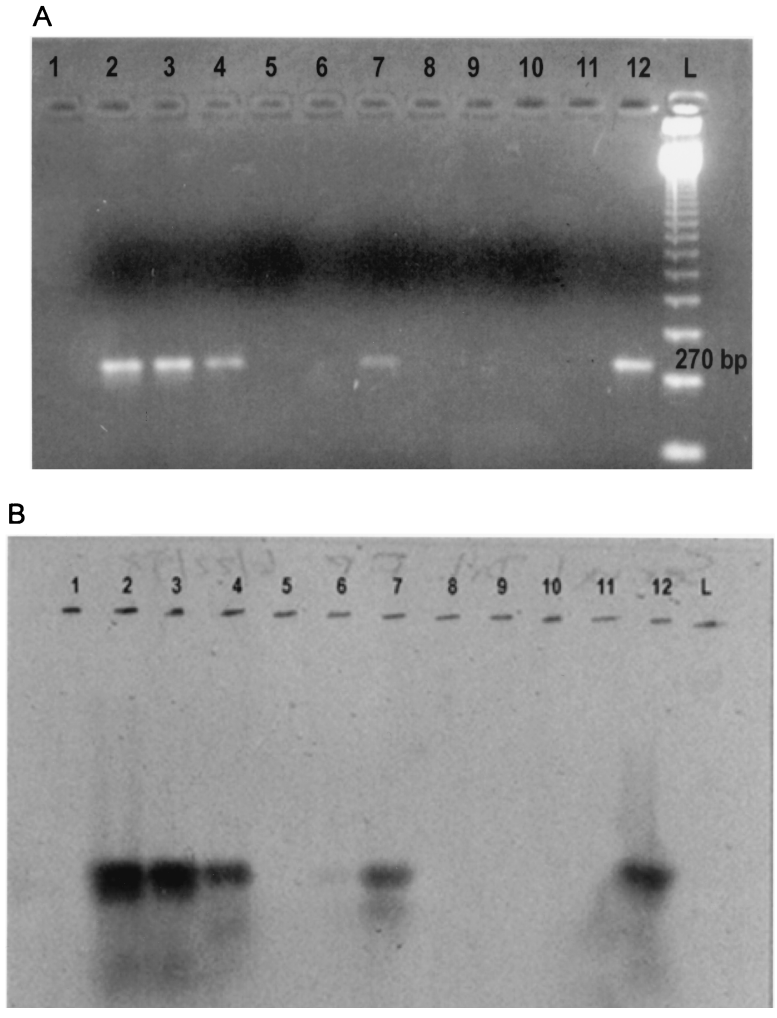

Fig. 3. Determination of the effect of filter paper on the sensitivity of HBV detection by PCR. Serial dilution of 1,500 pg/ml HBV DNA serum. Lanes 2–6 Serum. Lanes 7–11 serum on filter paper. (A) Agarose gel of PCR products; 270 bp band indicates positive samples. (B) Southern blot detection. Lanes: 1-empty, 2–1:10 (400 virions), 3–1:100 (40 virions), 4–1:1,000 (4.0 virions). 5–1:10,000 (0.4 virions), 6–1:100,000 (0.04 virions), 7–1:10 (400 virions), 8–1:100 (40 virions), 9–1:1,000 (4 virions), 10-1:10,000 (0.4 virions), 11-1:100,000 (0.04 virions), 12-positive control, L-123 bp lambda ladder.

bound to PCR product on nylon membranes, and visualized with a digoxigenin nucleic acid detection kit (Boehringer Mannheim, Kit #1175041).

Results

Sensitivity of PCR and Southern Hybridization Detection of HBV. PCR and gel electrophoresis yielded visible products of 270 bp after amplification from HBV-positive serum at the 1:1,000 dilution, equivalent to four viral particles; the presence of bed bug tissues mixed with positive serum decreased the sensitivity of the procedure 10-fold to the equivalent of 40 viral particles (Fig. 2A). PCR products blotted for Southern hybridization with the probe binding to the precore region of the viral genome were visible at the 1:10,000 dilution (equivalent to 0.4 viral particles); however, the presence of bed-bug tissue decreased the sensitivity of Southern blots 100-fold, to the equivalent of 40 viral particles (Fig. 2B). Adding filter paper on which feces had been deposited to HBV-positive serum decreased the sensitivity of the PCR and Southern detection methods also by 100-fold, such that PCR and the Southern blot had visible bands at the equivalent of 400 viral particles (Fig. 3).

