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PERSISTENCE OF THE HEPATITIS B VIRAL GENOME IN SELECTED HEMATOPHAGOUS INSECTS

By

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ABSTRACT

THE PERSISTENCE OF THE HEPATITIS B VIRAL GENOME
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Hepatitis B virus (HBV) infection is a major cause of acute and chronic hepatitis, cirrhosis, and primary hepatocellular cancer, with an estimated 300 million carriers world wide. In China, Southeast Asia, sub-Saharan Africa, parts of the Middle East, and South America the primary modes of transmission are perinatal infection of infants, vertical transmission, and horizontal transmission, however, 30-40% of people with hepatitis B virus have no identifiable risk factors.

Numerous researchers have investigated the possible role of hematophagous arthropods in the transmission of hepatitis B virus under varying conditions. All of the studies to date have examined the persistence of the HBsAg as the detection method for the virus. The surface antigen is found on all three viral particles produced by the virus, only one of which is infectious. Thus, the detection and persistence of the HBsAg is not a measure of infectiousness but an indicator that the arthropod ingested a potentially infectious blood meal.

The purpose of this research was to evaluate the persistence and dissemination of the hepatitis B viral genome, by ingestion and inoculation, in *Cimex lectularius* L. (Hemiptera: Cimicidae), *Rhodnius prolixus* Stal (Hemiptera: Reduviidae), *Aedes triseriatus* (Say) (Diptera: Culicidae), *Culex quinquefasciatus* Say (Diptera: Culicidae), and *Anopheles stephensi* Liston (Diptera: Culicidae).

The hepatitis B viral genome was detect by polymerase chain reaction with confirmation by Southern blot. The sensitivity of these detection methods was determined for each insect by serial dilution of a known serum with added insect tissue. The virus was detectable for 48 hours in *An. stephensi* and 72 hours in *Ae. triseriatus* ingesting the virus. Virus was not detected at 7 days in inoculated mosquitoes. The virus persisted in the bodies and feces of *C. lectularius* ingesting a infected blood meal for 35 days. Virus was also detectable in low levels for 21 days in bodies but not feces of inoculated *C. lectularius*. Hepatitis B virus also persisted trans-stadially through one molt in all instar stages, but trans-ovarial transmision was not shown. Virus was detectable in inoculated *R. prolixus* bodies only at 7 days.

The results obtained in this laboratory study indicates that mosquitoes, *C. lectularius*, and *R. prolixus* do not serve as biological vectors of the hepatitis B virus. However, the persistence of the virus for varying time period in the bodies and feces of the various insects does raise the question of potential for mechanical transmission. Future studies need to examine if crushed insects and defecated material from insects ingesting a infected blood meal are infectious to susceptible hosts.

This dissertation is dedicated to my parents, Donald and Jean Blow, who have supported and encouraged me in all my pursuits.

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I feel a strong attachment to Alma College where I did my undergraduate degree. A school steeped in academic excellence and tradition, Alma taught me to think outside the box. I have found this to be a valuable asset in the ensuing years. I will always remember Alma for the academic challenge, the chance to play college basketball, and most of all the deep friendships that still endure.

The Army has been a major influence in my life. From my time as an enlisted soldier in Germany to ROTC to today as an officer, the Army has taught me responsibility, integrity, and leadership. Thank you to everyone who has influenced me during my career, I only hope that I can be a positive influence for others as I continue my career.

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DNA	deoxyribonucleic acid
HBV	Hepatitis B virus
HBsAg	Hepatitis B surface antigen
HBeAg	Hepatitis B e antigen
HBcAg	Hepatitis B core antigen
HBIG	Hepatitis B immune globulin
Anti-HBs	Antibody to hepatitis B surface antigen
Anti-HBc	Antibody to hepatitis B core antigen
Anti-HBe	Antibody to hepatitis B e antigen
HBV-DNA	DNA of HBV
ORF	Open reading frame
pgRNA	pregenomic RNA
PCR	Polymerase chain reaction
PHC	Primary hepatocellular cancer
RNA	ribonucleic acid

INTRODUCTION

HEPATITIS B

Hepatitis is a disease of antiquity. Hippocrates recognized epidemic jaundice in the fourth century B.C. and incidences of jaundice possibly caused by viral hepatitis are described in the Babylonian Talmud (Purcell 1985, Krugman 1989). The contagious nature of jaundice was recognized as early as the eight century A.D. in Europe. The first recognized epidemic of what was probably hepatitis B, began in Bremen, Germany, in October, 1883, and ended in April, 1884 (Purcell 1985, Krugman 1989). The Bremen epidemic is interesting not only because it was the first report of presumed hepatitis B, but it also pointed to parenteral exposure to blood or blood components as an important vehicle of transmission.

During the first half of the twentieth century, outbreaks of "long incubation period" hepatitis were observed in many countries of the world among: patients who attended clinics for venereal disease, diabetes, and tuberculosis; persons who received blood transfusions; persons who were inoculated with mumps or measles convalescent serum; and military personnel who received yellow fever vaccine during World War II (Krugman 1989). The etiology of viral hepatitis was established in the early 1940's through transmission studies in volunteers (MacCallum 1947). More detailed studies followed which revealed that there were two types of hepatitis virus, original identified as serum hepatitis (hepatitis B virus) and infectious hepatitis (hepatitis A virus).

The modern era of viral hepatitis research began with the description by Blumberg et al. (1965) of an isoprecipitant in the serum of Australian aborigines,

termed Australia antigen, that was later shown by Prince (1968) and others to be related to the hepatitis B virus. The discovery of Australia antigen, now called hepatitis B surface antigen (HBsAg) (See Table 1 for current Nomenclature), and its association with post transfusion hepatitis resulted in the expansion of hepatitis research.

Dane et al. (1970) discovered virus like particles, about 42 nm in diameter, in multiple serum specimens from three Australia-antigen-positive hepatitis patients. He suggested that these particles were complete virus particles, HBV, and that the much more numerous 22 nm particles, HBsAg, were surplus viral coat material.

Etiology. Hepatitis B is a unique partially double-stranded DNA virus which has been classified as a hepadnavirus (Ganem and Varmus 1987), a group which includes woodchuck hepatitis virus (WHV) (Summers et al. 1978), Pekin duck hepatitis B virus (DHBV) (Mason et al. 1980), and Beechy ground squirrel hepatitis virus (GSHV) (Marion et al. 1980). Hepadnaviruses are typically restricted to species closely related to their natural host. The barrier to cross species infection is the lack of appropriate receptors to the virus (Galle et al. 1988, Shih et al. 1989). The only natural host for hepatitis B virus appears to be humans, although several higher nonhuman primates, such as, the chimpanzee (the primary experimental model) the gibbon, and the gorilla have been shown to be susceptible to persistent infection in the laboratory (Hadler and Margolis 1989).

Clinical Manifestation. Although persistent or chronic virus infection is a minority outcome of primary infection, it is important for several reasons.

Persistent infection with hepatitis B virus often results in morbidity and mortality (Ganem 1996). The incubation period of hepatitis B is long (45-160 days; average 120) and the onset of acute disease develops so gradually that infection is well established before becoming apparent symptomatically (CDC 1990). Clinical manifestations of infection vary greatly, with many patients being relatively symptom-free ("asymptomatic carriers"), while others have varying grades of chronic liver injury and inflammation (chronic hepatitis B). Acute hepatitis is subclinical in about two thirds of the cases and most people with chronic liver disease never experience symptoms of acute hepatitis with jaundice (Hollinger 1996). These asymptomatic carriers may remain healthy for years, or the disease may progress rapidly to cirrhosis and death. Asymptomatic carriers are the primary epidemiological reservoir for the spread of hepatitis B virus to susceptible hosts (Ganem 1996). In those with clinical illness the onset of is usually marked by anorexia, vague abdominal pain, nausea and vomiting, and occasionally a rash. The symptoms may progress to jaundice and a mild fever. Chronic liver disease occurs in about 1-8% of adult hepatitis B patients (Redeker 1975, Hoofnagle et al. 1978, Beasley et al. 1983).

TABLE 1: Currently accepted nomenclature and abbreviations for the viruses, antigens, and antibodies associated with Hepatitis B (CDC 1990, Hollinger 1996).

Abbreviation	Term	Description
HBV	Hepatitis B virus	42 nm double shelled particle, consists of a 7 nm outer shell and a 27 nm inner core. Core contains a small, circular, partially double stranded DNA molecule and an endogenouse DNA polymerase. The etiological agent of hepatitis B; also known as the Dane particle
HBsAg	Hepatitis B surface antigen	The complex of antigenic determinants found on the surface of HBV and on the 22 nm particles and tubular forms. Originally known as Australia antigen or hepatitis associated antigen (HAA)
HBeAg	Hepatitis B e antigen	The antigenic determinate that is closely associated with the nucleocapsid of HBV. Circulates as a soluble protien in serum and correlates with HBV replication and infectivity
HBcAg	Hepatitis B core antigen	The antigenic specificity assocated with the 27 nm core of HBV found within the core of the virus
Anti-HBs	Antibody to hepatitis B surface antigen	indicates immune response to HBV infection, passively acquired antibody, or immune response to vaccination
Anti-HBe	Antibody to hepatitis B e antigen	presence in serum of HBsAg carrier suggest low titer of HBV and low infectivity
Anti-HBc	Antibody to hepatitis B core antigen	indicates past or present infection with HBV; not present in persons with vaccine-induced immunity
HBV-DNA	DNA of HBV	detected by nucleic acid hybridization; indicates the presence of intact HBV

Particle Types. Hepatitis B virus is unusual among animal viruses in that infected cells produce multiple types of virus-related particles (Bayer et al. 1968, Dane et al. 1970, Gerin et al. 1971, Kim and Tilles 1973, Robinson and Li 1976). Ganem (1996) characterized hepatitis B virus as having three types of particles: (a) 42 to 47-nm double shelled infectious particles (Dane particles or HBV), (b) 22-nm spheres, and (c) smaller quantities of filaments of 22-nm diameter and variable length. The hepatitis B virus outer shell is a lipoprotein envelope containing the viral surface glycoproteins, originally detected serologically as HBsAg (Blumberg et al. 1965). The 22-nm spheres and filaments are composed exclusively of HBsAg and host-derived lipid; their principal lipids include phospholipids, cholesterol, cholesterol esters, and triglycerides (Peterson 1981, Gavilanes et al. 1982, Aggerbeck and Peterson 1985, Peterson 1987). The 42-nm diameter virion consists of an envelope and a nucleocapsid containing a circular 3200 nucleotide DNA molecule, a DNA polymerase, a protein kinase activity and a 'DNA-linked protein' (Tiollais et al. 1985). The envelope carries the HBsAg and the capsid, the hepatitis B core antigen (HBcAg) (Figure 1). The presence of complete virions in the serum is indicative of active viral multiplication in the liver (Tiollais et al. 1985).

Both complete HBV (Dane) particles and incomplete particles (HBsAg 22 nm spheres and filaments) are found in serum during the course of acute and chronic infection. The HBV particle, infectious particle of hepatitis B virus, titer ranges from less then 10^4/ml to greater than 10^9/ml (Blumberg et al. 1965). The concentration of non-virion associated HBsAg (spheres and filaments) greatly exceeds the concentration of complete virions by 10^4 or greater.

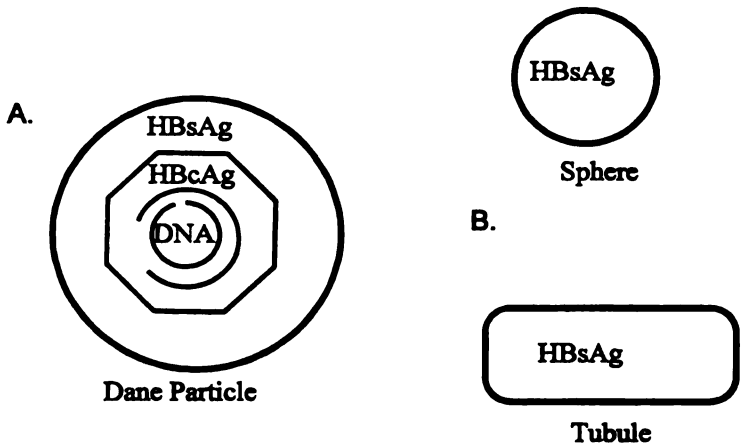

Figure 1. Hepatitis B viral particles. A) HBV DNA particle, envelope carries the HBsAg and the capsid, the hepatitis B core antigen (HBcAg) and viral DNA. B) 22 nm spheres and tubules consisting of HBsAg and host derived lipid.

Viral Genome. Hepadnavirus genomes consist of small (3000-3300 nucleotides), circular DNA that is partly single stranded (Tiollais et al. 1981, Summer 1984, Hadler and Margolis 1989). Although the viral DNA is circular, both strands of the duplex are linear, and the circular conformation is maintained by base pairing between 250 to 300 nucleotides located at the extreme 5' ends of the two strands. The minus strand is uniform in length (about 3200 nucleotides) and has a protein covalently bound at its 5' end (Gerlich and Robinson 1980).

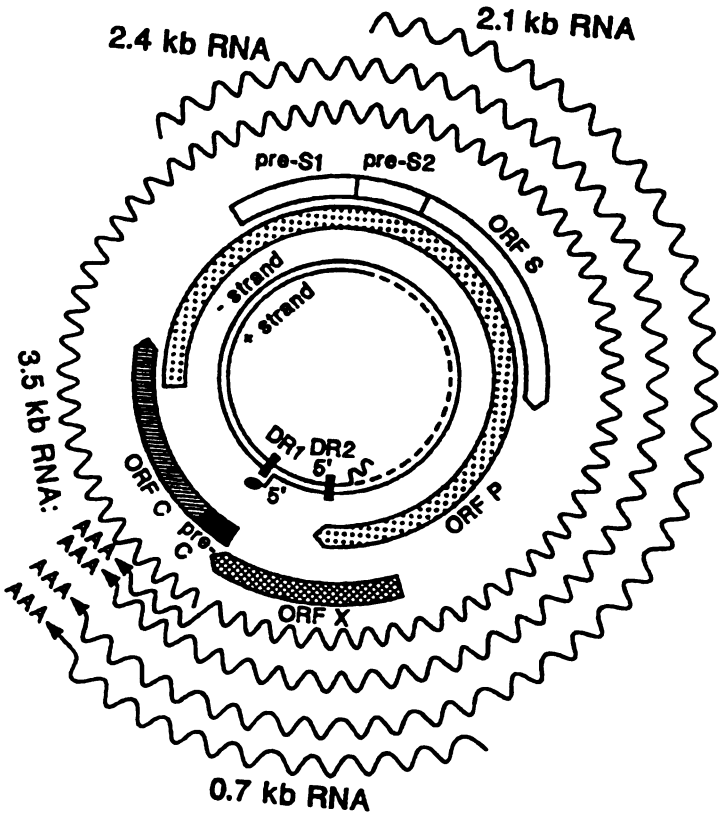

Figure 2. Hepatitis B viral genome. The inner circle represents virion DNA, with dashed lines signifying single-stranded regions. Boxes denote DNA codeing regions, arrows, indicating the direction of translation. Wavy lines depict viral RNA detected in cells (Ganem 1996).

The replication mechanism of hepatitis B virus, discovered by Summers and Mason (1982) for duck hepatitis B virus and confirmed later for human hepatitis B virus (Summer and Mason 1982, Blum et al. 1984, Fowler et al. 1984, Miller et al. 1984a, Miller et al. 1984b), differs strikingly from that of other DNA viruses. The central feature is the use of a RNA copy of the genome as an intermediate in replication. The current model for HBV replication (Tiollais et al. 1985)

consists of the following steps: (1) the virus penetrates a hepatocyte, the DNA reaches the nucleus and is converted to an open, circular, double-stranded DNA and then to supercoiled DNA; (2) the minus strand is transcribed by the cellular RNA polymerase to a 3.5 kb pre-genomic (pg) RNA; (3) the pgRNA is encapsulated with the DNA polymerase and the DNA-linked protein; (4) the minus strand DNA is then synthesized by reverse transcription of the pgRNA, with a covalently bound DNA-linked protein at 5' end of the minus strand acting as a primer. Hepatitis B virus DNA in the hepatocyte can exist free or integrated into the host cellular chromosome (Brechot et al. 1982, Shafritz 1982). Free hepatitis B virus DNA, which represents intermediate forms of replication, is detected during the acute and chronic stages of hepatitis B virus infection (Tiollais et al. 1985).

The HBV genome has four overlapping open reading frames (ORF) that code for four genes. The polymerase (P) gene encodes a protein that has four different activities: the terminal protein, reverse transcriptase, RNase-H, and HBV DNA polymerase (Thomas and Carman 1994). The core (C) gene contains 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 HBeAg. The second initiation codon product is the 21-kd core protein, that forms the viral nucelocapsid with the HBV DNA (Thomas and Carman 1994). The X gene's exact function is currently undefined. The surface (S) gene contains three in frame ATG's that produce the three envelope proteins, designated as HBsAg. The two larger proteins have either pre-S1 and

pre-S2 regions or only a pre-S2. The pre-S1 appears to be involved in binding to the hepatocytes (Thomas and Carman 1994).

Cell and Tissue Affinity. Hepatitis B virus enters the circulatory system by direct inoculation, passage through mucous membranes, or through breaks in the integument, and is then carried to the hepatocytes, where primary replication occurs (Hadler and Margolis 1989). An important step in tissue affinity is the binding of hepatitis B virus envelope proteins to specific receptors on the hepatocytes, however, the mechanism for attachment and penetration has not been elucidated (Neurath et al. 1986, Leender et al. 1992, Hertogs et al. 1993). Intensive investigations have focused on the pre-S1 sequence of the large HBsAg gene product for attachment and penetration of hepatitis B virus into the hepatocyte plasma membranes (Neurath et al. 1986, Pontisso et al. 1989, Pontisso et al. 1991, Leender et al. 1992, Hertogs et al. 1993). It has been postulated that hematopoietic cells may initially support viral replication (Ganem 1996). Species-specific receptors have been recognized on hepatocytes, monocytes, lymphocytes, fibroblasts, and in spleen tissue (Michalak et al. 1991, Pontisso et al. 1991, Franco et al. 1992, Leender et al. 1992, Neurath et al. 1992). The absence of the HBsAg receptor precludes the virus from gaining access to the cell and thus replicating.

Pathogenicity. Although HBsAg has been detected in a wide variety of body fluids, only semen, serum, and saliva have been demonstrated to be infectious (Alter and Mat 1994). To fully comprehend the infectiousness of the blood of an HBeAg positive carrier, it is important to realize that each milliliter of blood may contain greater than 10^8 infectious virions (Hollinger 1996). Hepatitis B virus

circulates at approximately 10^{10} virions per milliliter in the blood of an acutely infected or early-stage chronic carrier (Hadler and Margolis 1989). The quantity of viral particles at infection influences the incubation time to the onset of disease (Hollinger 1996). The greater the quantity of virus at infection the shorter the time before the onset of disease. The number of virus particles present even in small amounts of blood serum or other body fluids can be quite high. One picogram of HBV-DNA contains approximately 2.9×10^5 genomic copies of the virus per milliliter or 290 copies/ul (Hollinger 1996). Inoculation with one microliter of infectious blood may cause an infection (Alter 1982).

The stability of HBV does not always coincide with that of HBsAg. Various methods of inactivating the virus, such as ether, sodium hypochlorite, isopropyl alcohol, heating, and autoclaving have all been examined. HBV retained its ability to induce an immune response (immunogenicity) and to interact specifically with free antibody and/or with antigen-binding receptors on lymphocytes (antigenicity) after exposure to ether, acid (pH 2.4 for at least 6 hours), and heat (98 degrees C for 1 minute; 60 degrees C for 10 hours) (Shikata et al. 1976, Bond et al. 1977, Hollinger 1996). However, hepatitis B virus inactivation may be incomplete under these conditions if the concentration of virus is excessively high. Hepatitis B virus has been shown to retain infectivity when stored at 30 to 32 degrees C for at least 6 months, 60 degrees C for 4 hours, and frozen at -20 degrees C for 15 years (Bond et al. 1977, Hollinger 1996) .

Diagnosis. Serological tests allow for diagnosis of hepatitis B infection and differentiation between acute and chronic disease by serological markers, whose

appearance and disappearance signals a change in the clinical stage of the disease. A serum sample positive for HBsAg indicates that an active hepatitis B virus infection is present (Hollinger 1996) but does not determine the stage or infectivity of the host. In acute hepatitis B, HBsAg is detectable in the serum and usually persists through convalescence. The presence of HBeAg correlates with viral replication in the liver and is detectable in the early course of acute hepatitis B. As acute hepatitis B resolves, HBsAg and HBeAg become undetectable and neutralizing antibodies, anti-HBs, appear. The presence of anti-HBs is associated with the resolution of infection and correlates with immunity. However, the persistence of HBsAg and HBeAg in serum suggests a transition from acute to chronic hepatitis infection and indicates continuing viral replication. Patients who are HBeAg positive are more likely to transmit hepatitis B sexually, percutaneously, or perinatally.

Serological Assays. A number of serological assays are available commercially to detect the hepatitis B virus and aid the clinician, epidemiologist or researcher in differentiating acute from chronic infections, evaluating relative infectivity and prognosis, and assessing the immune status of the patient. Radioimmunoassays (RIA) and enzyme-linked immunoassays (EIA) are the most sensitive and specific of the various commercially licensed assays available. These assays, performed both as a "sandwich" and competitive binding assays, can detect HBsAg in the 0.25 to 0.5 ng/ml range (Hollinger 1996).

The infectiousness of blood or body fluids of a HBsAg positive patient is determined by testing for HBeAg, anti-HBe, or HBV DNA. HBeAg positive

samples may contain up to 10^8 infectious virions per milliliter of blood (Krugman et al. 1974, Krugman et al. 1979, van Ditzhuijsen et al. 1985, Hollinger 1996), whereas, patients who are anti–HBe positive circulate fewer than 100 infectious virions per milliliter of blood.