Infective Feedings. Legs and bodies were collected at 1, 7, 14, 21, 28, or 35 d after feeding. HBV was detected by PCR and Southern blot in one of one bed bugs on day 1; in 0 of five bugs by PCR, but three of five of the same bugs by Southern blot on day 7; in five of five bugs by both detection methods on days 14, 21, 28; and in three of three bugs by both methods on day 35 d. There were no positive paired, negative controls at these time points. Fig. 4 shows PCR and Southern blot detection of HBV in bugs from days 28 and 35 after-feeding to illustrate how these procedures were used to differentiate positive and negative samples in

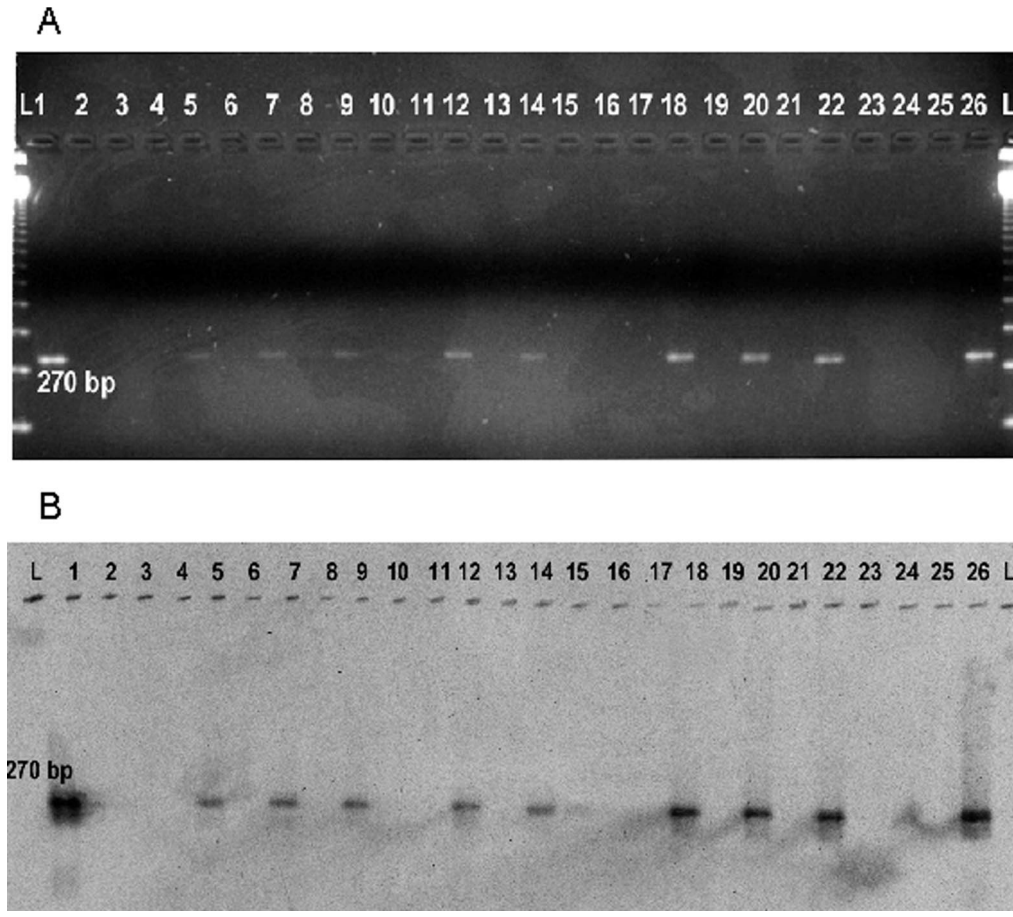

Fig. 4. Detection of HBV in *C. lectularius* bodies assayed at days 28 and 35 after an infectious blood meal. (A) Agarose gel of PCR products, 270-bp band indicates positive samples. (B) Southern blot products. Lanes L-123-bp lambda ladders. Lanes 1 and 26 are positive controls, lanes 2 and 25 are negative controls, and lane 23 is a reagent blank. Lanes 3, 4, 16, and 17 are control bed bugs. Lanes 5–15 were collected on day 28 and lanes 5, 7, 9, 11, and 13 are bodies. Lanes 18–24 were collected on day 35 and lanes 18, 20, and 22 are bodies.

the experiment. Legs from one bug were positive on day 21 and legs from another bug were positive on day 28 by Southern blot only; all other legs were negative.