The polymerase chain reaction (PCR) technique is a method for amplifying nucleic acid by repeated cycles of high temperature denaturization, oligonucleotide primer hybridization, and DNA polymerase extension. A PCR based assay is used for the detection of hepatitis B virus DNA in serum and tissue and can detect as few as 250 HBV DNA molecules/ml (Hollinger 1996). Due to its greater sensitivity and detection of specific nucleotide sequences of the viral genome, PCR has been important in elucidating the intricate immunopathogenic mechanisms of hepatitis B virus.

Epidemiology

Hepatitis B infection is a major cause of acute and chronic hepatitis, cirrhosis, and primary hepatocellular carcinoma (PHC) world wide (CDC 1990). It is estimated that there are as many as 300 million carriers world wide and an estimated 2 million people are killed each year by this virus (Hamilton 1994). There are three main outcomes when an individual is infected with the hepatitis B virus: (1) symptomatic acute hepatitis with development of anti-HBs antibody in the blood leading to long term immunity; (2) a chronic carrier state of HBV; (3) asymptomatic infection leading to either immunity or a chronic carrier state (Kane 1995). It is estimated that 6-10% of acute HBV infections become chronic (Mehaeus 1995).

Persons at high risk for exposure to the hepatitis B virus are: intravenous drug users; sexually active homosexual and bisexual men; sexually active heterosexual men and women; inmates of long term correction facilities; health care and public safety workers who are exposed to blood and bodily fluids; transfusion recipients; hemodialysis patients; household contacts and sexual partners of HBV carriers; and international travelers. An important feature of the epidemiology of hepatitis B in the United States is that 30% to 40% of patients cannot be definitively associated with an identifiable risk factor, which places them out of reach of immunization strategies that target high risk groups (Alter and Mat 1994).

Incidence. The frequency of hepatitis B infection and patterns of transmission vary markedly in different parts of the world. It is estimated that 300 million people across the globe carry the hepatitis B virus (Kuhns 1995) with three million cases of acute viral hepatitis diagnosed each year in Africa alone (Kuhns 1995). The number of reported cases of hepatitis B is only a fraction of the actual cases, since symptomatic cases make up only 33-50% of all hepatitis B virus infections (Mehaeus 1995). To obtain an estimate of the actual number of cases the reported number of cases must be multiplied by a factor between 2 and 10 depending on the country.

Beasley et al. (1982) conducted a study of 1,510 Chinese preschool children (1-4 years of age) and found that 118 (7.8%) were HBsAg positive and 122 (8.1%) were anti-HBs positive at enrollment. A year later 33 of the susceptible children had become HBsAg positive and 65 anti-HBs positive. In a subsequent follow up of 25 of the 33 HBsAg positive children, 17 (68%) were still positive.

The study found there was no difference in incidence of infection in relation to age but among children infected at older ages fewer children became carriers among (Table 2). Beasley et al. (1982) also noted a strong correlation between HBV markers in the child and the HBsAg positive mothers. Infants born to HBV carrier mothers had a 95% incidence of becoming infected with HBV (Beasley et al. 1981).

Table 2: The risk of developing chronic hepatitis based on the age of infection (Beasley et al. 1982, Hadler and Margolis 1989).

Age of Infection	Percentage of Chronic Infection
Infant	90-95 %
1-5	25-50%
Older children	5-10%
Adults	1-8%

Prevalence. The highest prevalence of hepatitis B virus infection is found among people born in areas of high endemicity and their descendants, intravenous drug users, and homosexual men (Alter and Mat 1994). The endemicity of hepatitis B infection is considered high in those areas of the world where the prevalence of HBsAg is 8-15% and where 70-90% of the population have serological evidence of previous infection (Table 3) (Hadler and Margolis 1989).

Public Health Implications. Primary hepatocellular carcinoma (PHC) is one of the most common cancers in Asia and sub-Saharan Africa (Beasley 1988). Epidemiological evidence has established a strong association between chronic

HBV infection and PHC (Rogers et al. 1997). Hepatitis B virus is the etiological agent for 70% - 90% of the cases of PHC world wide (Beasley 1988, Benenson 1995). An estimated 15-25% of people with chronic HBV infection die prematurely of either cirrhosis or PHC (Alter and Mat 1994).

Modes of Transmission. The efficiency of the various mechanisms of hepatitis B transmission is related to the amount or titer of circulating infectious virus in the blood of infected persons in either the acute phase of infection, or in chronic HBsAg carriers positive for hepatitis B e antigen (HBeAg) (Favero et al. 1979). Mechanisms of transmission, in order of efficiency, are: direct percutaneous inoculation by needle of contaminated serum or plasma; percutaneous transfer of infective serum or plasma in the absence of overt needle puncture; contamination of mucosal surfaces by infective serum or plasma; contamination of mucosal surfaces by infective secretions other than serum or plasma; and, transfer of infective material via vectors or inanimate environmental surfaces (Favero et al. 1979). Perinatal and transplacental transmission may represent the most important mechanism for the maintenance of a carrier population in certain regions of the world (Wright et al. 1970, Merrill et al. 1972, Ohbayashi et al. 1972, Schweitzer et al. 1973, Mazzur et al. 1974, Schweitzer 1975). Women with acute hepatitis late in pregnancy or during the neonatal period readily infect their infants (Mirick et al. 1965, Merrill et al. 1972, Schweitzer 1975). The infants are usually HBsAg negative at birth but develop antigenemia within 1 to 3 months (Hollinger 1996). This time period corresponds to the incubation period for HBV if the infection occurred during delivery, although the method of transmission during delivery is unknown. However, HBsAg has been detected

Table 3: Geographic patterns of the prevalence of hepatitis B markers (Maynard 1981, Alter and Mat 1994, Hamilton 1994).

	Low Endemicity	Intermediate Endemicity	High Endemicity
HBsAg	0.1 -0.5%	2 - 7%	8 - 15%
Anti-HBs prevalence	4 - 6%	20 - 55%	70 - 95%
Neonatal infection	Rare	Uncommon	Common
Childhood infection	Rare	Uncommon	Common
Distribution (estimated number infected)	United States (1.25 million) Western Europe Australia	Eastern Europe Mediterranean South America Middle East Russia	Southeast Asia (40 million) China (104.5 million) Tropical Africa (49.5 million) Pacific Island South America Middle East

in cord blood, amniotic fluid, breast milk and vaginal secretions as well as maternal blood (Lennemann and Goldberg 1974, Papaevangelou et al. 1974, Boxall 1975, Schweitzer 1975, Stevens et al. 1975, Derso et al. 1978, Lee et al. 1978) to that transmission could occur when the placental barrier breaks down, via amniotic fluid, minor abrasions, or mucous membranes.

Person to person transmission of HBV can occur in settings involving interpersonal contact over a long period of time via inapparent contact of infective secretions with skin lesions or mucosal surfaces (CDC 1990). Foster et al. (1984) found that exudates from skin lesions to be HBsAg positive and a history of skin disease is known to be a risk factor for infection (Bernier et al. 1982). In China, Southeast Asia, sub-Saharan Africa, parts of the Middle East, and South America, the primary modes of transmission are perinatal infection of infants from HBeAg positive mothers, vertical transmission, and horizontal transmission between children. Bond et al. (1981) found that inanimate objects contaminated with hepatitis B virus could contribute to disease transmission for periods of time up to one week and possibly longer. Tattooing, circumcision, ear piercing and bites of hematophagous arthropods have also been proposed as mechanisms of transmission although supporting evidence is weak (Fox et al. 1988). The HBsAg has been found to be very stable on surfaces and could be readily detected in an environment contaminated with blood or fluid containing high levels of surface antigen (Favero et al. 1973). The presence of HBsAg on a surface indicates contamination with infectious blood at some previous time; however, it does not prove the presence of viable HBV (Bond et al. 1977).

Methods of Control. The most effective control measure is to immunize susceptible populations. There are currently two inactivated vaccines licensed in the United States and Canada, both of which have been shown to be safe and effective in protecting against all serotypes of the hepatitis B virus. The vaccines are currently part of the childhood immunization program (CDC 1990) and are commonly administered in 3 injections over the course of 6 months (Benenson 1995). The vaccine is highly immunogenic in neonates, children, and immunocompetent adults with an immune response rate of 95-97.5% (Rogers et al. 1997). In persons not responding to the initial vaccine series, approximately 40% will acquire anti-HBs after revaccination with another three shot series.

Only wide scale immunization of infants and children can reduce the incidence of disease in endemic areas of the world. Infants who are infected vertically or perinatally have the greatest risk of becoming a carrier. The administration of hepatitis B immune globulin (HBIG) after birth is approximately 70-80% effective in reducing infant carrier rates, however, 20-25% of infants treated with HBIG became carriers (Beasley et al. 1981, Beasley et al. 1983). The administration of the hepatitis B vaccine to infants treated with HBIG increased the efficacy to 94% in the prevention of perinatal transmission of hepatitis B and in providing long term immunity (Beasley et al. 1983). The World Health Organization (WHO) (1997) has recommended the addition of hepatitis B vaccine in the national immunization programs for routine infant and/or adolescent immunization. In other areas with low prevalence, the initial strategy was the immunization of high risk groups with the first available vaccine. However, immunization of high-risk adults was not feasible and many of these

countries have begun to implement routine infant and adolescent immunization. Other methods of stopping the spread of HBV include: screening of all donated blood for the HBsAg; use of disposable needles and syringe or sterilization of reusable equipment; routine immunization of all persons falling into a high risk group; screening of all pregnant women; and surveillance for post-transfusion hepatitis.

HEPATITIS AND ARTHROPODS

Neefe (1949) was the first to consider the possibility that heamtophagous arthropods could transmit the hepatitis virus. Prince (1970) found an increased prevalence of SH (hepatitis B surface antigen) antigenemia in the tropics but was unable to account for this by known mechanisms of transmission, suggesting transmission in nature by nonparenteral routes. The subsequent detection of the hepatitis B surface antigen (HBsAg) by solid-phase radioimmunoassay in wild-caught mosquitoes supported this hypothesis (Prince et al. 1972). The detection of the hepatitis B surface antigen in wild caught mosquitoes (Prince et al. 1972, Blumberg et al. 1973, Brotman et al. 1973, Dick et al. 1974, Wills et al. 1976), *Cimex spp.* (Newkirk et al. 1975, Wills et al. 1977, Jupp et al. 1978, Jupp and McElligott 1979, Ogston et al. 1979, Jupp et al. 1980, Ogston and London 1980, Taylor and Morrison 1980, Jupp et al. 1983, El-Masry and Kotkat 1990), tampan tick, *Ornithodoros moubata* Murray, (Joubert et al. 1985, Jupp et al. 1987) and *Rhodnius prolixus* Stål (Villarejos et al. 1975) implicated these arthropods as potential vectors. Numerous studies have since been conducted utilizing both wild collected and laboratory-fed arthropods. However, all of these studies used

detection assays for the HBsAg, which is not indicative of viral replication or antigenicity.

Arboviral Transmission. Hundreds of species of hematophagous arthropods transmit viral, bacterial, protozoan, and filarial microogansims pathogenic to humans and animals. The relationship between arthropods and microorganisms has been, and still is, the subject of intensive research to define factors responsible for making the arthropod an efficient vector of the microorganism. The International Catalogue of Arboviruses (Karabatsos 1985) lists over 500 viruses that are definite, probable, or possible arboviruses. The World Health Organization (1985) based its definition of an arbovirus on the ecological interrelationships of virus, vertebrate host, and arthropod:

> "arboviruses are viruses which are maintained in nature principally, or to an important extent, through biological transmission between susceptible vertebrate hosts by hematophagous arthropods or through transovarian and possibly veneral transmission in arthropods; they multiply and produce viremia in the vertebrates, multiply in the tissues of arthropods, and are passed on to new vertebrates by the bites of arthropods after a period of extrinsic incubation."

This definition does not include viruses that are transmitted mechanically since they do not multiply in the arthropod during transmission. Arboviruses vary greatly in size and structure and belong to various families and genera, although most have RNA genomes and enveloped virions (Woodring et al 1996). They are united by their ability to replicate in hematophagous arthropods, to infect a vertebrate host when the arthropod vector feeds on that host, and to replicate in the vertebrate producing viremia (WHO 1985).

Arthropod-borne diseases have the following characteristics: the infectious agent is found in the blood of an infected vertebrate host; the agent can be

transmitted from the host to an arthropod in a blood meal; the arthropod can transmit the agent to another host; and, an effective vector must in some way support the pathogen it is transmitting (Harwood 1981). The relationship between arthropod and pathogen depends to a great extent upon the nature of the pathogen. It is not necessary for the pathogen to multiply within the vector, but it must be stabilized for a sufficient time to allow the vector to transport it to a susceptible host (Harwood 1981).

Two methods of transmission are recognized, mechanical and biological. Mechanical transmission occurs when the vector is merely a carrier, usually via contaminated mouthparts, from infectious source to a susceptible host [e.g., equine infectious anemia by stable flies (Hawkins et al., 1976)]. Biological transmission occurs when the pathogen multiplies in the vector and is subsequently transmitted following the invasion of the salivary glands (e.g., yellow fever virus by *Aedes* mosquitoes) (Mitchell 1983) or excreted in the feces during feeding (e.g., *Trypanosoma cruzi* by *Triatoma*). Biological and mechanical transmission are not mutually exclusive nor mutually inclusive. Vectors that transmit viruses biologically may be capable of mechanical transmission for a period immediately after the blood meal.

Harwood (1981) found that frequent feeding or probing could greatly increase the effectiveness of a vector. This is particularly true where mechanical contamination of the mouthparts is the means of transfer. The frequent interruption of feeding due to annoyance and restless movement of the host greatly aids the spread of the causal agents of anthrax, tularemia, equine infectious anemia, and anaplasmosis by tabanid flies (Harwood 1981).

Some viruses are transmitted vertically from parent organisms to progeny (Japanese encephalitis by *Cx. tritaeniorhynchus*, St. Louis encephalitis by *Cx. tarsalis*, Yellow fever by *Aedes aegyptii*)(Fine 1985). Vertical transmission includes transovarial, transovum, and transvenereal passage (Nuttal et al. 1991). To be transmitted vertically the virus has to persist throughout the embryonic, larval and pupal stages, withstanding the enzymatic autolysis of larval tissue during metamorphosis (Fouche et al. 1989). Vertical transmission of virus allows the arthropod to be the reservoir host for the virus, thereby maintaining a potential source of infection without a vertebrate host.

Arboviruses spend a significant portion of their life cycle in the arthropod. Their movement into vertebrate hosts is usually short-term for amplification and subsequent transmission to more vectors (Nuttal et al. 1991). Upon transmission to a susceptible vertebrate host, the virus usually undergoes rapid amplification causing a high viremia. This viremia is causes morbidity and mortality in the host, reduces host defenses making the host more vulnerable to vectors, and allows the high viral titers necessary to infect competent vectors feeding on the host. Many arboviruses are zoonotic (e.g., encephaitides) maintained in animal-mosquito cycles with humans being an incidental dead-end host (Leake 1998).

Erythrocyte Digestion. Blood borne pathogens gain access to hematophagous arthropods during the ingestion of the blood meal. Blood is pumped via the mouthparts from the host to the digestive tract. There are two basic types of digestive tracts found in hematophagous insects, a simple tube in which the blood is stored in the midgut or a complex gut having between one and three diverticulae which may be used in addition to the midgut for storage of the blood

meal (Lehane 1996). The blood meal is separated from the midgut epithelium by the peritrophic matrix. The function of the peritrophic matrix is not fully understood. It is composed of mucopolysaccharides, with some protein and lipid components (Lehane 1996) and is semi-permeable to allow the digestion and subsequent absorption of the blood meal. Two types of peritrophic matrices are recognized based on their method of production (Waterhouse 1953). Type I peritrophic matrices form from the secretions of cells along the complete length of the midgut and gradually develop into the peritrophic matrix and are found in adult mosquitoes, blackflies, sandflies, and tabanids. Type II peritrophic matrices are produced by the proventriculus, and pass backwards down the length of the midgut forming an unbroken cylinder which contains and separates the food from the midgut epithelium (Lehane 1996). Type II matrices are found in adult muscids, tsetse flies and hippoboscids. Hemipteran insects do not produce a peritrophic matrix but they do produce a extracellular coating in response to ingestion of the blood meal (Lane and Harrison 1979, Billingsley and Downe 1985, Billingsley and Downe 1986)

There are two general patterns of erythrocyte digestion (Vaughan and Azad 1993). The first pattern (Type I), observed for fleas and lice, is characterized by the rapid hemolysis and liquefaction of the blood meal within 6 hours after feeding. No peritrophic matrix was formed and the blood meal remained liquid throughout the 48 hour observation period. In the second pattern (Type 2), observed for bedbugs, sandflies, and mosquitoes, there was a time lag of 6-18 hours before substantial breakdown of erythrocytes began. A well-defined peritrophic matrix surrounded the blood meal of sand flies and mosquitoes at 18

to 24 hours after feeding, and blood meals were clotted. Peritrophic matrix formation and blood meal clotting was much less pronounced in bed bugs.

Vector Competency. Not all hematophagous arthropods are competent vectors for all pathogens. Vector competency, a term that has recently gained usage, is the intrinsic ability of a vector to biologically transmit a disease agent (Woodring et al. 1996). Intrinsic aspects of vector competency include susceptibility to infection, ability of the pathogen to reproduce or develop, and transmission efficiency. Four basic criteria have been used to incriminate vectors in the transmission of arboviruses: (1) the isolation of the virus from naturally infected vectors; (2) laboratory demonstration of vector infection by feeding on a viremic host; (3) laboratory demonstration of infected vectors transmitting the virus during blood feeding; and, (4) evidence of blood feeding contact between the suspected vector and host under natural conditions.

Vector competence is complex involving several barrier systems with in the arthropod. Chamberlain and Sudia (1961) recognized that one of the main factors in determining infection of the mosquito and its importance as a disease vector, was the susceptibility of the mid-gut area to infection. Ingested virus must first infect and replicate in the arthropod's midgut epithelial cells, and progeny virus must then disseminate via the hemocoel before the virus can be subsequently transmitted horizontally (via salivary glands) or vertically (via the egg or sperm) (Nuttal et al. 1991) (Figure 3). Although only a few days are required after virus ingestion for high viral concentrations to be attained in the mosquito, two to four times that incubation period are generally needed before efficient transmission by bite can occur (Chamberlain and Sudia 1961).

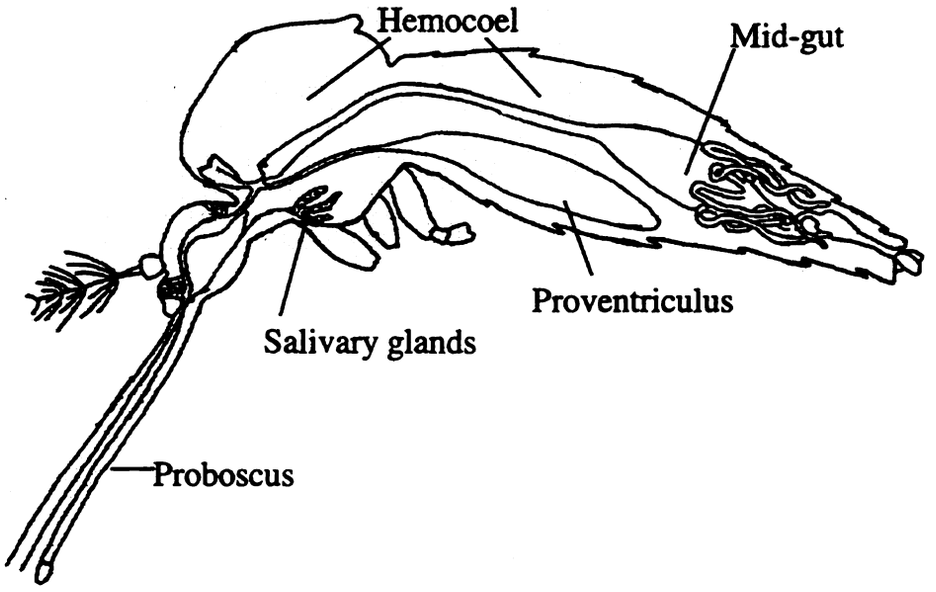

Figure 3. Mosquito Digestive System. Ingested blood passes through the proboscus into the mid-gut were digestion takes place. Virus must infect, reproduce and escape from the mid-gut cells to move into the hemocoel.

Successful infection of the midgut depends partly on the amount of virus in the host's blood and the volume of blood ingested (Nuttal et al. 1991). The concept of "minimum dose" or "infection thresholds" permeates the literature on virus-vector systems (Rehacek 1965, Burgdorfer and Varma 1967, Murphy et al. 1975, Nuttal 1984). The concentration of virus necessary to overcome the threshold of infection varies in different arthropod species, and for individuals of the same arthropod or virus (Hardy et al. 1983, DeFoliart et al. 1987). Studies with yellow fever showed that ingestion of a definite concentration of virus was required to infect *Aedes aegypti* and *Haemagogus* sp. (Anderson and Osorno-Mesa 1946, Bates and Roca-Garcia 1946a, Bates and Roco-Garcia 1946b,

Waddell and Taylor 1947, Anderson 1985). Mosquitoes fed virus in lower concentration failed to become infected but an increase in the viral concentration resulted in a higher proportion of infected specimens. Others (Chamberlain et al. 1954, Barnett 1956, Chamberlain et al. 1956, Hurlburt 1956, Chamberlain and Sudia 1957, Hale et al. 1957, Gresser et al. 1958, Chamberlain et al. 1959) working with a number of viruses demonstrated that the threshold virus concentration varied considerably between vector mosquito species for different viruses as measured by infection of susceptible hosts.

Once an infected blood meal has been ingested, studies indicate that the midgut is the anatomical site of viral interference (Nuttal et al. 1991). Following infection, viral titers in the midgut cells initially decrease during which time the virions penetrate the host cell becoming an intracellular (replication) stage know as the eclipse phase. Viral titers then increase rapidly to reach maximum levels within a few days as new virions are produced. The next barrier to infection is the basal lamina which is found in both the midgut and surrounding the salivary glands. This barrier is often referred to as the "midgut escape barrier" or "salivary gland infection barrier" (Hardy et al. 1983).

Salivary glands appear to become infected from viremic hemolymph (Chamberlain and Sudia 1961, Murphy et al. 1975, Beaty and Thompson 1978, McLintock 1978). Chamberlain and Sudia (1961) proposed that the absence of salivary gland infection was due to failure of virus to reach the salivary glands or, the inability to penetrate the glandular tissue to establish infection. Secondary amplification of virus in extramesenteronal cells and tissues is a prerequisite for infection of the salivary glands of some arboviruses (Nuttal et al. 1991) especially

when low concentrations of virus are ingested. In some virus-vector associations, a "leaky gut" has been described whereby the virus passes into the hemocoel more rapidly than replication could occur. If the virus is ingested in sufficient quantity when reaching the hemocoel it may infect the salivary glands without undergoing prior multiplication (Hardy et al. 1983). The passive movement of virus from the gut lumen to the hemocoel may relate to a potentially explosive cycling and vertebrate epizootics, and reflect a response of the virus to changes in selection pressures (Nuttal et al. 1991). Whatever the mechanism of the barrier to salivary gland infection, its presence or absence, together with the gut infection threshold, is of primary concern in determining the epidemiological importance of a vector species (Chamberlain and Sudia 1961).

The time interval between acquisition of virus in a blood meal and the ability to transmit the virus is known as the "extrinsic incubation period". During this period the vector is incapable of biological transmission because sufficient time has not elapsed for the virus to establish an infection of the salivary glands. Hence, the extrinsic incubation period is an important factor in the epidemiology and epizootiology of arthropod-borne viruses, as it dictates how long the vector must survive before it is a competent transmitter. Extrinsic conditions such as ambient temperature can affect the extrinsic incubation period (Turell et al. 1985, Watts et al. 1987, Kay et al. 1989). Intrinsic vector competence includes both physiological factors that govern infection of the vector and ability to transmit the virus, and innate behavioral traits such as host preference and probing activity (Nuttal et al. 1991).

The interplay of both intrinsic and extrinsic factors in vector disease transmission is termed the vectorial capacity, which is the overall ability of the vector at a given place and time to transmit a pathogen (Woodring et al. 1996). It encompasses the interactions between the vector, pathogen and vertebrate host, as well as ecological and behavioral factors such as vector population size, longevity, length and number of gonotrophic cycles, feeding behavior, and diel activity. Vector competency is the component of vectorial capacity that is restricted to the vector-pathogen relationship.

Vectors characteristically exhibit much lower levels of infection than the primary host in endemic regions. There are several possible explanations for this observation. One likely possibility is that the rate of transmission from primary host to vector is low in comparison to the transmission between two host species (Anderson 1985). High population densities in endemic regions often result in frequent contacts between vectors and susceptible hosts (Harwood 1981) which permits some vectors that are otherwise poor hosts of a pathogen to be of epidemiological significance. A striking feature of the epidemiology of indirectly transmitted disease agents is that the prevalence of infection within intermediate host (or vector) population is characteristically low, even in areas where the primary host population is heavily infected (endemic regions) (Anderson 1985).

DISEASE VECTORS

The ability of various Diptera to transmit disease pathogens has been extensively researched and studied. Several species of Hemiptera are also

known to transmit disease or have been implicated in the transmission of disease due to their intimate relationship with man.

Culicidae. There are approximately three and a half thousand species of mosquitoes, or Culicidae, in the order Diptera (Clements 1992). The family Culicidae is subdivide into 3 subfamilies: Culicinae, Toxorhynchitinae, and Anophelinae. The subfamilies Culicinae and Anophelinae contain primarily hematophagous species which are of medical importance as disease vectors. The subfamily Toxorhynchitinae consists of non-blood feeding mosquitoes.

Biology. All mosquitoes undergo complete metamorphosis. Females mosquitoes lay between 50 and 500 eggs at one time. The eggs are deposited on water or other damp substrates that are likely to be flooded. Eggs laid in the water hatch within 1-3 days while eggs laid on damp substrates diapause until appropriate conditions signal hatching. Mosquito larvae are legless, with a well formed head capsule and siphon for breathing. Larvae feed by either filtering microorganisms and detritus from the water column or scraping material off surfaces. A pupae is formed after four larval instars. During the pupal stage the larval organs degenerate and are replaced with adult organs from undifferentiated cells in the imaginal discs. When metamorphisis is complete the pupae swallows air to increase internal pressure causing the pupal cuticle to split along the cleavage lines. Adults slowly emerge and unfold the legs and body to stand on the water surface. The soft adult cuticle sclerotizes within 10-15 mintues and the adult is able to fly away. The time from pupation to eclosion is between 2-4 days.

Mosquitoes do not associate with a vertebrate host except for feeding and some use the host as a mating site. Mosquitoes actively search with variety of sensory stimuli for a suitable host from which to obtain a bloodmeal. After locating a host the mosquito begins to search for a suitable site to probe for blood. Chemoreceptors are used to locate the appropriate site to insert the proboscus. Mosquitoes have piercing sucking mouthparts which they use for feeding either from subdermal hematomas caused by the action of the mouthparts or directly from blood vessels. During probing the mosquito releases saliva through the proboscis. The saliva in some species contains anticoagulants, anti-platelet, and aggregation factors that keep the blood from clotting, counteracting the host's immune response to feeding. Mosquitoes withdraw and move to a new site to probe if unable to locate an appropriate feeding site.

Rearing. The reproductive ability of female mosquitoes is dependent on their nutrition in both the larval and adult stages. Large, well fed larvae have the greatest fecundity as adults. The actual fertility of the female is dependent on her ability to locate a blood source, obtain an adequate blood meal, and find a mate to produce eggs. There is great variation in fecundity and fertility between genera and species. The number of eggs produced by female mosquitoes has been shown to be influenced by maternal body size (Hawley 1985, Briegel 1990a, Briegel 1990b), the number of previous gonotrophic cycles (Roubaud 1934, Detinova 1949, Detinova 1955, Hien 1976, Hawley 1985), seasonal variation (Shannon and Hadjinicalao 1941, Detinova 1955), and blood meal type (Shelton 1972, Jalil 1974, Downe and Archer 1975, Nayar and Sauerman 1975).

Laboratories rearing mosquitoes for research all use some type of larval diet. Many use highly specific food that is mixed for optimum concentration of protein, carbohydrates and other essential amino acids and nutrients while others utilize ground fish food or other dry food. Larva need to be kept in rearing pans that are age specific and not crowded to limit competition for food resources. Different mosquitoes require different larval densities, nutritional requirements, and water quality. Larval rearing temperature is important in producing optimum size adults. The relationship between larval growth and temperature has been investigated extensively and can be summarized as: larval growth and development occur within a temperature range, with a lower developmental threshold and an upper lethal temperature; temperature ranges vary with species; larval growth and development is positively correlated with an increase in temperature for most of the temperature range (Clements 1992).

Feeding Mechanisms. Most female mosquitoes require blood, for egg production, and plant juices, for energy. Plant sugars are a major food source providing an important energy source during the adults life span. Ingested plant juices are stored in the crop and digested in the midgut. Mosquitoes commonly ingest more than their own weight in blood, the mean blood meal weight can be from two to four times the weight of the female (Nayar and Sauerman 1975). The mosquito abdomen is able to stretch to accommodate the ingestion of the large quantity of blood. Anopheline mosquitoes are unable to grossly distend their midgut and abdomens with ingested blood. Certain species use diuresis to increase the protein intake of the limited blood meal. Certain species discharge fluid from the anus during feeding. Since the erythrocytes contain the protein

requried for egg production, the discharge of excess fluid and hence the concentration of the erythrocyctes increases the amount of protein a female ingests in a single blood meal.

Disease Transmission. Anopheline mosquitoes are vectors of malaria parasites of humans, human filarial nematodes and very few arboviruses (Gillies and DeMeillon 1968, Gillies and Coetzee 1987). The relationship between Anopheline mosquitoes and the human malaria parasites is exclusive. The malaria parasite undergoes sexual reproduction within the mosquito, thus without this intimate relationship the malaria cycle would be broken. Human pathogens vectored by members of the Culicinae subfamily include: filarial nematodes and arboviruses.

Cimicidae. The family Cimicidae includes bed bugs, swallow bugs, poultry bugs, bat bugs, and others. The wings are reduced to inconspicuous pads. The bodies are broad and flat, enabling the bugs to creep into narrow crevices. These insects are night prowling and hematophagous, feeding on birds and bats, but some occasionally fed on humans. Usinger (1966) described 74 species representing 22 genera and 6 subfamilies. Others have been described since then (Harwood and James 1979). Many are local in distribution and are of little medical importance, but two have followed man, a unusual host for the family, over a large part of the world. These are *Cimex lectularius*, the common bed bug, a cosmopolitan species of both hemispheres but particularly occurring in the temperate regions, and *Cimex hemipterus*, the tropical bed bug, found in both hemispheres but essentially a species of the tropics (Harwood and James 1979).

Biology. The bed bug is nocturnal in its feeding habits, hiding in cracks and crevices during the day. In its nighttime activity it leaves its hiding place, often traveling considerable distances to feed on hosts. Ordinarily, the bugs stay close to wooden bedsteads. Seams in mattresses and box springs commonly afford harborage. Bed bugs are gregarious; great assemblages may be found in some convenient crevice or beneath some nearby loose wallpaper. Infestations of bed bugs are traceable to introductions of eggs, young, or adults. One impregnated female might furnish the nucleus for a well developed colony within a few months. Bugs in all stages are easily carried in clothing, traveling bags, suitcases, or laundry, and they may be introduced with second hand beds, bedding and furniture. They may pass from the clothing of one person to another on crowded public vehicles (Harwood and James 1979).

The adult *C. lectularius* is reddish brown in color, whereas the young are yellowish white. The female deposits eggs in batches of 10 to 50, totaling 200 to 500, spread out in a yellowish patch (Harwood and James 1979). The eggs are relatively large and yellowish white in color. The young hatch in 4 to 21 (usually about 10) days. The time required for development from egg to maturity is 37 to 128 days, under favorable laboratory conditions; these, however, are seldom, if ever, realized in nature. All instars, particularly the later ones, can endure prolonged starvation (80 to 140 days, depending on the instar and sex; the higher figure being for adult males); this of course lengthens the life cycle (Harwood and James 1979). Bed bugs molt five times, and the minute wing pads characteristic of the adult insect make their appearance with the last molt. Ordinarily one meal is taken between each molt and one before egg deposition;

an average of 8 days is required between each molting (Harwood and James 1979).

Rearing. For maintenance purposes colonies may be limited to one blood meal per week. However, for maximum egg production, rapid development of the nymphal stage and availability of large numbers of bugs for experiments, blood meals should be offered two or three times per week (Harwood and James 1979). After a full blood-meal has been ingested the time required until molting depends on the temperature of the environment, the amount of blood ingested above the minimum required for molting, the particular instar, individual variations of the insects themselves, and perhaps also on the amount of activity subsequent to feeding: for the more active insect will utilize a greater proportion of blood to supply energy for movement and less will remain for growth (Johnson 1941). The lower threshold for hatching, nymphal development, and adult activity in *C. lectularius* is between 13 degrees C and 15 degrees C (Hase 1930, Mellanby 1935, Kemper 1936, Johnson 1941), although the insect can tolerate extremes as low as -15 degrees C for brief periods (Usinger 1966). The thermal death point is between 44 degrees C and 45 degrees C (Usinger 1966), but Omori (1941) found that development ceased, especially with *C. lectularius*, at 36 to 37 degrees C. Johnson (1941) found the total period of development to range from 36.6 days at 33 degrees C to 127.9 days at 18 degrees C. Burden (1966) found in searching the literature that temperatures of 23.9 to 29.4 degrees C and relative humidities of 26% to 70% have been used for colonization.

In *C. lectularius* the frequency of food intake is closely correlated with egg-laying, molting, longevity, and temperature, which affects all other processes including rate of digestion (Usinger 1966). Larger bugs take larger blood meals and produce more eggs. A mated female will feed to repletion in about 5 to 10 minutes and then retreat to a harborage to remain quiescent while digesting the blood meal and developing eggs. At 23 degrees C, the time after feeding until the first eggs were laid was 5 to 6 days, and oviposition lasted for 6 days, producing 6 to 10 eggs (Johnson 1941, Usinger 1966). Kemper (1936) found that young adults, at room temperatures 18 to 20 degrees C, were stimulated to search for another blood meal at approximately weekly intervals, while at 27 degrees C the time was reduced to about 3 days.

Various warm-blooded animals and feeding methods (Girault 1910, Janisch 1933, Rendtorff 1938, Busvine 1958, Gratz 1958, Adkins and Arant 1959, Gilbert 1964) have been used in the laboratory colonization of bed bugs. The relative values of the blood of man, fowl, rabbit, and mouse have been discussed by Johnson (1937, 1940). Johnson (1941) found that under laboratory conditions larger meals were taken from mice (probably on account of its high body temperature) than from man or fowl, and it was this, presumably, that induced a relatively rapid rate of development. Adult bugs bred on mice, perhaps because of the larger meals taken, are heavier than those bred on man or fowl and associated with this is a slight increase in fecundity (Johnson 1941).

Feeding Mechanism. The bite of the bed bug is produced by piercing organs of the hemipteran type. It is probable that punctures by these stylets, unattended by contamination or specific poisons, would produce little pain.

Dickerson and Lavoipierre (1959) described the feeding mechanics of the bed bug. The labium, a sheath around the fascile of piercing stylets, does not enter the skin, although it plays an important part in the act of feeding, as its lip-like tips grasp the fascicle in a pincer-like grip and helping to steady it as it is thrust into the tissues. Having entered the skin, the fascicle, which is extremely flexible, readily probes in various directions, moving forwards and backwards and at times bending well over 90 degrees. While probing, the fascicle often pierces, cuts across, and sometimes enters, minute capillaries and larger vessels, without ceasing its restless movement. This active probing results in the formation of small and large hemorrhages in the tissues, but bugs have seldom been observed to feed from them. The fascicle continues its active movements in the tissues until it encounters and enters a vessel of suitable calibre, from which the blood meal is taken up. The bed bug is able to engorge itself completely with blood in 3 to 10 minutes (Harwood and James 1979).

Persons bitten by bed bugs are affected in various ways. In some the bite produces marked swellings and considerable irritation; in others not the slightest inconvenience is caused (Harwood and James 1979). The welts and local inflammation are caused by allergic reactions to the saliva that is introduced early in the act of feeding.

Disease Transmission. Bed bugs meet all the criteria for human disease transmission, i.e., they are obligate bloodsucking parasites that have close and frequent contact with humans (Gubler 1991). The fact that bed bugs must feed at least four and usually five times, upon either the same or different hosts, to reach maturity, has placed these insects under suspicion as potential vectors of

disease causing organisms (Harwood and James 1979). Bed bugs have been suspected in the transmission of 41 human diseases, the agents, or causes of which coincide with 10 disease categories (Burton 1963, Usinger 1966). Many of the causative agents of disease have developed or remained alive within the bed bug after experimental infection for varying numbers of days (Burton 1963). This does not necessarily mean that these organisms are transmitted by bed bugs under natural conditions. Experimentally they have become infected with a variety of pathogens, but to date, they have not been incriminated in transmission of any human disease (Strickland 1991).

Reduviidae. The family Reduviidae is composed of two biological groups, the hematophagous species of the subfamily Triatominae which are ectoparasites on vertebrates, and the nineteen or more additional subfamilies which are predaceous on arthropods (Ryckman and Ryckman 1966, Gubler 1991). The subfamily Triatominae consists of 100 species in 15 genera (McClelland 1992). Most species of bugs are sylvatic and live in close association with a variety of wild animals, e.g., armadillos, opossums, mice, rats, bats, and squirrels. Others live in close association with humans and domestic animals. The bugs usually lay their eggs in or near the habitation of the host. *Rhodnius prolixus* is the most commonly studied species of Triatominae.

Biology. The Triatominae have five nymphal instars and need to feed only once to repletion before each molt. The life cycle of *R. prolixus* takes about six months at 28C (Gardiner and Maddrell 1972). In instars, one blood meal up to nine times their body weight is taken per molt cycle; adults take meals of up to three times their body weight each time they feed (Friend et al. 1965b) A blood

meal of this size will cause the insect to molt if it is a instar, or to produce eggs if it is a fertilized female adult; it will also sustain the insect for periods of at least 90 days (Friend 1965a).

Rearing. Laboratory culture methods have been described by Larrousse (1927), Galliard (1935), Dias and Philip (1938), Usinger (1944), Grundemann (1947), Abalos and Wygodzinsky (1951), Ames et al. (1954), and Ryckman (1952, 1954, 1965, 1966). Artificial feeding experiments by Núñez and Segura (1987) observed no significant difference between heparinized guinea pig or sheep blood and excluded citrate as an anticoagulant. Greater adult size and consequently higher fecundity resulted when feeding occurred on two successive days (Patterson 1979). The mechanical damage to erythrocytes and the potential alteration of the ionic balance probably essential for this type of hematophagous insect must be taken into account when using defibrinated blood (McGuire et al. 1973).

The voluminous meal needs to be initially digested before being absorbed, as in other animals. Blood is the only nutrient the triatomines need for molting, metabolism, and egg production (Buxton 1930). Since more than 50% of the blood proteins are inside the erythrocytes, the mechanism of hemolysis is an important step for digestion (Garcia 1987). Normally, within 4 days after the meal, most erythrocytes are lysed and the hemoglobin is crystallized in the crop. This process is observed in all instars and adults (Baur et al. 1979, Smit 1983). Garcia (1987) found free amino acids are found in small quantities in the blood meal. Amino acids are constituents of proteins, which must be hydrolyzed before the amino acids are available to insect cells. Protein digestion occurs in

the anterior intestine of the triatomines, which has a quite different mixture of proteolytic enzymes with pH optima in the acidic range. The proteolytic enzymes (proteases) attack peptide bonds in proteins and peptides. They are usually divided in proteinases (endopeptidases) and peptidases (exopeptidases). By the sequential action of these proteases, ingested proteins are hydrolyzed and their complete breakdown is ensured to yield a mixture of free amino acids which are then rapidly absorbed and incorporated into insect proteins.

Feeding Mechanism. Triatomines are bloodsucking bugs of immoderate feeding habits. Given the opportunity, these insects quickly, skillfully, and painlessly obtain a blood meal from a host (Garcia 1987). Factors known or suspected to affect feeding behavior in *R. prolixus* include: (a) external cues (e.g., temperature gradients, carbon dioxide, odors, movement, vibration, surface texture, standing liquid) (Wigglesworth and Gillett 1934a, Wigglesworth and Gillett 1934b, Lavoipierre et al. 1959, Friend 1965a, Friend and Smith 1971, Friend and Smith 1972, McGuire et al. 1973); (b) diet composition (e.g., the nature and concentration of phagostimulatory nucleotides, osmotic pressure, pH, ion species and concentrations), which determines whether or not engorging occurs (Friend 1965a, Friend and Smith 1971, Friend and Smith 1972, Smith and Friend 1972, Smith and Friend 1976a, Smith and Friend 1976b, Friend and Smith 1977a); (c) length of time since previous meal (Friend and Smith 1975); and (d) degree of abdominal stretch (Maddrell 1963, Anwyl 1972). Consequently rigidly controlled and uniform conditions of rearing and handling need to be employed in the rearing and handling of *R. prolixus*.

The orientation and feeding process in *R. prolixus* has been described (Wigglesworth and Gillett 1934b) as a "chain of reflexes," in which the initiation of the response is conditioned by the physiological state of the insect. The insect is aroused from a state of akinesis by the antennae responding to air currents or heat (Friend and Smith 1977a). It is then attracted to the host, natural or artificial, by heat and perhaps odor stimuli perceived by receptors on the antennae. During probing, the tip of the labium is applied to the skin surface. The mandibles penetrate the surface, and the maxillae, which form the food and salivary canals, are then inserted in a series of thrusts and withdrawals during which saliva is continuously ejected from between their tips (Friend and Smith 1977a). After one or more samplings of the available fluid, ingestion of the diet commences in the presence of suitable phagostimulants. Feeding stops when a critical abdomen volume is reached; this is controlled by abdominal stretch receptors (Maddrell 1963, Anwyl 1972) rather than by back pressure on the pharyngeal pumps.

Disease Transmission. Triatomine are known to transmit *Trypanosoma cruzi*, causative agent of Chagas' Disease, by the defecation of infective material on a susceptible host. All known vectors of *T. cruzi* comprise about 50 species (out of 100) in nine (of 15) genera in the subfamily Triatominae of the family Reduviidae(McClelland 1992). Transmission is usually associated with the bite of the bug, which often takes 10 to 20 minutes to engorge and frequently defecates during or shortly after the feeding process (Gubler 1991). Infection of the new host is usually accomplished by scratching the infective trypanosomes into the bite wound or into other skin abrasions or, most commonly, by

transferring the agents on fingers to the highly receptive conjunctiva of the eye or to the mucosa of the mouth or nose (Gubler 1991). Bugs which are slow to defecate will leave the host before excreting urine rich in infective metacyclic trypanosomes (McClelland 1992).

PURPOSE OF RESEARCH

The overall goal of this research project was to determine the persistence of HBV genome in several hematophagous arthropods. Previous studies, utilized primarily EIA/RIA methodologies testing for the presence of HBsAg. The detection of HBsAg showed ingestion of the viral particles during feeding on either a viremic hosts or artificial feeders. However, the significance of the continued detection of the HBsAg over varying time periods is debatable. Is it an artifact of the original blood meal? or, New viral particles? Due to the unique constraints of the virus, a vector competency study was impractical as it would require an animal model with a hepadnavirus. With the use of molecular detection methods regions of the HBV genome can be detected. By testing for pre-C and C regions of the genome, only regions of the genome coding for the infectious particle regions would be detected. However, the detection of the viral genome does not signify infectivity or transmissibility. The persistence of the virus and its dissemination in the insect may signify that the vector has been infected. The insect must then be able to mechanically or biologically transmit the virus to another susceptible host to be a competent vector.