HBV DNA was not detected by PCR from any of the 20 fecal spots collected 0 or 1 d after infectious feeding and only one of 20 fecal spots was positive by Southern hybridization. However, detection rates by both assays increased with increasing time after the infectious blood meal, and all fecal spots tested ≥21 d after the infectious blood meal were positive for HBV by both detection methods (Fig. 5).

HBV was detected by both PCR and Southern blot in all nymphs (1 second instar, 4 third instars, 8 fourth instars, 1 fifth instar) and in eight of eight adults, where each individual molted once after an infectious blood meal. There was no HBV detected in corresponding negative controls. HBV was not detected in any of the nymphs that hatched from eggs oviposited on days 7 or 14 after the infectious blood meal.

Intrathoracic Inoculations. For bugs that were inoculated intrathoracically, HBV was detected by Southern blot (but not by PCR) in four of five of the bugs collected on day 7, four of five bugs collected on day 14, and two of five of the bugs collected on day 21 after inoculation. Legs of two individuals were positive by Southern blot on day 7. In contrast to bugs that had fed on an infectious blood meal, HBV was not detected by either PCR or Southern blot in the feces from bed bugs inoculated with HBV.

Discussion

Our results document the persistence of HBV after an infectious blood meal in bed-bug bodies, transtadial passage, and viral shedding in feces, but not transovarial transmission. These results are similar to previous studies where the HBsAg was used for virus detection (Jupp and McElligott 1979, Ogston et al. 1979, Ogston and London 1980, Taylor and Morrison 1980, Jupp et al. 1983). Our use of PCR and Southern hybridization provided a sensitive method for detecting HBV nucleic acids in our samples. The oligonucleotide primers and the probe we used hybridized with the conserved precore and core regions of the genome (Lauder et al. 1993). The core region contains

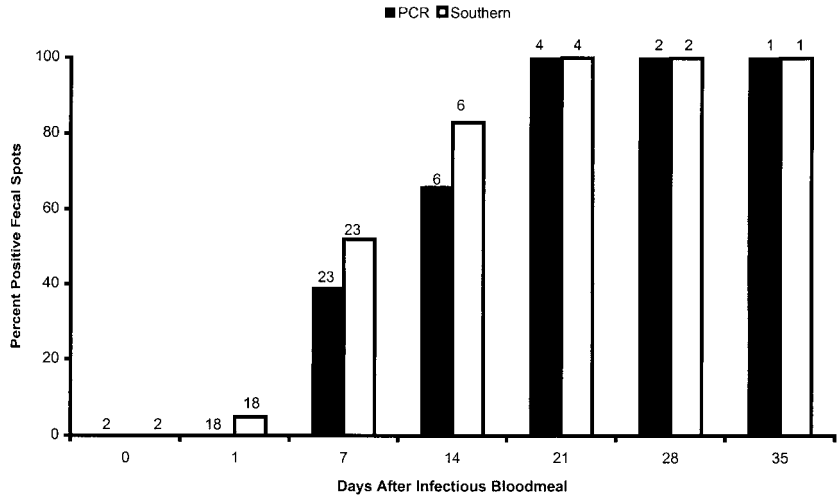

Fig. 5. Detection of HBV by PCR and Southern hybridization in feces shed from *C. lectularius*, after an infectious blood meal. Hepatitis B viral genome was detected in fecal spots extracted from filter paper substrates. PCR detection sensitivity was ≈400 virions and Southern blot was ≈40 virions. Percentage is calculated from the number of positive samples over the total number of samples collected for each time point (number on the top of each bar).

two initiation codons that encode the structural proteins of the nucleocapsid (Pasek et al. 1978). The first initiation codon product is the 25-kd precore/core protein that produces the HBsAg. The second initiation codon product is the 21-kd core protein that forms the viral nucleocapsid with the HBV DNA (Thomas and Carman 1994). Thus, detecting this region of the genome provides indirect but strong evidence that the bed-bugs in our experiments ingested an infectious blood meal and not merely noninfectious particles with surface antigen, and potentially infectious particles were detected in subsequent samples. Certainly, carefully controlled transmission studies would be needed to answer the lingering question of whether HBV, detected by the PCR and Southern blot methods, is actually infectious.

One of the most important findings from our experiments was that HBV was shed stercorarially for up to 35 d from bugs that had fed on an HBV-positive volunteer. These results expand previous observations where HBsAg was detected for up to 6 wk in bed-bug feces (Taylor and Morrison 1980, Jupp et al. 1983). The sensitivity of these methods theoretically allowed us to detect as few as 40 HBV particles in the bed bug and 400 HBV particles in the feces. Consequently, at least 400 potentially infectious particles may have been present in each fecal spot that we examined. These spots were small (<1 mm in diameter) and consisted of dried feces that had been shed by the bugs from one collection time point to the next on the filter paper substrate. Although the alimentary physiology of bed bugs is poorly known, our observations and those of others (Usinger 1966, Taylor and Morrison 1980, Jupp et al. 1983) indicate that after feeding, bed bugs retain blood in a partially digested state for several weeks, and over time, excrete fecal drops consisting of the unassimilated blood meal. These spots are common in heavy aggregations of bed bugs in human dwellings, and can be used as visible sign of their presence (Usinger 1966). In a review of experiments on the vector competence of bed bugs (both *C. lectularius* and *Cimex hemipterus* [F.]), Burton (1963) documented 'survival time' postblood feeding of the following disease-causing agents shed in bed-bug feces (in parentheses): *Bacillus anthracis* (4 d), *Franciscella tularensis* (>250 d), *Salmonella paratyphi* (21 d), *Coxiella burnettii* (285 d), yellow fever virus (15 d), lymphocytic choriomeningitis virus (85 d), and *Borrelia recurrentis* (28 d). Apparently, the gut environment with its aged blood meal is suitable for survival of these pathogens, and in some instances they are shed in feces in a potentially infectious form. However, there appear to be no studies that assay the rate of blood meal digestion relative to fecal production, nor studies that document whether bugs shed feces while feeding on a host (we did not attempt to observe it).

Detecting virus in *C. lectularius* legs 21 and 28 d after HBV-infectious bloodmeal raises the possibility that HBV disseminated to the hemolymph from the gut. The fact that HBV nucleic acids were detected by PCR for 35 d postfeeding and 21 d postinoculation in *C. lectularius* indicates that the virus persisted for several weeks in the gut, and probably persisted for at least 3 wk circulating in hemolymph. We cannot rule out the possibility of contamination due to sample processing during removal of the legs or by defecation, nor does the small sample size here allow a determination of the significance of these positive samples. However, the presence of detectable virus in or on the bodies and feces of the *C. lectularius* raises the question as to whether the virus may be mechanically transmitted. Bed bugs live in close proximity to a host, often found in bedding, furniture, and cracks and crevices in walls. Thus, they may defecate virus into the host's environment and provide a potential source for an atypical route of infection. Jupp et al. (1983) concluded

that there was no indication of virus replication in *C. lectularius* (i.e., no evidence of biological transmission) but that mechanical transmission from insects to humans could occur by contamination if a person crushed HBV-positive bugs; by contamination from infected feces deposited onto skin; or by infection by bite due to regurgitation or interrupted feeding. These modes of infection occur in at least two other arthropod-borne disease systems: body lice (*Pediculus humanus humanus* L.) and the rickettsiae that cause epidemic louse-borne epidemic typhus (*Rickettsia prowazekii*), which is transmitted stercorarially and by crushing; and with the transmission of *Trypanosoma cruzi* by kissing bugs (Reduviidae) through stercorarial routes (Edman 2000). Separately, studies indicate that inanimate objects contaminated with HBV may contribute to disease transmission for up to 1 wk and possibly longer (Bond et al. 1977, 1981). The virus has been reported to be viable after storage at room temperature for 6 mo (Havens and Paul 1965) and exposure to UV irradiation (Barker 1970). Therefore, it is possible that HBV shed onto surfaces with bed-bug fecal material could remain viable and infectious.

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