The purpose of this research is to evaluate the persistence and dissemination of, ingested and inoculated, hepatitis B virus, by PCR and Southern blot, in *Cimex lectularius* L. (Hemiptera: Cimicidae), *Rhodnius prolixus* Stål (Hemiptera:

Reduviidae), *Aedes triseriatus* (Say) (Diptera: Culicidae), *Culex quinquefasciatus* Say (Diptera: Culicidae) and *Anopheles stephensi* Liston (Diptera: Culicidae). The specific objectives to be tested are: (1) persistence of hepatitis B virus in the saliva or on mouthparts of *R. prolixus, Ae. triseratus, Cx. quinquefasciatus* and *An. stephensi*; (2) persistence of hepatitis B virus in the legs of *R. prolixus, Ae. triseriatus, Cx. quinquefasciatus An. stephensi.* and *C. lectularius*; (3) persistence of hepatitis B virus in the feces of *C. lectularius, R. prolixus, Ae. triseriatus, Cx. quinquefasciatus* and *An. stephensi*; (4) persistence of hepatitis B virus in the bodies of *C. lectularius*, *R. prolixus, Ae. triseriatus, Cx. quinquefasciatus* and *An. stephensi*; and (5) persistence of hepatitis B virus trans-stadially and trans-ovarially in *C. lectularius.*

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Chapter 1

Persistence of the Hepatitis B viral genome in Three Species of Diptera: Culicidae

INTRODUCTION

Hepatitis B infection is a major cause of acute and chronic hepatitis, cirrhosis, and primary hepatocellular carcinoma (PHC) world wide (CDC 1990) killing an estimated 2 million people each year (Hamilton 1994). Primary hepatocellular cancer is one of the most common cancers in Asia and sub-Saharan Africa (Beasley 1988) where 8-15% of the population carry markers of HBV infection (Maynard 1981). Persons known to be at risk of contracting hepatitis B include: sexually active men and women; prison inmates; health care and public safety workers; hemodialysis patients; international travelers; and household contacts of HBV carriers. Primary modes of transmission are parenteral, perinatal, and sexual contact, although, 30 - 40% of people infected with HBV have no identifiable risk factor (Alter and Mat 1994).

Neefe (1949) was the first to consider the possibility that hematophagous arthropods could transmit the hepatitis virus. Prince (1970) found an increased prevalence of SH (hepatitis B surface antigen) antigenemia in the topics but was unable to account for this by known mechanisms of transmission, and suggested transmission in nature by nonparenteral routes. Of the potential routes discussed, the possibility of transmission by hematophagous arthropods was thought the most probable. The subsequent detection of the hepatitis B surface

antigen (HBsAg) by solid-phase radioimmunoassay in wild caught mosquitoes supported this hypothesis (Prince et al. 1972).

Numerous researchers have since investigated the possible role of mosquitoes in the transmission of hepatitis B virus under varying conditions. However, all of these studies used detection assays for the hepatitis B surface antigen (HBsAg) which is present on the surface of all three particles produced by the hepatitis B virus. Only one of the three particles contains virions and is thus infectious (Blumberg et al. 1965). The concentration of non-virion associated HBsAg greatly exceeds the concentration of complete virions by 10^4 per milliliter or greater. The results and interpretations from these experiments are contradictory. Some authors (Hawkes et al. 1972, Leevy et al. 1972, Prince et al. 1972, Brotman et al. 1973, Muniz and Micks 1973, Ishii et al. 1974, Wills et al. 1976, Chen et al. 1987, Fouche et al. 1989, Fouche et al. 1990, Jupp et al. 1991) considered mosquitoes to be potential mechanical vectors of hepatitis B virus, while others (Smith et al. 1972, Metselaar et al. 1973, Wills et al. 1976) claimed that they are biological vectors of the virus. No evidence implicating mosquitoes as potential vectors of hepatitis B virus could be found by other authors (Skinhoj 1972, Byrom et al. 1973, Newkirk et al. 1975, Berquist et al. 1976). Still other authors (Blumberg et al. 1973, Tin et al. 1973, Dick et al. 1974, Papaevangelou and Kourea-Kremastinou 1974, Hyams 1989) found the results to be inconclusive, requiring more in detailed study.

Several investigators (Leevy et al. 1972, Brotman et al. 1973, Newkirk et al. 1975, Chen et al. 1987) found a direct correlation between antigen disappearance and blood meal digestion. Other investigators have detected

HBsAg after 6 days (Muniz and Micks 1973), 7 days (Tin et al. 1973), 10 days (Smith et al. 1972), and 5 weeks (Metselaar et al. 1973).

Due to the host specificity of the virus few transmission studies have been conducted. The results of the studies that have been conducted are contradictory. No evidence of mechanical or biological transmission was found in studies using human volunteers (Neefe 1949) and chimpanzees (Berquist et al. 1976), however, evidence of transmission was found in studies using chickens (Smith et al. 1972) and monkeys (Yuhuang et al. 1995). Smith (1972) detected HBsAg by immunofluorescent staining in chicken subcutis immediately after the chickens were bitten by infected mosquitoes. Yuhuang et al. (1995) reported that 9 of 29 monkeys bitten by infected mosquitoes were positive for HBV serum markers. The validity of this study, however, is questionable. The mosquitoes were fed an infectious blood meal via an artificial feeder and after blood meal digestion were allowed to feed on the abdomen of a Tupaia Belangeri monkey. The study gives the detection of hepatitis markers for the monkeys but not for the mosquitoes. Nor does the study address the question of potential mechanical transmission of the virus due to infectious blood on the mosquito cages. To date a conclusive study has not been published that explicitly shows mosquitoes are capable of transmitting the hepatitis B virus.

Still other researchers (Whittle et al. 1983, Foster et al. 1984, Tabor et al. 1985, Mayans et al. 1990) have examined the problem from an epidemiological perspective. Hepatitis B infections in Africa commonly occur after one year of age (Tabor et al. 1985), whereas, in Asia many children are infected at birth by their mother (Stevens et al. 1975). Tattooing, circumcision, ear piercing,

traditional scarring, exudative skin lesions, and hematophagous arthropods have all been proposed as mechanisms of transmission. Mayans (1990) found a significant association between hepatitis e (HBe) antigenaemia and the presence of bed bugs in children's beds. However, a later intervention study (Mayans et al. 1994) found little epidemiological evidence to support bedbugs as the major mode of transmission of hepatitis B virus in Gambian children.

The purpose of this study was to determine the persistence of the hepatitis B virus in *Anopheles stephensi*, *Aedes triseriatus*, and *Culex quinquefasciatus* that had ingested or been inoculated intrahemocoelly with the virus. Polymerase chain reaction and Southern blotting was used to detect a segment of the hepatitis B viral genome.

MATERIALS AND METHODS

Insect Rearing. *Anopheles stephensi* and *Aedes triseriatus* colonies were reared at 24 degrees C, with 30% humidity and a photoperiod of 16:8 (L:D) h. Stock colony adults were held in 12 X 12 X 12 screened cages with raisin water for a sugar source, and blood fed weekly on anesthetized rats. Larvae were reared in 500 ml plastic pans with lids and given ground fish food.

Anopheles stephensi: Three days post blood feeding, a wet oviposition paper was placed in the cage. Eggs were rinsed off the paper into a pan of distilled water to hatch. First and second instars were fed a diet of suspended yeast. Older larvae were fed Tetra Min Baby fish Food "E." Larval density was monitored in the pans and larvae were sorted into new pans when they were gauged to be too dense. Pupae were collected and placed in an emergence cage at 6 days prior to the start of an experiment. Females, one to six days old,

were transferred to experimental feeding containers on the day prior to an experiment and wet gauze was placed on the mesh lids to provide humidity.

Aedes triseriatus : A oviposition cup with egg paper as a liner was placed in the stock colony cage and left for 3-5 days after blood feeding. The cup was removed from the cage and allowed to sit with water for another 1-3 days to allow embryonation to occur. The egg paper was removed from the cup and allowed to dry. Dried egg papers were stored at room temperature with low humidity. Egg papers were placed on wet paper towels 24 hours prior to hatching. The egg paper was then placed in deoxygenated water, as a hatching stimulus, and suspended liver powder was added. Newly hatched larvae were transferred to 500 ml pans the next day and fed the suspended liver powder until pupation. Larval density was monitored in the pans and larvae were sorted into new pans when they were gauged to be too dense. Pupae were collected and placed in an emergence cage at 6 days prior to the start of an experiment. Females, one to six days old, were transferred to experimental feeding containers on the day prior to an experiment. Wet gauze was placed on the mesh lids of these containers to provide humidity.

Culex quinquefasciatus: The *Culex quinquefasciatus* were obtained from the stock colony at the U.S. Army Medical Research Institute for Infectious Disease (USAMRIID), Ft. Detrick, Maryland. Adult females were collected and placed in an experimental container immediately prior to intrathoracic inoculation.

Blood Feeding on Animals. Stock colonies were fed weekly on rats. The All University Committee on Animal Use and Care approved the use of rats to feed the insect colonies, AUF# 05/96-053-00. Rats where selected based on

willingness of both Diptera and Hemiptera to feed on them and the ability to maintain a sufficient number of rats to feed the all the insect colonies necessary to conduct this research.

Cages of *An. stephensi* and *Ae. triseriatus* were transported from the Natural Science Building to the Giltner animal room, a University Laboratory Animal Resource (ULAR) facility. Cages were placed directly in front of the room vent. Rats were restrained in surgical stocking and wrapped loosely in a towel and placed under the scavenger hood. Methoxyflurane was administered with a nose cone until the rat became fully relaxed at which time the towel and surgical stocking were removed. The abdominal area was shaved and sterile eye ointment administered to the eyes in preparation for feeding. The rat was then laid on its stomach on the mosquito cage with its nose in the nose cone for approximately 20 minutes. Rats' respiration rate was monitored throughout the feeding period. Rats were removed from the cage and allowed to expire the anesthetic before being placed in a recovery cage. Recovering rats' respiration and state of consciousness were monitored until each rat was awake, then it was returned to its own cage.

Rats were used once every two weeks for feeding. Any rat not appearing active and healthy was removed from the feeding rotation until examined by ULAR personnel. Any rat that developed a suspected tumor or possible irritation due to feeding was removed from the feeding rotation and the ULAR veterinarian notified immediately.

Artificial Blood Feeding. Stock mosquito colonies were fed by the artificial feeder and all infective feedings were done using the artificial feeder. A

Hemotek 5W1 artificial feeding system (Discovery Workshop, England) was used for all feedings. The system consists of 5 independently controlled, heated feeding chambers. Each feeding chamber holds 5 milliliters of blood. Three different types of feeding membranes (parafilm, latex, and cellulose) were used for colony maintenance. A cellulose membrane was used exclusively during infected feedings. The membrane was stretched across the feeding chamber and secured with an O-ring. The chambers were filled with blood, capped and screwed into the heating element, then placed on the cage for feeding.
unit can supply power to five independently controlled heating elements.

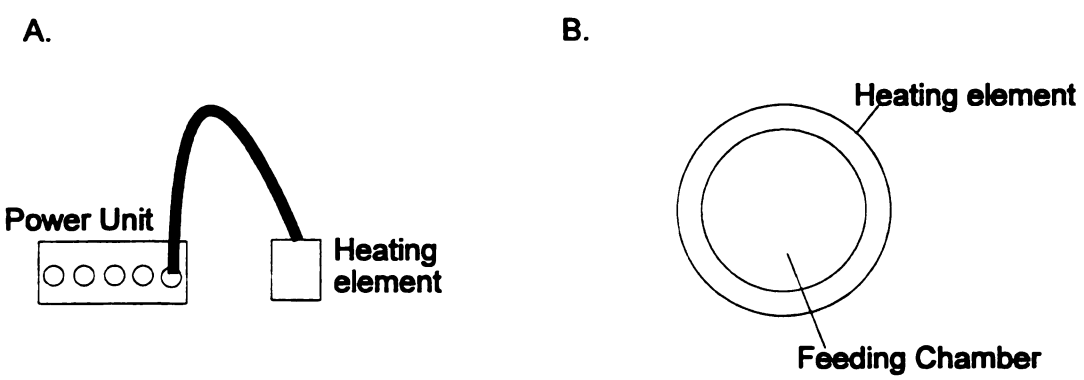

Figure 1. Diagram of Hemotek 5W1 artificial feeding system. A) The power unit can supply power to five independently controlled heating elements. B) Each feeding chamber was covered with a membrane and filled holds 5 milliliters of blood.

Bovine or equine blood was used for all feedings. Mature bovine blood preserved with heparin was obtained in 100 ml aliquots from Pel-Freez Biologicals, (P.O. Box 68, Rogers AR 72757). Mature equine blood preserved with heparin was obtained in 100 ml aliquots from Veterinary Dynamics Inc., (1535 Templeton Road, Templeton CA 93465). Blood was collected and shipped over night on wet ice at 4 degrees C and then stored at 4 degrees C in the laboratory. Blood was kept no longer than 30 days from date of drawing from the bovine or equine.

Hepatitis B Virus Infected Blood Collection and Transport. Blood was collected by venipuncture from a HBV positive patient by the Department of Gastroenterology-Hepatology, William Beaumont Hospital, Royal Oak, Michigan, after obtaining informed consent. The collection has been approved by both William Beaumont Hospital and Michigan State University, University Committee on Research Involving Human Subjects (UCRIHS) IRB # 96-039. Non-infected blood was collected by the Medical Entomology Laboratory, Department of Entomology, Michigan State University after receiving informed consent. The blood was transported on ice from William Beaumont Hospital to Michigan State University. The blood was centrifuged and serum removed and frozen at -70 degrees C until use.

Quantitative hepatitis B viral levels were determined by the Abbott hepatitis B viral DNA Assay (Abbott Laboratories, Abbott Park, IL) a liquid phase molecular hybridization assay for quantifying hepatitis B virus DNA in human serum. Results are reported in picograms of DNA per milliliter (pg/ml) of serum.

Experimental Insect Feeding, Handling and Containment. All cohort feeding was conducted using a single Hemotek feeding chamber in a Labconco glove box rated to Biosafety Level III (inspected by Michigan State University, ORCBS, Environmental Services 17 April 1996). The chamber was loaded with infectious blood in the same manner as in routine feeding. The chamber used for the infected feeding was soaked in 10% bleach solution and then autoclaved to sterilize it after use. Engorged mosquitoes were transferred by bulb aspirator from the feeding container to a clean container with filter paper in the bottom. All feeding containers were autoclaved, placed in biohazard bags, and autoclaved again prior to disposal. Infected cohorts were kept in the sealed Labconco glove box with humidity pads for the duration of the experiment.

All cohorts, control and infected, were handled in the same manner. Control samples were always collected before the infected samples. A mechanical aspirator was used to collect the sample from the infected cohort containers. The aspirator chamber was sealed with tape and placed in a -4 degrees C freezer to chill the mosquitoes. Immobilized mosquitoes were transferred to a labeled petri dish in the freezer. The petri dish was then placed on ice and taken to the bench top work space. Individual mosquitoes were removed from the petri dish with heat sterilized forceps and placed on a clean microscope glass slide. Wings and legs were removed with a sterile scalpel and the mosquito was then placed on a sticky piece of tape to restrain movement. Legs were collected and placed in a labeled 1.5 ml eppendorf tube. The proboscis was then inserted into a microcapillary tube containing tissue culture broth, either E-199 or TM100, to stimulate salivation. After 5 minutes the microcapillary tube was removed from

the proboscis and the tissue culture broth was expelled into a labeled 1.5 ml eppendorf tube. Finally, the body was placed in a labeled 1.5 ml eppendorf tube. All samples were then frozen at -70 degrees C until DNA extraction. Filter paper used to collect feces was removed at the designated time points, placed in labeled petri dishes, and frozen at -70 degrees C. Clean filter paper was then placed in the containers.

Experiments

Sensitivity of PCR and Southern blot assays for the hepatitis B virus. To determine the sensitivity of the PCR and Southern blot assays a ten-fold serial dilutions of the HBV serum (1485 pg/ml) were made. One picogram (pg) of HBV DNA equates to 2.8×10^5 genomic equivalents thus, approximately 1 picogram of HBV DNA per milliliter of serum equals 2.9×10^5 copies/ ml or 290 copies/ul (Hollinger 1996). Therefore, 1 ul of the 1485 pg/ml serum used in the dilution contained approximately 400 copies of the HBV DNA. This serum was then serially diluted beyond the theoretical end point of one viral particle. A second set of serial dilutions was made and a whole mosquito was added to each tube to determine the level of detection in mosquitoes. A third set of serial dilutions was made and dispensed onto a piece of filter paper to determine the level of detection from filter paper. The dilution tubes were then handled in accordance with extraction and detection methods.

Persistence of ingested Hepatitis B virus. *Anopheles stephensi* and *Aedes triseriatus* cohorts were fed either an infective or control blood meal by artificial feeder. The HBV blood meal consisted of 1 ml HBV serum (1495 pg/ml HBV-DNA), 2 ml horse serum and 2 ml horse red blood cells. The control blood meal

consisted of 1 ml HBV negative human sera, 2 ml horse serum and 2 ml horse red blood cells. Both mixtures were loaded into separate Hemotek W51 feeding chambers and respective mosquito cohorts allowed to feed.

Experiment 1: Persistence of ingested HBV in mosquitoes. *An. stephensi* and *Ae. triseriatus* that ingested a HBV infected blood meal were collected at 3, 7 and 14 days post infected feeding. Collections were made within 2 hours of the time of the experimental feeding.

Experiment 2: Persistence of ingested HBV in mosquito feces. Collection of fecal paper from *An. stephensi* and *Ae. triseriatus* cohorts from experiment 1. Filter paper was identified by its collection time. The fecal filter paper contained feces only from the previous collection time to the labeled collection time.

Experiment 3: Persistence of ingested HBV in mosquitoes. *An. stephensi* and *Ae. triseriatus* that engorged on a HBV infected blood meal were collected immediately after feeding (time point 0), 6, 12, 24, 48, and 72 hours post feeding.

Experiment 4: Persistence of ingested HBV in mosquitoes feces. Collection of fecal paper from *An. stephensi* and *Ae. triseriatus* cohorts from experiment 3. Filter paper was identified by its collection time. The fecal filter paper contained feces only from the previous collection time to the labeled collection time.

Experiment 5: Transmission potential of refeeding *An. stephensi*. *An. stephensi* that engorged on a HBV infected blood meal were held for 3 days and then offered the opportunity to fed on a negative blood meal. Engorging mosquitoes, remaining blood, feeding membrane, and fecal paper were all collected.

Persistence of inoculated hepatitis B virus. *Anopheles stephensi, Aedes triseriatus*, and *Culex quinquefasciatus* were given intrathoracic inoculations of 0.3 ul of either an HBV or control suspension. The HBV suspension consisted of 1 part HBV serum (>2000 pg/ml HBV-DNA) and 5 parts E-199 tissue culture broth mixture. Thus, each 0.3 ul HBV inoculation theoretically contained 30 viral particles. The control suspension consisted of 1 part negative serum and 5 parts E-199 tissue culture broth mixture. Inoculations were performed by Dr. Michael Turrell, USAMRIDD, Ft. Detrick, Maryland.

Experiment 6: Inoculated *Anopheles stephensi, Aedes triseriatus*, and *Culex quinquefasciatus* were collected at time 3, 7, 14 and 21 days as described above.

Experiment 7: Collection of fecal paper from *Anopheles stephensi, Aedes triseriatus*, and *Culex quinquefasciatus* cohorts from experiment 6. Filter paper was identified by its collection time. The fecal filter paper contained feces only from the previous collection time to the labeled collection time.

Methods

DNA Extraction and Detection Procedure. DNA was extracted from the insect using DNAzol™ and Polyacryl Carrier (Molecular Research Center). DNAzol™ is a ready to use reagent for the isolation of genomic DNA. The DNAzol™ procedure is based on the use of a novel guanidine-detergent lysing solution which hydrolizes RNA and allows the selective precipitation of DNA from a cell lysate. Polyacryl Carrier is a molecular biology grade solution of acryl polymer designed for use in the isolation of small amounts of DNA with DNAzole™.

Polyacryl carrier does not affect the activity of restriction enzyme, reverse transcriptases, TAQ polymerases, DNA polymerase, ligase and other enzymes use for DNA analysis.

Two hundred fifty microliters (ul) of DNAzole™ and 2.5 ul of Polyacryl Carrier™ were added to the tubes containing mosquito legs and bodies. The samples were homogenized with a pestle. Five hundred microliters of DNAzole™ and 5 ul of Polyacryl Carrier™ were added to the tubes containing tissue culture broth and saliva. Individual fecal spots were cut out of the filter paper. Filter paper that contained numerous spots was cut into pieces no larger than 2 mm X 2 mm. Four to five fecal spots or one piece of paper were placed in a labled eppendorf tube. Seven hundred and fifty microliters of DNAzole™ and 15 ul of Polyacryl Carrier™ were added to tubes containing fecal filter paper, tubes were centrifuged for 10 seconds at 10,000 rpms, and placed in a 4 degrees C refrigerator for overnight extraction of the feces from the filter paper. Genomic DNA was precipitated from the lysate with an equal amount of ice cold 100% ethanol. The precipitate was then washed twice with 95% ethanol and allowed to dry. Precipitated DNA was reconstituted with 10 ul HPLC water, concentrated by centrifugation and frozen at -20 degrees C until use. In this condition the isolated DNA was ready for analysis without additional purification. (See Appendix A for detailed procedure).

Oligonucleotide primers for PCR. The majority of the primers used for polymerase chain reaction (PCR) provide amplification of the C (HBc) and S (HBs) genome regions. Analysis of the hepatitis B virus genome revealed regions that appear to be highly conserved (nucleotides 241 to 270, 1891 to

1920, 2431 to 2460). (Robertson 1995). The oligonucleotide primers used in these experiments amplify the conserved pre-core and core region, nucleotides1891 to 1920 (Lauder et al. 1993). Primer 1763 5'-GCTTTGGG-GCATGGACATTGACCCGTATAA-3' begins at map position 1763 and primer 2032R 5'-CTGACTACTAATTCCCTGGATGCTGGGTCT-3' (from the complementary reverse [R] DNA strand) begins at map position 2032 of the hepatitis B virus genome (Fujiyama et al. 1983) producing a 270 bp band fragment (Figure 2).

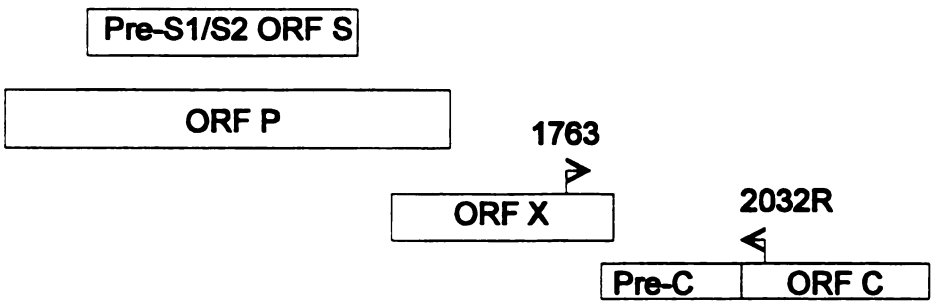

Figure 2. Diagram of primer location in hepatitis B viral genome. Four overlapping open reading frames are present in the DNA. The ORF C (core) region encodes the structural protein of the nucleocapsid and the ORF S/pre-S encodes the viral surface glycoproteins. Primers amplify a conserved core region, producing a 270 bp size fragment.

Polymerase Chain Reaction. Polymerase chain reactions were run in accordance with the procedures outlined by Kaneko et al. (1989). Each run contained at least one positive control, negative control and a blank. A 2.5uL aliquot of DNA extracted from each sample was amplified in a 50µL reaction volume containing 2.5 U of Taq DNA polymerase (Perkin Elmer), 200 µmol/L each of the four deoxyribonucleic triphosphates (Boehringer Mannheim, 100mM pH7, dNTP's Li-salt), 1 µmol/L of the primer pair (primers 1763 and 2032R), 50 mmol/L Tris-HCl (pH 8.3) (Perkin Elmer10X PCR Buffer II), 1.5 mmol/L $MgCl_2$, and 0.01% (wt/vol) gelatin (25mM $MgCl_2$, Perkin Elmer). The reaction was performed for 30 cycles in a programmable DNA Thermal Cycler (Perkin Elmer GeneAmp® PCR System 2400). Samples were heated at 94 degrees C for 1.5 minutes , cooled to 42 degrees C for 1.5 minutes and extended for 3 minutes at 72 degrees C (DNA polymerase extension).

Contamination of the PCR reaction mixture with minute amounts of recombinant hepatitis B virus DNA can result in spurious positive results. To eliminate potential sources of DNA contamination, all reagents were prepared with pipettors designated for reagent use only and dispensing was done in a laminar flow hood. No infectious samples were processed in the hood, and the ultra-violet light was turned on prior to preparing PCR reagents in the hood. The PCR reaction mixture was dispensed into PCR tubes in the hood and the tubes were closed. Tubes were then taken to the bench top and sample DNA was added there. Counter tops were disinfected with 10% bleach solution before and after any procedures. Autoclavable Oxford® Benchmate digital pipettors were used with aerosol resistant tips to dispense reagents and specimen.

All PCR products were separated on 2% agarose gels with 0.5 µg/ml ethidium bromide in a electrophoresis chamber containing 1X TBE (0.9 mM Tris, 0.9 mM boric Acid, .02 mM EDTA) with 0.5 µg/ml ethidium bromide. Amplified DNA was mixed by gently aspirating with the pipettor. Eight microliters of the PCR reaction mixture was mixed with 2 µL of gel loading buffer III 6X (0.25% bromophenol blue, 0.25% xylene cyanol FF, and 30% glycerol in H_2O) and dispensed into the appropriate gel well. Two microliters of a 123bp lambda DNA ladder (Gibco BRL) was mixed with 2 uL of gel loading buffer III and 6uL of HPLC water. The electrophoresis apparatus was adjusted to a voltage of 100 between 60-80 amps of current, allowed to run for at least 1.5 hour, and then the DNA was visualized under ultraviolet light.

Southern Analysis. DNA products were transferred from the 2% agarose gels to nylon membranes by a BioRad Trans-Blot SD semi dry electrophoretic transfer cell (Cat. Nos. 170-3940, 170-3948, 170-3949). Two pieces of extra thick BioRad blotting paper and one piece of nylon membrane (Boehringer Mannheim Cat Nos. 1209-272, 1209-299) were cut to the size of the gel. The gel, membrane, and blotting paper were soaked in 0.5X TBE with no ethidium bromide for 10 minutes then formed into a sandwich built on the anode plate. Each layer was rolled to remove air bubbles prior to adding the next layer in the sandwich. The sandwich, from bottom to top, was composed of blotting paper, nylon membrane, gel, and blotting paper. The sandwich was then saturated with 0.5X TBE and excess TBE was wiped up. The gel frame was placed around the sandwich, the top plate latched down, and the top cover secured completing the circuit. The Trans-blotter was then plugged into the power pack.

The amperage required for blotting was calculated by taking the product of: (length of the gel) X (width of gel) X 0.3. The power pack was set to run on constant amperage and the calculated amperage was set at a 40 minute run time. Voltage was monitored throughout the transfer. A voltage that increased over 25 indicated that the buffer capacity had expired and the run should be stopped. When this occurred, the amperage was either reduced or the process was halted depending upon the amount of transfer time remaining.

When the transfer was completed, the system was disassembled in the reverse order. A pencil was used to mark the wells on the nylon membrane before removing the gel. The membrane was then removed, rinsed in 2X SSC (3mM NaCl and .3 mM sodium citrate), and placed on a piece of extra thick filter paper soaked in 0.4 N NaOH for 5 minutes to break the DNA bonds. The membrane was rinsed again in fresh 2 X SSC and then placed on piece of filter paper that was folded into a pocket and labeled. The DNA was then fixed to the membrane by drying at either 80□C for 2 hours or 120□C for 1 hour. After drying all membranes were stored in a ziplock bag in a 4C refrigerator.

Digoxigenin oligonucleotide labeling and detection. A oligonucleotide internal to the PCR oligonucleotides was used to probe PCR products. Primer 1913 5' -TGTTCACCTCACCATACAGC- 3' began at map position 1913 of the hepatitis B viral genome and was 3'-end labeled with digoxigenin-ddUTP (Boehringer Mannheim Cat. No. 1362-372). The steroid hapten digoxigenin (DIG) was linked via a spacer arm to the primer. The oligonucleotide was enzymatically labeled at its 3' end with terminal transferase using a single DIG-labeled dideoxyuridine-triphosphate (DIG-ddUTP).

The digoxigenin labeled probe was used with the digoxigenin nucleic acid detection kit (Boehringer Mannheim Cat. No. 1175 041) to detect hepatitis B nucleic acids on the Southern blots, by enzyme linked immunoassay using an anti-digoxigenin alkaline phosphatase conjugated antibody. The nylon membranes were labeled and placed in pre-hybridized solution (concentrations) in a shaker over at 39 degrees C for 6 hours. One picomole of DIG-labeled probe was added for every 10 ml's of pre-hybridization solution. The membranes were returned to the 39 degrees C shaker oven for overnight hybridization. After blocking of the membrane, binding of antibody-conjugate to hybrized DIG labeled nucleic acid occurs. The color reaction is initiated alkaline pH by the addition of BCIP and NBT. A blue precipitate starts to form within a few minutes in the positive control bands. Color development was stopped after 1 hour and the membranes were photographed with 35mm 160 Tungsten film on a copy table (See Appendix B for complete protocol).

RESULTS

Sensitivity of PCR and Southern blot detection of HBV.

The addition of mosquito bodies had no effect on the sensitivity of the PCR or Southern detection methods. The PCR had visible bands present at the 1:1000 dilution, 4.0 viral particles, in both the serum and serum plus mosquito lanes (Figure 3). The Southern blot had visible bands at the 1:10000 dilution, 0.4 viral particles, in both the serum and serum plus mosquito lanes. This level of sensitivity was considered adequate as theoretically we were able to detect a single viral particle. The addition of fecal filter paper decreased the sensitivity of the PCR and Southern detection methods hundred fold. The PCR and Southern

blot had visible bands at the 1:10 dilution, 400 viral particles (Figure 4). Nested PCR was not done due to a high rate of false positives and the inability to establish a consistent lower limit of viral detection.

Determination of Virus Persistence and Dissemination. Viral persistence was determined by the detection of virus in the mosquito bodies. A mosquito that had virus detected in its body, but not its legs, was considered to have a nondisseminated viral persistence. The detection of virus in the legs of the mosquitoes indicated that the virus had disseminated (Turrell et al. 1984).

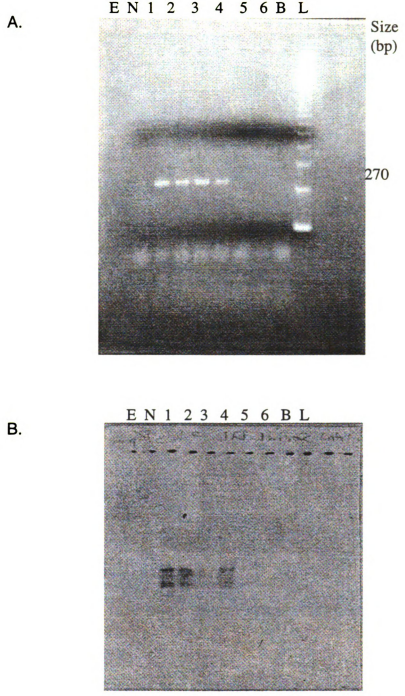

Figure 3. Determination of the effect of mosquito tissue on the sensitivity of HBV detection using PCR. Serial dilution of 1485 pg/ml HBV DNA serum plus mosquito. A) PCR detection. B) Southern blot detection. Lanes: E- empty, N- negative control, 1- straight serum (400 virions), 2- 1:10 (400 virions), 3- 1:100 (40 virions), 4-1:1000 (4 virions), 5-1:10000 (0.4 virions), 6-1:100000 (0.04 virions), B - reagent blank, L-123 bp lambda ladder .

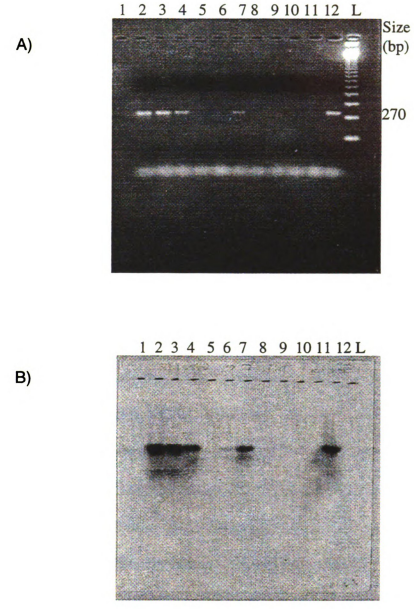

Figure 4. Determination of the effect of filter paper on the sensitivity of HBV detection using PCR. Serial dilution of 1485 pg/ml HBV DNA serum. Lanes: E- empty, A) Agarose gel of PCR products, 270 bp band indicates positive samples. B) Southern blot detection. Lanes:1- empty, 2-1:10 (400 virions), 3-1:100 (40 virions), 4-1:1000 (4.0 virions), 5-1:10000 (0.4 virions), 6-1:100000 (0.04 virions), 7- 1:10 (400 virions), 8- 1:100 (40 virions), 9-1:1000 (4.0 virions), 10-1:10000 (0.4 virions), 11-1:100000 (0.04 virions), 12- positive control, L-123 bp lambda ladder.

HBV Infected Feeding Results. In the first experiment all results for both *An. stephensi* and *Ae. triseriatus* were negative at days 3, 7 and 14 (Table 1). Therefore, a second experiment was conducted to examine the HBV persistence at shorter intervals after feeding. Mosquito legs, saliva, bodies and fecal paper were collected immediately after feeding, 6,12, 24, 48 and 72 hours post feeding. Virus was detected by PCR in 100% of *An. stephensi* bodies at 0 and 6 hours. At 12 hours 60% of the bodies had detectable virus by PCR but 100% of bodies were positive by Southern blot. At 24 hours 70% of the bodies were positive by PCR and Southern blot. At 48 hours only 40% of the bodies were positive by PCR and Southern blot, however, one mosquitoes legs were also positive by PCR and Southern blot (Figure 5 and Figure 7). All mosquitoes were negative by 72 hours and all subsequent collection times (Figure 5 and Figure 8).

Virus was detected by PCR in 100% *Ae. triseriatus* bodies immediately after feeding. The 2 mosquitoes collected at 6 hours were both negative. At 12, 24, 48 and 72 hours, 100% of the mosquitoes had virus detectable by PCR and Southern blot. One 12 hour mosquito saliva and legs and one 48 hour mosquito legs were positive by Southern blot (Appendix C, Table 2).

Persistence of HBV in Feces. In the first experiment, hepatitis B virus was detected on 27% of the *An. stephensi* pieces of 3 day fecal filter paper by PCR and 45% by Southern blot. Hepatitis B virus was detected on 3% of the *Ae. triseriatus* pieces of 3 day fecal filter paper by PCR and Southern blot, and 23% of the pieces of 7 day fecal filter paper by PCR and and 30% by Southern blot (Figure 6).

In the second *An. stephensi* feeding experiment, 33% of the pieces of fecal filter paper collected immediately after feeding were positive by PCR and 44% by Southern blot. No virus was detected in the feces at 6 hours by either PCR or Southern blot. At 12 hours, 25% of the pieces of fecal paper were positive by Southern blot only. At 24 hours, 16% of the pieces of fecal filter paper were positive by PCR and Southern blot. At 48 hours, 100% of the pieces of fecal filter paper were positive by PCR and Southern blot. At 72 hours, 71% of the pieces of fecal filter paper were positive by PCR and 86% by Southern blot (Figure 6).

In the second *Ae. triseriatus* feeding experiment, no virus was detected on the fecal filter paper immediately after feeding, 12 hours and 48 hours. There was no feces on the 6 hour filter paper. At 24 hours, 16% of the pieces of fecal filter paper were positive by PCR and Southern blot and at 72 hours 11% of the pieces of fecal filter paper were positive by PCR and Southern blot (Figure 6).

Transmission potential of *An. stephensi* during refeeding. All mosquitoes that refed were negative for the virus by both PCR and Southern blot. The membrane and all blood used in the refeeding negative by PCR and Southern blot for virus and virus was not detected on the filter paper collected after immediately after refeeding. (Appendix C, Table 5).

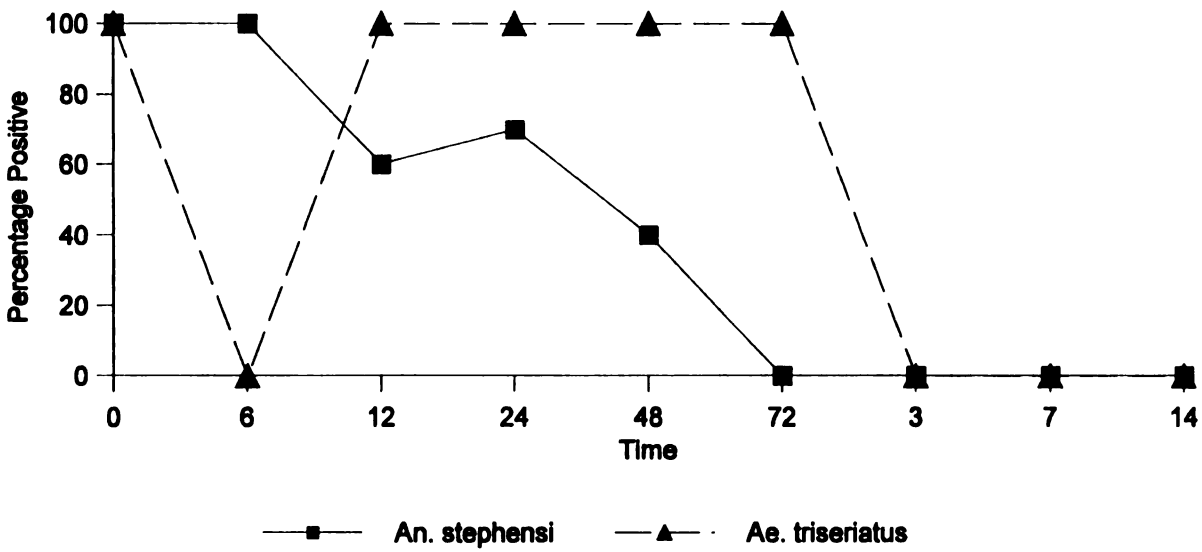

Figure 5. The persistence of ingested hepatitis B virus in the bodies of *An. stephensi* and *Ae. triseriatus*. Time points 0, 6, 12, 24, 48 and 72 hours are from the experiment 3 and 3,7 and 14 days are from experiment 1. Data used to construct tables is found tables 1 and 2 in Appendix C. Data shows that virus is undetectable by PCR by 72 hours in *An. stephensi* and 3 days in *Ae. triseriatus*.

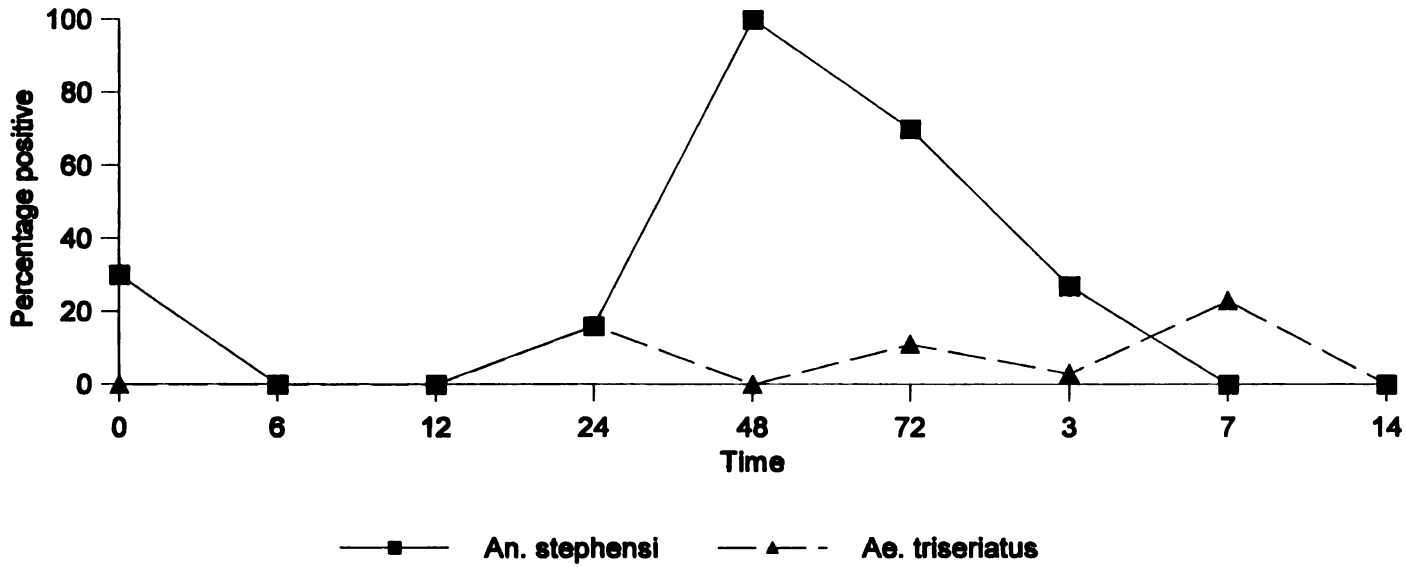

Figure 6. Persistence of ingested hepatitis B in the feces of *An. stephensi* and *Ae. triseriatus*. Time points 0, 6, 12, 24, 48 and 72 hours are from experiment 4 and 3, 7 and 14 days are from experiment 2. Data used to construct tables is found tables 3 and 4 in Appendix C. Data shows that virus is undetectable by PCR by 72 hours in *An. stephensi* and 3 days in *Ae. triseriatus*.

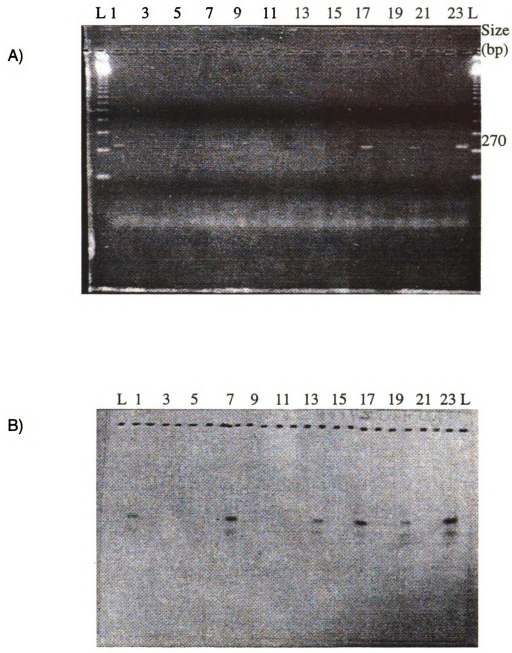

Figure 7. Persistence of HBV in 24 and 48 hour *An. stephensi.* A) Agarose gel of PCR products, 270 bp bands indicate positive samples. B) Southern blot detection. Lanes L 123 lambda ladder, Lane 1 and 23 positive controls, Lanes 2 and 22 negative controls, lane 21 reagent blank. All control and infected sample run in the order legs, saliva, and body. Lanes 3-5 48 hour control, lanes 6-8 one 24 hour infected sample, lanes 9-20 four 48 hour infected samples. Lanes 8, 11, 14, 17, and 20 contain bodies.

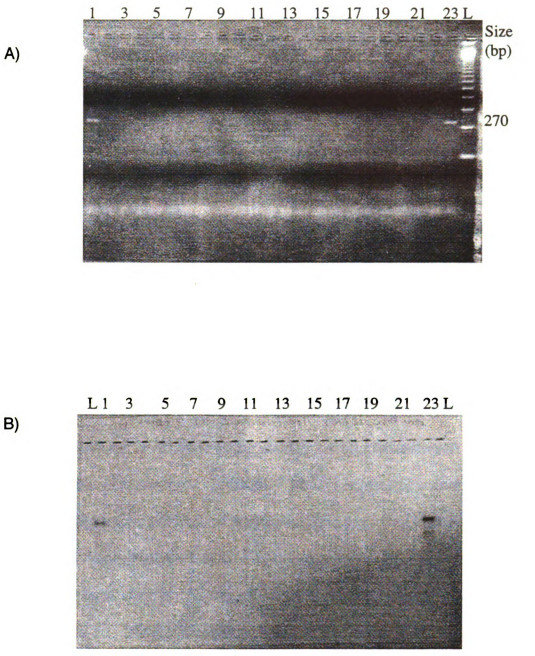

Figure 8. Persistence of HBV in 48 and 72 hour *An. stephensi.* A) Agarose gel of PCR products, 270 bp bands indicate positive samples. B) Southern blot detection. Lanes L - 123 bp lambda ladder, Lanes 1 and 23 positive controls, Lane 2 and 22 negative controls, lane 21 reagent blank. All control and infected sample run in the order legs, saliva, and body. Lanes 3-5 72 hour control, lanes 6-8 one 48 hour infected sample, lanes 9-20 four 72 hour infected samples. Lanes 8, 11, 14, 17, and 20 contain bodies.

HBV intrathoracic inoculation results. No virus was detected, by PCR or Southern blot, in any samples of saliva, legs, bodies or feces of *Ae. triseriatus*, *An. stephensi*, and *Cx. quinquefasciatus* collected at 3, 7,and 14 days post HBV intrathoracic inoculation.

All raw data from this results section are shown in Appendix C, Tables 1-5.

DISCUSSION

The results obtained in this laboratory study indicate that the mosquitoes studied are unlikely to serve as biological vectors of the hepatitis B virus. Viral nucleic acid was detected in *An. stephensi* for 48 hours post infected feeding and in *Ae. triseriatus* for 72 hours post infected feeding, indicating that the disappearance of the virus correlates with the digestion of the blood meal. This observation agrees with the findings of Brotman (1973), Chen (1987), Leevy (1972), and Newkirk (1975), who found a direct correlation between HBsAg disappearance and blood meal digestion. The lack of reappearance of virus in the later infected feeding collection time points and the lack of detectable virus in the inoculated mosquitoes indicates that the virus does not infect mosquitoes.

The detection of virus on the legs of one 48 hour *An. stephensi* legs, saliva and legs of one 12 hour *Ae. triseriatus*, and the legs of one 48 hour *Ae. triseriatus* poses several questions. The detection of virus in the legs of 3 mosquitoes may indicate virus dissemination, however, the legs were positive prior to the digestion of the blood meal so it is unlikely that there was sufficient time for the virus to disseminate into the legs. One possible explanation is the contamination of the legs and saliva during the collection process. The handling

and removal of the legs and wings from the body may have resulted in the rupture of the gut or defecation of virus, contaminating the legs and saliva during collection. Due to the sensitivity of the PCR method these results need to be examined carefully for potential contamination. The small sample size does not allow a determination of the significance of these positive samples.

The presence of detectable virus in the feces of the mosquitoes raise the question as to whether the virus may be mechanically transmitted. Mosquitoes only associate with a host during feeding, therefore, the significance of virus in the feces is debatable. Our results indicate that anopheline mosquitoes defecate virus during feeding on an infected host, however, the host is the source of the virus and thus cannot be reinfected. There is potential that a anopheline mosquito ingesting a partial infected blood meal and subsequently feeding on a susceptible host may transmit the virus during feeding by excretion of virus during pre-diruess. A second potential means of mechanical transmission is when a partially engorged infected mosquito is killed during a subsequent feeding attempt resulting in deposition of virus on a susceptible host. A third possibility is that mosquitoes are resting in human dwellings after feeding and excreting virus into the environment, providing a potential route of contact infection.

This study agrees with earlier studies (Hawkes et al. 1972, Leevy et al. 1972, Prince et al. 1972, Brotman et al. 1973, Muniz and Micks 1973, Ishii et al. 1974, Wills et al. 1976, Chen et al. 1987, Fouche et al. 1989, Fouche et al. 1990, Jupp et al. 1991) in finding no evidence of biological transmission by mosquitoes but they may potential be mechanical vectors. The use of PCR and Southern blot

techniques, allowed us to detect a segment of the viral genome and not just the surface antigen which is found on all three particle types. The specificity and sensitivity of these methods, theoretically allowed us to detect as few a 0.4 hepatitis B virions in the mosquito and 40 hepatitis B virions in the feces. The detection of a segment of the pre-core and core region of the viral nucleic acid does not correlate to infectivity. Hence, no definitive conclusions can be drawn from the detection of viral genome in the feces of the mosquitoes. A carefully controlled transmission study is needed to answer these lingering questions. However, due to the host specificity of the hepatitis B virus and the cost associated with working with primates, the use of a related animal hepadnavirus and its animal host would be recommended to address the question of mechanical transmission of the virus by mosquitoes.

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CHAPTER 2

PERSISTENCE OF THE HEPATITIS B VIRAL GENOME IN *CIMEX LECTULARIUS* L. AND *RHODNIUS PROLIXUS* Stal

INTRODUCTION

Hepatitis B infection is a major cause of acute and chronic hepatitis, cirrhosis, and primary hepatocellular carcinoma (PHC) world wide (CDC 1990) killing estimated 2 million people each year (Hamilton 1994). Primary hepatocellular cancer is one of the most common cancers in Asia and sub-Saharan Africa (Beasley 1988) where 8- 15% of the population carry markers of HBV infection (Maynard 1981). Persons known to be at risk of contracting hepatitis B include: sexually active men and women; prison inmates; health care and public safety workers; hemodialysis patients; international travelers; and household contacts of HBV carriers. Primary modes of transmission are parenteral, perinatal, and sexual contact, however, 30 - 40% of people infected with HBV have no identifiable risk factor (Alter and Mat 1994).

Neefe (1949) was the first to consider the possibility that hematophagous arthropods could transmit the hepatitis virus. Prince (1970) found an increased prevalence of SH (hepatitis B surface antigen) antigen in the Tropics. Unable to account for the increase by known mechanisms of transmission, Prince (1970) suggested transmission in nature by nonparenteral routes. Of the potential routes discussed, the possibility of transmission by hematophagous arthropods was thought the most probable. The subsequent detection of the hepatitis B

surface antigen (HBsAg) by solid-phase radioimmunoassay (RIA) in wild caught mosquitoes supported this hypothesis (Prince et al. 1972) and stimulated research on other anthropophilic hematophagous arthropods.

The detection of HBsAg in unengorged nymphs and adult *Cimex hemipterus* and *C. lectularius* from huts in Africa (Jupp et al. 1978, Ogston et al. 1979, Wills et al. 1977) suggested that bed bugs could be potential mechanical or biological vectors of hepatitis B virus. Bed bugs must feed at least four and usually five times, upon either the same or different hosts, to reach maturity. Trans-stadial transmission of the hepatitis B virus was shown for one molt but not two molts and detected 52 days in bugs only undergoing a single molt (Jupp and McElligott 1979). Neither Jupp (1979) nor Taylor (1980) found evidence of transovarial transmission of the virus. Laboratory experiments showed that HBsAg could be detected in the insect bodies for 6-7.5 weeks post infected feeding (Jupp and McElligott 1979) and in the feces for 5-6 weeks (Jupp et al. 1983, Taylor and Morrison 1980). Jupp et al. (1980) and Jupp et al. (1983) demonstrated that the virus was not replicating by serial passage of the virus through three successive cohorts of bed bugs during which hepatitis B surface antigen was detected in the first and second passages but not the third. Jupp et al. (1983) concluded that there was no indication of virus multiplication in *C. lectularius* but that mechanical transmission from insects to man could occur by contamination of a person when crushing infective bugs; contamination from infected feces; or, infection by bite due to regurgitation or interrupted feeding.

Villajeros et al. (1975) examined the vector potential of *Rhodnius prolixus* for hepatitis B virus in South and Central America by feeding colony cohorts infected

blood and collecting them at weekly intervals. Antigen was detected in 100% of the stomachs and feces for the first two weeks and persisted up to four weeks in 50% of the insects. At no stage during the 6 week experiment did they detect HBsAg in the hemolymph or salivary glands.

All of these studies used detection assays for the hepatitis B surface antigen (HBsAg) which is present on the surface of all three particles produced by the hepatitis B virus. Of the three particles, only the Dane particle contains virions and is infectious (Blumberg et al. 1965). The concentration of non-virion associated HBsAg greatly exceeds the concentration of complete virions by 10^4 or greater.

The epidemiological significance of the association between suspected arthropod vectors, hepatitis B virus, and humans provides a different perspective of the potential importance of arthropod vectors. Hepatitis B infections in Africa commonly occur after one year of age (Tabor et al. 1985), whereas, in Asia many children are infected at birth by their mother (Stevens et al. 1975). Tattooing, circumcision, ear piercing, traditional scarring, exudative skin lesions, and hematophagous arthropods have all been proposed as mechanisms of transmission. Mayans (1990) found a significant association between hepatitis e (HBe) antigenaemia and the presence of bed bugs in children's beds. However, a later intervention study (Mayans et al. 1994) found no significant association to support bed bugs as a major mode of transmission of hepatitis B in Gambian children.

Due to the host specificity of the virus few transmission studies have been conducted. No evidence of mechanical or biological transmission was found in

studies using mosquitoes and human volunteers (Neefe 1949) or chimpanzees (Berquist et al. 1976) but evidence of transmission was found in studies using mosquitoes and chickens (Smith et al. 1972) or monkeys (Yuhuang et al. 1995). Jupp et al. (1991) found no evidence of transmission by *C. lectularius* with chimpanzees however, he did not test for mechanical transmission by interrupted feeding or fecal contamination.

The purpose of this study was to determine the persistence of the hepatitis B virus in *Cimex lectularius* and *Rhodnius prolixus* that had ingested or been inoculated with the virus. Polymerase chain reaction and Southern blotting was used to detect a segment of the hepatitis B viral genome.

MATERIALS AND METHODS

Insect Rearing

Cimex lectularius were obtained from a colony maintained by LTC Harold Harlan, MSC, USA (Ret.) that was originally collected from a basic training billet at Fort Dix, N.J., on 20 March 1973 (Bartley 1974). One liter Nalgene jars with mesh screened lids and pieces of file folder for harborage were used for rearing. The cohort jars were maintained at 24C with 30% humidity and a 16:8 (L:D) h photoperiod When container density became to great to safely fed on a rat, the jar was opened and part of the paper substrate removed and placed in a new jar. Fresh paper substrate was added to both jars and the jars closed. Colony jars were not age graded due to the difficulty in handling and containing the larvae.

Rhodnius prolixus were obtained from the colony maintained by Dr. John Edman, University of Massachusetts. Age cohorts are maintained in 1 liter Nalgene jars with mesh screened lids and folded Whitman's number 1 filter

paper to provide harborage and egg laying substrate. The jars are surrounded by a dark material cover to minimize light. Cohort jars were maintained at 28 degrees with 84% humidity and a 12:12 (L:D) h photoperiod. Adults were removed from immature cohort jars and placed into adult cohort jars to promote mating and egg laying. A few adults were left in the jars with the eggs and larva to provide digestive symbionts. Larvae were sorted into age graded jars when jar density and age range became to great . Exuviae and dead insects were removed and fresh filter paper placed in the jars.

Blood Feeding on Animals. Stock colonies of bugs were fed weekly on rats. The All University Committee on Animal Use and Care approved the use of rats to feed the insect colonies, AUF# 05/96-053-00. Rats where selected based on willingness of both Diptera and Hemiptera to feed on them and the ability to maintain a sufficient number of rats to feed the all the insect colonies necessary to conduct this research.

Jars of *C. lectularius* and *R. prolixus* were transported from the Natural Science Building to the Giltner animal room, a University Laboratory Animal Resource (ULAR) facility. Rats were restrained in surgical stocking and wrapped loosely in a towel and placed under the scavenger hood. Methoxyflurane was administered with a nose cone until the rat became fully relaxed at which time the towel and surgical stocking were removed. The abdominal area was shaved and sterile eye ointment administered to the eyes in preparation for feeding. The rat was then laid on its back on a rolled towel with its head in the scavenger hood and its nose in the nose cone. The colony jars were placed on the abdomen supported by the rolled towel for approximately 20 minutes. Rats' respiration

rate was monitored throughout the feeding period. Rats were removed from the cage and allowed to expire the anesthetic before being placed in a recovery cage. Recovering rats' respiration and state of consciousness were monitored until each rat was awake, then it was returned to its own cage.

Rats were used once every two weeks for feeding. Any rat not appearing active and healthy was removed from the feeding rotation until examined by ULAR personnel. Any rat that developed a suspected tumor or possible irritation due to feeding was removed from the feeding rotation and the ULAR veterinarian notified immediately.

Artificial Blood Feeding. Stock *R. prolixus* colonies were also fed by artificial feeder, however, *C. lectularius* would not fed on the artificial feeder. A Hemotek 5W1 artificial feeding system (Discovery Workshops, England) was used for all feedings. The system consists of 5 independently controlled, heated feeding chambers. Each feeding chamber holds 5 milliliters of blood. Three different types of feeding membranes (parafilm, latex, and cellulose) were used for colony maintenance. A cellulose membrane was used exclusively during infected feedings. The membrane was stretched across the feeding chamber and secured with an O-ring. The chambers were filled with blood, capped and screwed into the heating element, then placed on the cage for feeding (Figure 1).

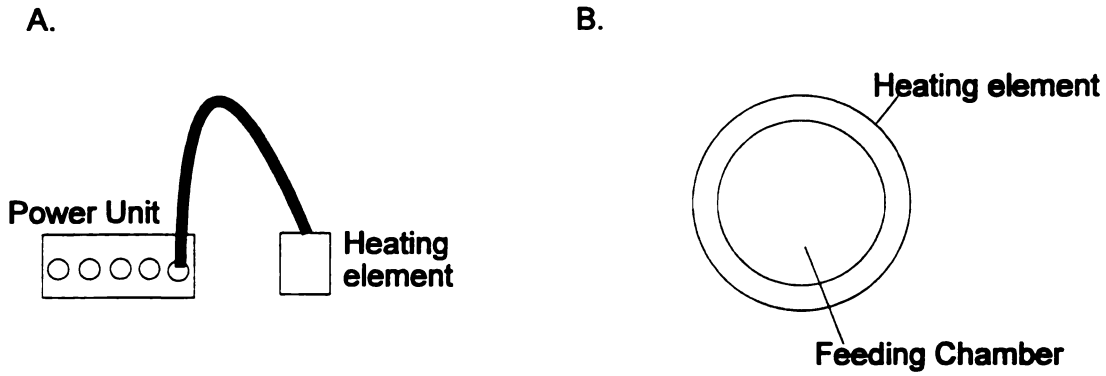

Figure 1. Diagram of Hemotek 5W1 artificial feeding system. A) The power unit can supply power to five independently controlled heating elements. B) Each feeding chamber was covered with a membrane and filled holds 5 milliliters of blood.

Bovine or equine blood was used for all feedings. Mature bovine blood preserved with heparin was obtained in 100 ml aliquots from Pel-Freez Biologicals,(P.O. Box 68, Rogers AR 72757). Mature equine blood preserved with heparin was obtained in 100 ml aliquots from Veterinary Dynamics Inc., (1535 Templeton Road, Templeton CA 93465). Blood was collected and shipped over night on wet ice at 4 degrees C and then stored at 4 degrees C in the laboratory. Blood was kept no longer than 30 days from date of drawing from the bovine or equine.

Experimental Insect Transport, Feeding, Handling and Containment. A HBV positive volunteer, HBV DNA titer 1485 pg/ml, was arranged by the Department of Gastroenterology-Hepatology, William Beaumont Hospital, Royal Oak, Michigan and the negative volunteer was the researcher transporting experimental insects. The human feeding was approved by both William Beaumont Hospital and Michigan State University, University Committee on Research Involving Human Subjects (UCRIHS) IRB # 96-039. *Cimex lectularius* adults and larva were collected from the stock colonies and placed in two clean jars with fresh filter paper one week prior to the infected feeding. The filter paper was changed the morning of the infected feeding and the *Cimex* transported to William Beaumont Hospital for experimental feeding. The HBV volunteer was shown the insects and the feeding procedure explained prior to obtaining informed consent. The control and infected feeding were then done on the forearm of both volunteers. The feeding time was approximately one hour to allow for a high engorgement rate.

Quantitative hepatitis B viral levels were determined by the Abbott hepatitis B viral DNA Assay (Abbott Laboratories, Abbott Park, IL) a liquid phase molecular hybridization assay for quantifying hepatitis B virus DNA in human serum. Results are reported in picograms of DNA per milliliter (pg/ml) of serum.

Hepatitis B virus infected insect processing and collection. Experimental cohort jars were placed in a white enamel lab pan with petroleum gel applied around the lip of the pan. That pan was then placed in a larger pan containing ice. Three empty clean1 liter Nalgene jars and the experimental feeding jar were placed in the first pan. The feeding jar was opened and engorged *C. lectularius*

were collected with fine tipped forceps and placed into either the adult or immature cohort jars. Non-engorged insects were placed in the third jar. After all the bed bugs were sorted into the labeled jars, fresh filter paper was placed in engorged adult and immature jars so that the paper did not reach the screened lid. The non-engorged insects were killed by freezing and then autoclaved prior to disposal. Infected cohorts were kept in the sealed Labconco glove box with humidity pads for the duration of the experiment. All cohorts, control and infected, were handled in the same manner. Control samples were always collected before the infected samples.

Immature *C. lectularius* were held for 14 days to allow for molting. The cohort jar was then placed in the freezer to chill the insects. Individuals were then removed from the jar, the instar noted, and placed in an appropriately labeled 1.5 ml eppendorf tube. All samples were frozen at -70 degrees C at the end of the collection.

Five adult *C. lectularius* were collected with fine tip forceps at each collection time and placed in labeled individual 1.5 ml eppendorf tubes. The remaining insects were gently removed from the filter paper and placed back in the jar. The filter paper was carefully examined eggs and a portion containing eggs was placed in a clean 100 ml Nalgene jar to hatch. The remaining filter paper was placed in a labeled petri dish and frozen at -70 degrees C until analysis. A fresh piece of filter paper was then placed in the cohort jar.

The infected insects were then placed on ice to chill them prior to processing. Individual bed bugs were removed from the eppendorf tube with heat sterilized forceps and placed on a clean microscope glass slide. The legs were removed

with a sterile scalpel and collected and placed in a labeled 1.5 ml eppendorf tube. The body was the returned to the labeled 1.5 ml eppendorf tube. All samples were then frozen at -70 degrees C until DNA analysis.

Experiments

Sensitivity of PCR and Southern blot assays for the hepatitis B virus. To determine the sensitivity of the PCR and Southern blot assays, PCR and Southern blot was done on a series of different template concentrations. One picogram (pg) of HBV DNA equates to 2.8 x 10^5 genomic equivalents thus, approximately 1 picogram of HBV DNA per milliliter of serum equals 2.9 x 10^5 copies/ ml or 290 copies/ul (Hollinger 1996). Therefore, 1 ul of the 1485 pg/ml serum used in the dilution contained approximately 400 copies of the HBV DNA. This serum was then serially diluted beyond the theoretical end point of one viral particle. Whole bed bugs or kissing bugs were added to separate replicate serial dilutions to investigate the effect of insect tissue on the sensitivity of the testing A fourth set of serial dilutions was made and dispensed onto a piece of filter paper to determine the level of detection from filter paper. The dilution tubes were then handled in accordance with extraction and detection methods.

Persistence of ingested Hepatitis B virus. *C. lectularius* were fed on the forearm of either a HBV positive (1495 pg/ml HBV-DNA) or negative volunteer. The experimental cohorts were then handled in the manner described above.

Experiment 1: Persistence of HBV genome in adult C. lectularius. One engorged adult *C. lectularius* was collected during the sorting process to serve as a control. Cohorts of 5 adults were then collected on days, 3, 7, 14, 21, 28, and 35 post HBV infected feeding.

Experiment 2. Persistence of HBV genome in C. lectularius feces. The HBV infected feeding paper was collected and then the filter paper was collected on days 3, 7, 14, 21, 28, and 35 post HBV infected feeding. The fecal filter paper contained feces only from the previous collection time to the labeled collection time.

Experiment 3. Trans-stadial Transmission of ingested hepatitis B virus. Immature *C. lectularius* were placed in a separate jar and held 14 days to allow for molting. At the time of collection, their instar stage was noted they were placed into individually labeled 1.5 ml eppendorf tubes and frozen at -70 degrees C until analysis.

Experiment 4. Trans-ovarial Transmission of ingested hepatitis B virus. A portion of the fecal filter that contained eggs was placed into a separate 100 ml Nalgene jar to hatch. All larva were collected and placed in one 1.5 ml labeled eppendorf tube and frozen at -70 degrees C until analysis.

Persistence of inoculated hepatitis B virus. *C. lectularius* were given a intrathoracic inoculation with 0.5 ul of either an HBV or control suspension and *R. prolixus* with 1.0 ul of either an HBV or control suspension in a ratio of 1 part HBV serum (>2000 pg/ml HBV-DNA) or control serum and 5 parts E-199 tissue culture broth mixture. Thus, each 0.5 ul HBV inoculation theoretically contained 45 viral particles and each 1.0 ul HBV inoculation theoretically contained 90 viral particles. The control suspension consisted of 1 part negative serum and 5 parts E-199 tissue culture broth mixture. Inoculations were performed by Dr. Michael Turell, USAMRIDD, Ft. Detrick, Maryland. The experimental cohorts were then handled in the manner described above.

Experiment 5. Persistence of inoculated HBV in C. lectularius. Cohorts of 5 adults were then collected on days 7, 14, and 21 post HBV inoculation.

Experiment 6. Detection of inoculated HBV in C. lectularius feces. The filter paper was collected on days 7, 14, and 21 post HBV inoculation. Filter paper was identified by its collection time. The fecal filter paper contained feces only from the previous collection time to the labeled collection time.

Experiment 7. Persistence of inoculated HBV in R. prolixus. Cohorts of 5 adults were then collected on days 7, 14, 21, 28, and 35 post HBV inoculation.

Experiment 8. Detection of inoculated HBV in R. prolixus feces. The filter paper was collected on days 7, 14, 21, 28, and 35 post HBV inoculation. The fecal filter paper contained feces only from the previous collection time to the labeled collection time.

Methods

DNA Extraction and Detection Procedure. DNA was extracted from the insect using DNAzol™ and Polyacryl Carrier (Molecular Research Center). DNAzol™ is a ready to use reagent for the isolation of genomic DNA. The DNAzol™ procedure is based on the use of a novel guanidine-detergent lysing solution which hydrolizes RNA and allows the selective precipitation of DNA from a cell lysate. Polyacryl Carrier is a molecular biology grade solution of acryl polymer designed for use in the isolation of small amounts of DNA with DNAzole™. Polyacryl carrier does not affect the activity of restriction enzymes, reverse transcriptases, TAQ polymerases, DNA polymerase, ligase and other enzymes use for DNA analysis.

Table 1. Quantity of DNAzol and Polycarrier gel added to each sample type for extraction of DNA.

Sample	DNAzol (ul)	Polycarrier Gel (ul)
Cimex body	250	5
Cimex legs	250	5
Rhodnius body	750	15
Rhodnius legs	500	10
Rhodnius saliva	500	10
Fecal Filter Paper	750	15

DNAzole™ and Polyacryl Carrier™ were added to the tubes containing saliva, bodies, and legs and the samples were homogenized with a pestle (Table 1). Individual fecal spots were cut out of the filter paper. Filter paper that contained numerous spots was cut into pieces no larger than 2 mm X 2 mm. Four to five fecal spots or one piece of paper was placed in a labeled eppendorf tube. DNAzole™ and Polyacryl Carrier™ were added to tubes containing fecal filter paper, tubes were centrifuged for 20 seconds at 10,000 rpm and placed in a 4□C refrigerator for overnight extraction of the feces from the filter paper. Genomic DNA was precipitated from the lysate with an equal amount of ice cold 100% ethanol. The precipitate was then washed twice with 95% ethanol and allowed to dry. Precipitated DNA was reconstituted with 10 ul HPLC water, concentrated by centrifugation and frozen at -20 degrees C until use. In this condition the isolated DNA was ready for analysis without additional purification. (See Appendix A for detailed procedure).

Oligonucleotide primers for PCR. The majority of primers used for PCR provide amplification of the C (HBc) and S (HBs) genome regions. Analysis of the hepatitis B virus genome revealed regions that appear to be highly conserved (nucleotides 241 to 270, 1891 to 1920, 2431 to 2460) (Roberston 1995). The primers used in these experiments amplify the conserved pre-core and core region, mucleotides 1891 to 1920 (Lauder et al. 1993). Primer 1763 5'-GCTTTGGGGCATGGACATTGACCCGTATAA-3' begins at map position 1763 and primer 2032R 5'-CTGACTACTAATTCCCTGGATGCTGGGTCT-3' (from the complementary reverse [R] DNA strand) begins at map position 2032 of the hepatitis B virus genome (Figure 2) (Fujiyama et al. 1983).

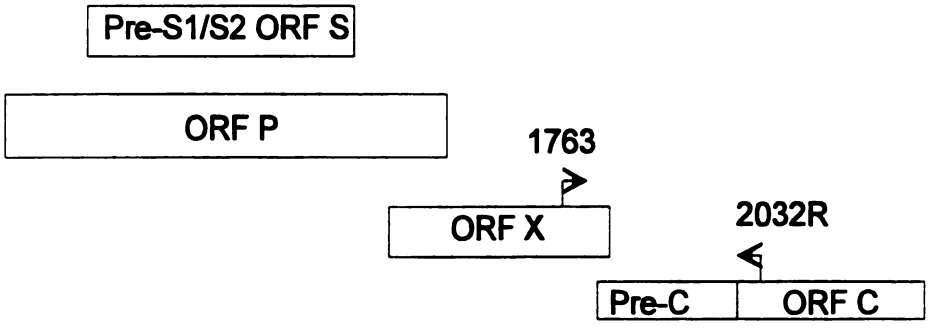

Figure 2. Diagram of Primer location in Hepatitis B viral genome. Four overlapping open reading frames are present in the DNA. The ORF C (core) region encodes the structural protein of the nucleocapsid and the ORF S/pre-S encodes the viral surface glycoproteins. Primers amplify a conserved core region, producing a 270 bp size fragment.

Polymerase Chain Reaction. Polymerase chain reactions were run in accordance with the procedures outlined by Kaneko (1989). Each run contained at least one positive control, negative control and a blank. A 2.5µL aliquot of DNA extracted from each sample was amplified in a 50µL reaction volume containing 2.5 U of Taq DNA polymerase (Perkin Elmer), 200 µmol/L each of the four deoxyribonucleic triphosphates (Boehringer Mannheim, 100mM pH7, dNTP's Li-salt), 25 pmol of each of the primer pair (primers 1763 and 2032R), 50 mmol/L Tris-HCl (pH 8.3)[10X PCR Buffer II (Perkin Elmer)] , 1.5 mmol/L $MgCl_2$, and 0.01% (wt/vol) gelatin (25mM $MgCl_2$, Perkin Elmer). The reaction was performed for 30 cycles in a programmable DNA Thermal Cycler (Perkin Elmer GeneAmp® PCR System 2400). Samples were heated at 94 degrees C for 1.5 minutes (denaturization), cooled to 42 degrees C for 1.5 minutes (oligonucleotide primer hybridization) and incubated for 3 minutes at 72 degrees C (DNA polymerase extension). Contamination of the PCR reaction mixture with minute amounts of hepatitis B virus DNA can result in spurious positive results (i.e., false positives). To eliminate potential sources of DNA contamination, all reagents were prepared with pipettors designated for reagent use only and dispensing was done in a laminar flow hood. No infectious samples were processed in the hood, and the ultra-violet light was turned on prior to preparing PCR reagents in the hood. The PCR reaction mixture was dispensed into PCR tubes in the hood and the tubes were closed. Tubes were then taken to the bench top and sample DNA was added there. Counter top's were disinfected with 10% bleach solution before and after any procedures. Autoclavable Oxford®

Benchmate digital pipettors were used with aerosol resistant tips to dispense reagents and specimen.

All PCR products were separated on 2% agarose gels with 0.5 µg/ml ethidium bromide in an electrophoresis chamber containing 1X TBE (0.9 mM Tris Base, 0.9 mM Boric Acid, .02 mM EDTA) with 0.5 µg/ml ethidium bromide. Amplified DNA was mixed by gently aspirating with the pipettor. Eight microliters of the PCR reaction mixture was mixed with 2 µL of gel loading buffer III 6X (0.25% bromophenol blue, 0.25% xylene cyanol FF, and 30% glycerol in H_2O) and dispensed into the appropriate gel well. Two microliters of a 123 bp lambda DNA ladder (Gibco BRL) was mixed with 2 uL of gel loading buffer III and 6 uL of HPLC water. The electrophoresis apparatus was adjusted to a voltage of 100 between 60-80 amps of current, allowed to run for at least 1.5 hour, and then the gel was examined for separation of DNA fragments. More time was occasionally required to obtain complete DNA fragment separation.

After electrophoresis the gel was examined with ultraviolet light fluorescence. The presence of a 270 bp band in the positive control lanes indicated that the PCR amplification had performed as expected. The presence of 270 bp bands in other lanes on the gel indicated positive samples. Gels were viewed and photographed on an ultraviolet light box.

Southern Analysis. DNA products were transferred from the 2% agarose gels to nylon membranes by a BioRad Trans-Blot SD semi dry electrophoretic transfer cell (Cat. Nos. 170-3940, 170-3948, 170-3949). Two pieces of extra thick BioRad blotting paper and one piece of nylon membrane (Boehringer Mannheim Cat Nos. 1209-272, 1209-299) were cut to the size of the gel. The

gel, membrane, and blotting paper were soaked in 0.5X TBE with no ethidium bromide for 10 minutes, then formed into a sandwich built on the anode plate. Each layer was rolled to remove air bubbles prior to adding the next layer in the sandwich. The sandwich, from bottom to top, was composed of blotting paper, nylon membrane, gel, and blotting paper. The sandwich was then saturated with 0.5X TBE and excess TBE was removed. The gel frame was placed around the sandwich, the top plate latched down, and the top cover secured completing the circuit. The Trans-blotter was then plugged into the power pack. The amperage required for blotting was calculated by taking the product of: (length of the gel) X (width of gel) X 0.3. The power pack was set to run on constant amperage and the calculated amperage was set at a 40 minute run time. Voltage was monitored throughout the transfer. A voltage that increased over 25 indicated that the buffer capacity had expired and the run should be stopped. When this occurred, the amperage was either reduced or the process was halted depending upon the amount of transfer time remaining.

When the transfer was completed, the system was disassembled in the reverse order. A pencil was used to mark the wells on the nylon membrane before removing the gel. The membrane was then removed, rinsed in 2X SSC (3mM NaCl and .3 mM sodium citrate), and placed on a piece of extra thick filter paper soaked in 0.4 N NaOH for 5 minutes to break the DNA bonds. The membrane was rinsed again in fresh 2 X SSC and then placed on piece of filter paper that was folded into a pocket and labeled. The DNA was then fixed to the membrane by drying at either 80 degrees C for 2 hours or 120 degrees C for 1

hour. After drying, all membranes were stored in a ziplock bag in a 4 degrees C refrigerator.

Digoxigenin oligonucleotide labeling and detection. A oligonucleotide internal to the PCR oligonucleotides was selected to confirm that the product amplified by PCR was actually part of the hepatitis B genome and not a random DNA amplification. Primer 1913 5'-TGTTCACCTCACCATACAGC- 3' began at map position 1913 of the hepatitis B viral genome and was 3'-end labeled with digoxigenin-ddUTP (Boehringer Mannheim Cat. No. 1362-372). The steroid hapten digoxigenin (DIG) was linked via a spacer arm to the primer. The oligonucleotide was enzymatically labeled at its 3' end with terminal transferase using a single DIG-labeled dideoxyuridine-triphosphate (DIG-ddUTP). The labeled oligonucleotide was then used in the DIG nucleic acid detection.

The digoxigenin labeled probe was used with the digoxigenin nucleic acid detection kit (Boehringer Mannheim Cat. No. 1175 041) to detect hepatitis B nucleic acids on the Southern blots, by enzyme linked immunoassay using an anti-digoxigenin alkaline phosphatase conjugated antibody. The nylon membranes were labeled, placed in pre-hybridized solution in a 39 degree C shaker oven, and then hybridized with the DIG-labeled 1913 oligonucleotide at 39 degree C in the shaker oven overnight. After blocking the membrane, binding of antibody-conjugate to hybrized DIG labeled nucleic acid occurs. The color reaction is initiated alkaline pH by the addition of BCIP and NBT. A blue precipitate starts to form within a few minutes in the positive control bands. Color development was stopped after 1 hour and the membranes were

photographed with 35mm 160 Tungsten film on a copy table (See Appendix B for complete protocol).

Determination of Virus Persistence and Dissemination. Viral persistence was determined by the detection of virus in the bugs bodies. A bed bug that had virus detected in its body, but not its legs, was considered to have a nondisseminated viral persistence. The detection of virus in the legs indicated that the virus had disseminated (Turrell et al. 1984).

Adult bed bugs ingest 5 -10 µl (Newkirk et al. 1975) of blood during feeding. The amount of virus ingested during the HBV infected blood meal was estimated by multiplying 400 (number of viral copies/ul of serum) by amount of blood ingested (5 or 10) and then multiplying the product times 55% (the estimated percentage of serum in blood).

RESULTS

Sensitivity of PCR and Southern blot detection of HBV.

The addition of bed bug bodies decreased PCR sensitivity 10 fold and Southern blot sensitivity 100 fold. The PCR had visible bands present at the 1:1000 dilution, 4 viral particles, in the serum and 1:100 dilution, 40 viral particles, in the serum plus bed bug lanes (Figure 3). The Southern blot had visible bands at the 1:10000 dilution, 0.40 viral particles, in the serum lanes and 1:100 dilution, 40 viral particles, in the serum plus bed bug lanes. The addition of kissing bodies decreased PCR and Southern blot sensitivity 100 fold. The PCR had visible bands present at the 1:10 dilution, 400 viral particles, in serum plus kissing bug lanes (Figure 4). The Southern blot had visible bands at the 1:100 dilution, 40 viral particles, in serum plus kissing bug lanes. The addition of fecal filter paper decreased the sensitivity of the PCR and Southern detection methods 100 fold. The PCR and the Southern blot had visible bands at the 1:10 dilution, 400 viral particles (Figure 5). Nested PCR was not done due to a high rate of false positives and the inability to establish a consistent lower limit of viral detection.

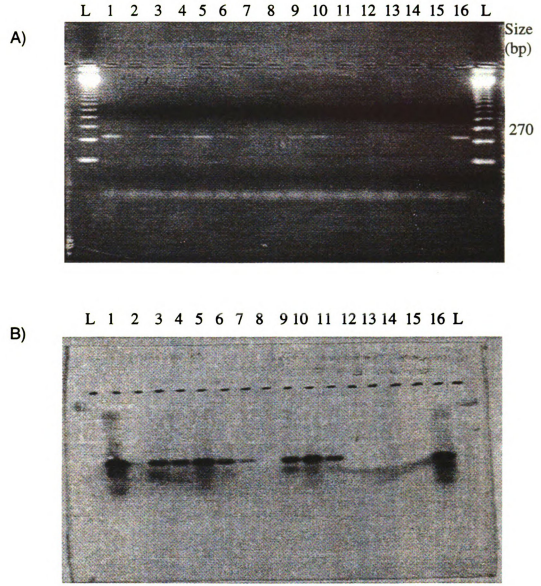

Figure 3. Determination of the effect of bed bug tissue on the sensitivity of HBV detection. Serial dilution of 1485 pg/ml HBV DNA serum A) Agarose gel of PCR products, 270 bp bands indicate positive samples. B) Southern blot detection. Lanes: 1-positive control, 2 Negative control, 3 -straight serum (400 virions), 4- 1:10 (400 virions), 5- 1:100 (40 virions), 6-1:1000 (4.0 virions), 7-1:10000 (0.4 virions), 8-1:100000 (0.04 virions), 9 -serum plus bug (400 virions), 10- 1:10 (400 virions), 11- 1:100 (40 virions), 12-1:1000 (4.0 virions), 13-1:10000 (0.4 virions), 14-1:100000 (0.04 virions), 15-negative control, 16-positive control, L-123 bp lambda ladder.

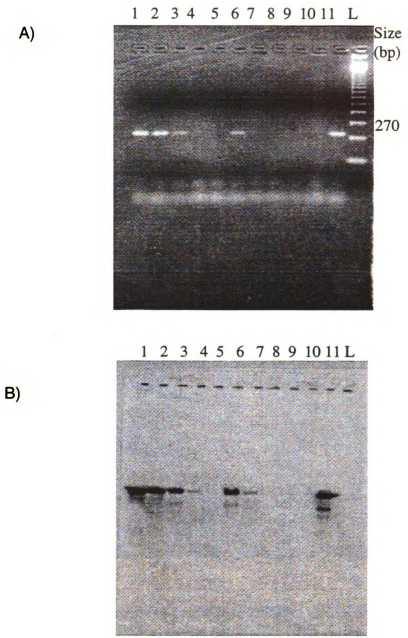

Figure 4. Determination of the effect of kissing bug tissue on the sensitivity of HBV detection. Serial dilution of 1485 pg/ml HBV DNA serum. A) Agarose gel of PCR products, 270 bp band indicates positive samples. B) Southern blot detection. Lanes: 1-1:10 (400 virions), 2-1:100 (40 virions), 3-1:1000 (4.0 virions), 4-1:10000 (0.4 virions), 5-1:100000 (0.04 virions), 6- 1:10 (400 virions), 7- 1:100 (40 virions), 8-1:1000 (4.0 virions), 9-1:10000 (0.4 virions), 10-1:100000 (0.04 virions), 11- positive control, L-123 bp lambda ladder.

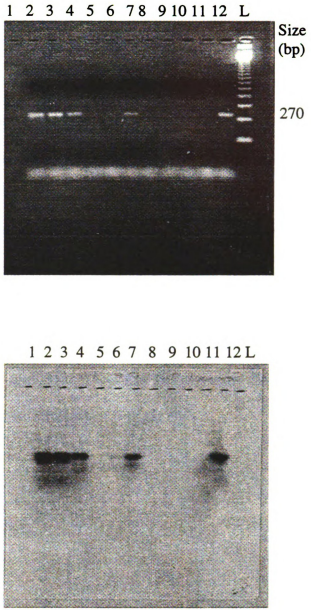

Figure 5. Determination of the effect of filter paper on the sensitivity of HBV detection using PCR. Serial dilution of 1485 pg/ml HBV DNA serum. Lanes: E- empty, A) Agarose gel of PCR products, 270 bp band indicates positive samples. B) Southern blot detection. Lanes:1- empty, 2-1:10 (400 virions), 3-1:100 (40 virions), 4-1:1000 (4.0 virions), 5-1:10000 (0.4 virions), 6-1:100000 (0.04 virions), 7- 1:10 (400 virions), 8- 1:100 (40 virions), 9-1:1000 (4.0 virions), 10-1:10000 (0.4 virions), 11-1:100000 (0.04 virions), 12- positive control, L-123 bp lambda ladder.

***Cimex lectularius* HBV Infected Feeding Results.** Legs and bodies were collected at 1, 7, 14, 21, 28 and 35 days post infected feeding (Table 2). Virus was detected by PCR and Southern blot in the single bed bug collected on day 1. Virus was not detected by PCR on day 7 but 60% were positive by Southern blot. At 14, 21, 28 and 35 days, virus was detected in 100% of the bodies by both PCR and Southern blot (Figure 6 and Figure 7). Legs from one bug were positive on day 21 and legs from another bug were positive on day 28 by Southern blot.

Persistence of inoculated HBV in *C. lectularius* bodies. Hepatitis B virus was not detectable by PCR at any collection time but was detectable in 80% of the day 7 and 14 bodies and 40% of the day 21 bodies by Southern blot. Two day 7 legs were positive by Southern blot (Figure 6).

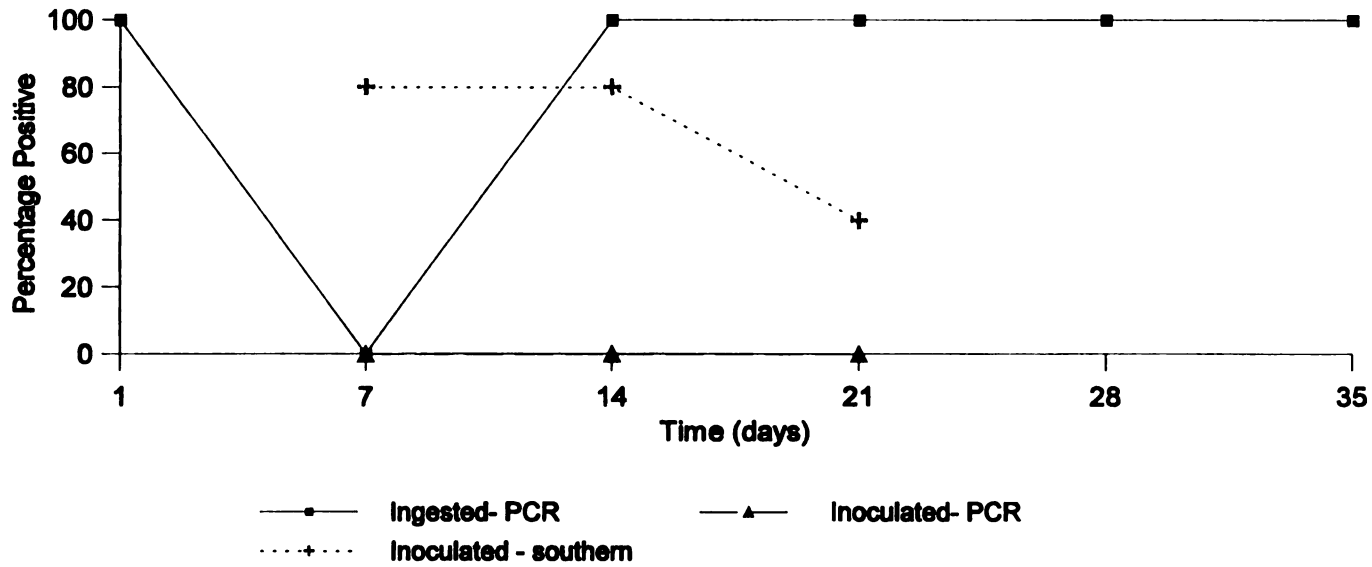

Figure 6. Persistence of ingested and inoculated hepatitis B virus in *C. lectularius*. Virus is detectable by PCR in bugs ingesting infected blood through day 35. Virus was not detectable by PCR (40 virions) in inoculated bugs, but is detected by Southern blot (<4 virions) through day 21.

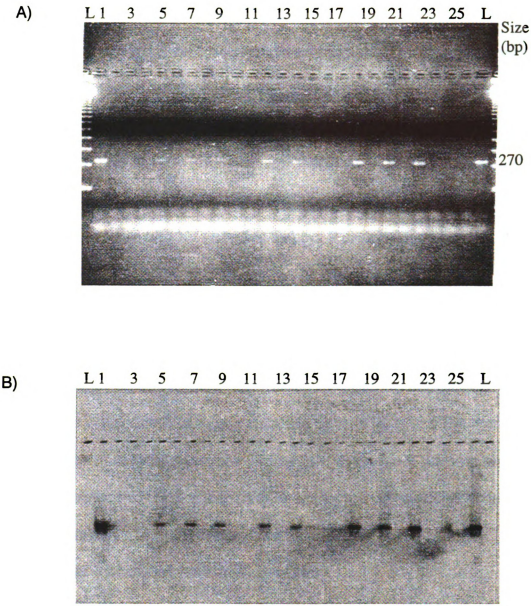

Figure 7. Persistence of ingested HBV in day 28 and 35 *C. lectularius* bodies. A) Agarsose gel of PCR products, 270 bp band indicates positive samples. B) Southern blot products. Lanes L are 123 bp lambda ladders. Lanes1 and 26 are positive controls, lanes 2 and 25 are negative controls and lane 23 is a reagent blank. Each sample was run body then legs. Lanes 3 and 4 and 16 and 17 are control 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.

Persistence of HBV in *C. lectularius* feces.

Ingested. Hepatitis B virus was not detected on the filter paper that was in the jars during infected feeding. Virus was detected in 5% of the filter paper samples by Southern blot on Day 1. On day 7, 39% of the fecal samples were positive by PCR and 52% by Southern blot. Sixty-seven percent of the day 14 fecal samples were positive by PCR and 83% by Southern blot. Day 21, 28 and 35 fecal samples were all 100% positive by both PCR and Southern blot (Figure 8 and Figure 9).

Inoculated. Hepatitis B virus was not detected by either PCR or Southern blot in the feces from bed bugs inoculated with hepatitis B virus.

Transstadial and Transovarial transmission of ingested hepatitis B virus in *C. lectularius*. Hepatitis B virus was detected by both PCR and Southern blot in all larval stages undergoing one molt (Figure 10). Hepatitis B virus was not detected, by either PCR or Southern blot, in any of the larvae that hatched from eggs laid on day 7 or 14 after the infected blood meal.

Persistence of inoculated HBV in *R. prolixus*.

Bodies. Hepatitis B virus was detected in 60% of bodies, 40% of saliva, and 20% of legs by Southern blot in day 7 samples. Virus was not detected, by either PCR or Southern blot, in any sample at any of the later collection times .

Feces. Hepatitis B virus was not detected by PCR or Southern blot in any fecal samples at any of the sampling times.

All raw data from this results section are shown in Appendix C, Tables 6-10.

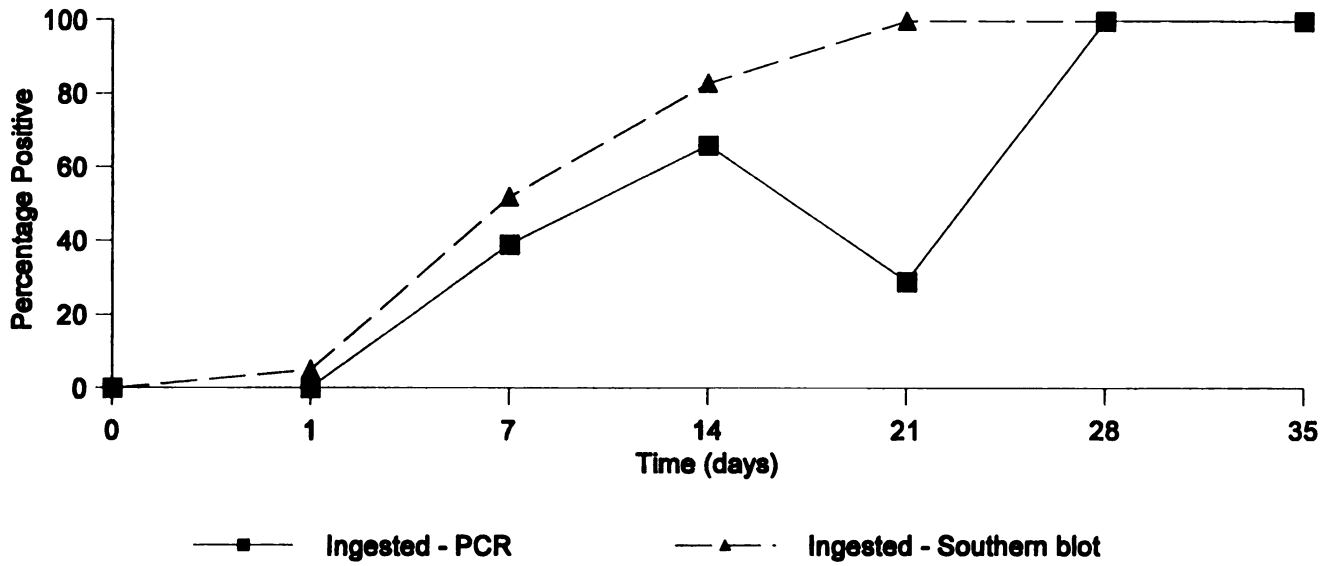

Figure 8. Persistence of ingested hepatitis B virus in the feces of *C. lectularius*. Hepatitis B viral genome detected in feces extracted from filter paper used to collect the feces. PCR detection sensitivity is 400 virions and Southern blot is <40 virions. Percentage is calculated from the number of positive samples over the total number of samples collected.

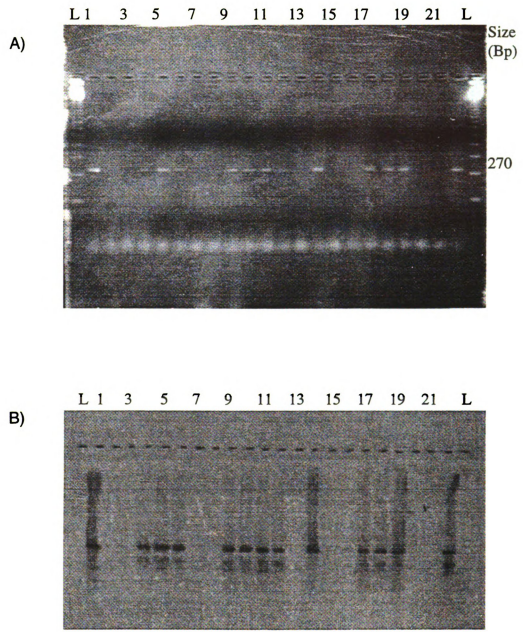

Figure 9. Persistence of HBV in day 14, 21, 28 and 35 *C. lectularius* feces. A) Agarose gel of PCR products, 270 bp band indicates positive samples. B) Southern blot detection. Lanes L- 123 bp lambda ladders, lanes 1 and 22 positive controls, lanes 2 and 21 negative controls, and lane 21 reagent blank. Lanes 3, 7,8, and 13 control feces. Lane 4- day 35, Lanes 5 and 6 day 28, Lanes 9-12 day 21, and lanes 14-19 day 14 feces.

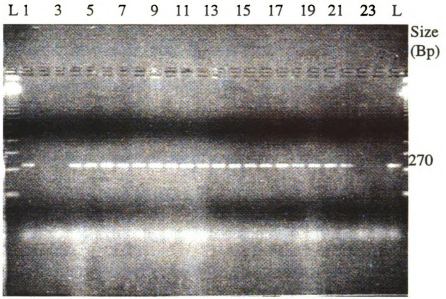

Figure 10. Trans-stadial transmission of HBV. Agarsoe gel of PCR products. Lanes 1 and 24 positive controls, lanes 2 and 23 negative controls, lane 22 reagent blank, lane 3 sample control, lane 4-21 infected instars from youngest to oldest.

DISCUSSION.

All the studies to date examined the persistence of the hepatitis B surface antigen in *Cimex* spp. and *R. prolixus*. The surface antigen is found on all three viral particles produced by the virus, only one of which is infectious. The use of PCR and Southern blot detection methods here allowed for the detection of the viral genome. Analysis of the hepatitis B virus genome revealed regions that appear to be highly conserved (nucleotides 241 to 270, 1891 to 1920, 2431 to 2460). (Robertson 1995). The oligonucleotide primers used in these experiments amplified the conserved pre-core and core region, from nucleotides1891 to 1920 (Lauder et al. 1993). The core gene contains 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 HBeAg. The second initiation codon product is the 21-kd core protein, that forms the viral nucelocapsid with the HBV DNA (Thomas and Carman 1994). Thus, the detection of this gene region indicates that the insects in my experiments did ingest an infectious blood meal and not just noninfectious particles with surface antigen.

The results obtained in this laboratory study indicate that *C. lectularius* and *R. prolixus* are unlikely to serve as biological vectors of the hepatitis B virus. Viral nucleic acid was detected for 35 days post feeding and 21 days post inoculation in C. lectularius and 7 days in inoculated *R. prolixus*, however, no corresponding infected feeding study was done due to colony numbers. The detection of inoculated virus only by Southern blot, indicates that the less than 40 viral copies

were present. Since there was no subsequent detection by PCR at later time points it is unlikely that the virus had infected and replicated in the insects.

The detection of virus from *C. lectularius* legs on day 21 and 28 post infected feeding poses several questions. The positive legs from inoculated insects would be expected since hemolymph circulates throughout the insects body and into the legs and the virus was inoculated into the insects body. The detection of virus in day 7 *R. prolixus* saliva suggest the virus was able to penetrate the salivary glands. The detection of virus in the legs of bed bugs ingesting the virus, may indicate viral dissemination or possibly contamination during collection, sample processing, or defecation. The removal of the legs from the body may have resulted in the rupture of the gut or defecation of virus, thus contaminating the legs during collection. Due to the sensitivity of the PCR method these results need to be considered carefully for potential contamination and false positive results. The small sample size does not allow a determination of the significance of these positive samples.

The presence of detectable virus in 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 and thus defecate shedding virus in the hosts environment, providing a potential source for inapparent infection. A second potential means of mechanical transmission is when a partially engorged or unengorged infected bed bug is killed during a subsequent feeding attempt, resulting in deposition of virus on a susceptible host.

The persistence of the virus after an infected blood meal in bed bug bodies and feces agrees with studies that found the persistence of the HBsAg in bed bugs ingesting infected blood meals (Jupp and McElligott 1979, Jupp et al. 1983, Ogston and London 1980, Ogston et al. 1979, Taylor and Morrison 1980). Further, the virus was shown to persist trans-stadially, in all stages of *C. lectularius,* confirming an earlier studies by Jupp and McElligott (1979). However, trans-ovarian transmission was not detected, also confirming earlier studies (Jupp and McElligott 1979, Taylor and Morrison 1980). The use of PCR and Southern blot techniques, allowed me to detect a segment of the viral genome and not just the surface antigen which is found on all three particle types. The specificity and sensitivity of these methods, theoretically allowed detection of as few a 4.0 hepatitis B virions in the bed bug, 40 virions in the kissing bug, and 40 virions in the feces. It is important to note here that the detection of a segment of the pre-core and core region of the viral nucleic acid does not correlate to infectivity. Hence, no definitive conclusions can be drawn about infectivity potential from the detection of viral genome. A carefully controlled transmission study is needed to answer these lingering questions. However, due to the host specificity of the hepatitis B virus and the cost associated with working with primates, the use of a related animal hepadnavirus and its animal host would be recommended to address the question of mechanical transmission of the virus by bed bugs and kissing bugs.

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CHAPTER 3

RESEARCH SUMMARY AND CONCLUSIONS

Hepatitis B virus (HBV) infection is a major cause of acute and chronic hepatitis, cirrhosis, and primary hepatocellular cancer world wide (CDC 1990). In the United States 30-40% of patients with hepatitis B infection cannot be definitively associated with an identifiable risk factor. Neefe (1949) was the first to consider the possibility that hematophagous arthropods could transmit the hepatitis virus. The subsequent detection of the hepatitis B surface antigen (HBsAg) by radioimmunoassay in wild caught mosquitoes supported this hypothesis (Prince et al. 1972). The detection of the HBsAg in wild caught mosquitoes (Berquist et al. 1976, Blumberg et al. 1973, Brotman et al. 1973, Dick et al. 1974, Prince et al. 1972, Wills et al. 1976), *Cimex spp.* (El-Masry and Kotkat 1990, Jupp and McElligott 1979, Jupp et al. 1983, Jupp et al. 1980, Jupp et al. 1978, Newkirk et al. 1975, Ogston and London 1980, Ogston et al. 1979, Taylor and Morrison 1980, Wills et al. 1977), tampan tick, *Ornithodoros moubata* Murray, (Joubert et al. 1985, Jupp et al. 1987) and *Rhodnius prolixus* Stål (Villarejos et al. 1975) implicated these arthropods as potential vectors.

The purpose of this research project was to study the persistence of HBV genome in several species of hematophagous arthropods. Previous studies utilized primarily EIA/RIA methodologies to test for the presence of HBsAg. The detection of HBsAg by those methods showed ingestion of the viral particles during feeding on either a viremic host or artificial feeder. However, the medical and epidemiological significance of the persistence of the HBsAg over varying

time periods post infected feeding is debatable. Is it a mere artifact of the original blood meal or, does it represent viral replication and generation of new viral particles? The use of molecular methods allowed for the detection of specific regions of the HBV genome. Specifically, by testing for pre-C and C regions of the genome, using PCR, only regions of the genome coding for the infectious particle regions would be detected. The persistence of the virus and its dissemination in the insect may indicate that the vector has been infected and may be able to mechanically or biologically transmit the virus.

I studied the persistence and dissemination of ingested and inoculated hepatitis B virus in *Cimex lectularius* L. (Hemiptera: Cimicidae), *Rhodnius prolixus* Stål (Hemiptera: Reduviidae), *Aedes triseriatus* (Say) (Diptera: Culicidae), *Culex quinquefasciatus* Say (Diptera: Culicidae) and *Anopheles stephensi* Liston (Diptera: Culicidae). The hepatitis B viral genome was detected by polymerase chain reaction with confirmation by Southern blot. The sensitivity of these detection methods was determined by serial dilution of a known serum with the addition of insect tissue or filter paper and was shown to vary with sample source (Table 1).

Viral nucleic acid was detected in *An. stephensi* for 48 hours and *Ae. triseriatus* for 72 hours post infected feeding, indicating that the disappearance of the virus correlates with the digestion of the blood meal (Brotman et al. 1973, Chen et al. 1987, Leevy et al. 1972, Newkirk et al. 1975). The lack of reappearance of virus in the later time points and the lack of detectable virus in the intrathoracically inoculated mosquitoes indicated that the virus did not infect mosquitoes. The presence of detectable virus in the feces of those mosquitoes

Table 1. Sensitivity of PCR and Southern blot detection methods for hepatitis B viral genome. Sensitivity determined by serial dilution of known serum with corresponding sample. Results are given in viral particles detected.

Sample	PCR	Southern blot
Mosquito	4	0.4
Cimex	40	< 4
Rhodnius	400	40
Filter paper	400	< 40

that ingested an infected blood meal raises the possibility that the virus may be mechanically transmitted by mosquitoes by excretion of infectious material.

Viral nucleic acid was detected in *C. lectularius* bodies and feces at 35 days post infected feeding and at 21 days post inoculation. The persistence of the virus after an infected blood meal in bodies and feces agrees with earlier studies that found persistence of HBsAg in bed bugs that had ingested an infected blood meal (Jupp and McElligott 1979, Jupp et al. 1983, Taylor and Morrison 1980). Further, the virus was shown to persist trans-stadially, in all stages of *C. lectularius*, confirming an earlier study by Jupp and McElligott (1979). However, trans-ovarian transmission was not detected, again confirming earlier studies (Jupp et al. 1978, Taylor and Morrison 1980).

The presence of detectable virus in the bodies and feces of bed bugs raises the question as to whether the virus may be mechanically transmitted. Bed bugs live in close proximity to a host and are often found in bedding, furniture, and cracks and crevices in walls. Thus, they may defecate infectious virus into the human living environment, providing a potential source for inapparent infection.

A second potential means of mechanical transmission could be when a partially engorged or unengorged infected bed bug is killed during a subsequent feeding attempt, resulting in deposition of virus on a susceptible host.

It is important to note that the detection of a segment of the pre-core and core region of the viral nucleic acid does not correlate to infectivity. Hence, no definitive conclusions can be drawn about potential infectivity from the detection of the viral genome. Further studies need to be conducted to examine the infectivity of feces and bodies of the various insects. However, due to the host specificity of the hepatitis B virus and the cost associated with working with primates, the use of a related animal hepadnavirus and its animal host would be recommended to address the question of mechanical transmission.

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APENDICES

APPENDIX A

DNAzol Extraction Protocol

1. Add DNAzol and Poly Carrier gel to samples.

Table 1: Quantity of DNAzol and Poly Carrier gel to add each sample tube.

Sample type	DNAzol (ul)	Carrier Gel (ul)
serum, blood	250	5
saliva	500	10
mosquito bodies and legs	250	5
fecal filter paper	750	15
bed bugs bodies and legs	250	5
kissing bugs bodies and legs	750	15

1. Homogenize all samples except bed bug and kissing bug bodies in tube with sterile pestle. Bed bug and kissing bug bodies are soaked in DNAzol and carrier gel first then homogenized. Soaking softens the exoskeleton allowing the bug to be homogenized. Samples were then soaked a second time.

2. Incubate samples:

Table 2: Time and temperature to incubate samples.

Sample type	Time	Temperature
serum, blood	10 minutes	room
saliva	10 minutes	room
mosquito bodies and legs	10 minutes	room
fecal filter paper	overnight	4 degrees C
bed bugs bodies and legs	1 hour	4 degrees C
kissing bugs bodies and legs	1 hour	4 degrees C

3. Centrifuge samples for 15 minutes at 4 degrees C and 10,000 x g.

4. While centrifuging, label another set of tubes with corresponding sample labels. Add the same amount of ice cold 100% ethanol as DNAzol to each tube.

5. Replace tubes in freezer until centrifugation is complete.

6. Transfer supernatant from specimen tubes to corresponding ETOH tubes.

7. Invert tubes 3-4 times and incubate for 5 minutes at room temperature.

8. Centrifuge 5 minutes at 4 degrees C and 1,000 x g.

9. At end of centrifugation, examine tubes for presence of pellet or precipitate in tube. If no visible pellet/precipitate centrifuge the tubes a second time.

10. Pour off supernatant, being careful not to lose the pellet/precipitate.

11. Add 750 ul of ice cold 95% ETOH, vortex the samples.

12. Centrifuge 5 minutes at 4 degrees C and 1,000 x g.

13. Pour off supernatant, being careful not to lose the pellet/precipitate.

14. Add 500 ul of ice cold 95% ETOH, invert samples 3-4 times.

15. Centrifuge 5 minutes at 4 degrees C and 1,000 x g.

16. Pour off supernatant, set tubes upside down on kim wipes until dry.

17. When dry, add 10 ul of deionized water to re-suspended the DNA.

18. Spin at room temperature for 20 seconds at 10,000 rpm to concentrated the DNA in the bottom of the tube.

19. Incubate 3-5 minutes to allow DNA to dissolve.

20. DNA can be stored at -20 degrees C for 1 month, or kept on ice for immediate use.

APPENDIX B

SOUTHERN DETECTION PROTOCOL

Table 1. Additionally required solutions for Southern blotting.

Additionally Required Reagents	Description
HCL	250 mM HCL
H_2O	Sterile, Distilled water
Denaturation solution 1	0.5 M NaOH, 1.5 M NaCl
Neutralization solution 1	0.5 M Tris-Hcl, pH7.5; 3 M NaCl
20 X SSC Buffer	3 M NaCl, 75 mM Sodium Citrate, pH 7.0
5 X SSC Buffer	750 mM NaCl, 75 mM Sodium Citrate, pH 7.0
Prehybridization Solution	5 X SSC, 1% Blocking Reagent, 0.1% N-lauralysarconsine, 0.02% SDS (Store at -20□C
Hybridization solution	DIG-labeled probe, diluted in Prehybridization solution
2 X Wash solution	2 X SSC, containing 0.1% SDS
0.5 X Wash solution	0.5 X SSC, containing 0.1% SDS

DENATURATION, NEUTRALIZATION AND BLOTTING BY TRANS BLOTTER

1. Cut a piece of nylon membrane and two pieces of extra thick blotting paper to the dimensions of the gel with a scalpel blade or scissors. The transfer membrane and blotting paper must have the same dimensions as the gel for proper transfer to occur.

2. Calculate the power conditions for transfer. For my samples the transfer conditions are 3.00mA/cm2 of gel agarose for 40 minutes. Preset the BioRad power supply to the calculated mA's and time. Run the power pack on constant Amps.

3. Saturate two pieces of precut Extra thick blot paper and precut nylon membrane in 0.5 X TBE buffer. Equilibrate the transfer membrane for at least 10 minutes.

4. Remove one piece of blotting paper from 0.5 X TBE and allow the excess buffer to drain off. Lay the blotting paper flat on the platinum anode. Using a

clean pipet roll out any air bubbles that may be trapped under the blotting paper with a top to bottom, left to right rolling motions.

5. Place the equilibrated transfer membrane on top of the blotting paper and roll out air bubbles.

6. Carefully place the agarose gel on top of the membrane, well side up. Make sure all the edges are aligned and air bubbles are rolled out.

7. Take the other piece of wetted blot paper and allow excess buffer to drain off, place it on top of the agarose gel. Roll out the blot paper to remove air bubbles and add approximately 15 ml's of 0.5 X TBE to top of sandwich to saturate. Remove any excess buffer that is present on the anode surface.

8. Place the safety cover onto the unit and plug the Trans-Blot SD cell into the power supply. Plug red into red and black into black.

9. Turn on the power supply and verify your calculated settings are pre-set. Press the run button and verify that the gel is running at your preset conditions. Monitor the voltage during the transfer for any significant fluctuations. The voltage will slowly increase during the transfer to maintain constant mA. If the voltage is lower, increase the length of the transfer time. If the voltage increases significantly (>25V) the buffer capacity has expired and the run should be stopped.

10. Following transfer, turn off the power supply and disconnect the leads. Remove the safety cover and the cathode electrode. Remove and discard the top blot paper. Using a fine tip pencil mark the wells on the blot paper through the gel. Remove the gel and restain with EtBr to check for complete DNA transfer.

11. Rinse the nylon membrane in 2x SSC.

12. To fix the DNA, saturate a piece of blot paper with 0.4 N NaOH. Place the transfer membrane on top of the saturated pad (DNA side up) for 5 minutes. Rinse the membrane briefly in 2 X SSC, and bake for 30 minutes at 80 degrees C in a vacuum oven or 1 hour at 120 degrees C with no vacuum.

PREHYBRIDIZATION AND HYBRIDIZATION:

1. Place the blot in a hybridization tube containing 20 ml prehybridization solution per 100 cm^2 of membrane surface area. Seal the tube, place in the rotator, and prehybridize at 39 degrees C (T_m = 47.9 degrees C) for 6 hours. Longer hybridization times are acceptable. Several membranes can be processed in the same tubes as long as there is sufficient prehybridization solution ot cover all the membranes and the membranes can move freely in the bag.

2. Add the DIG labeled 1913 probe (concentration 10 pmol/ul) to prehybridization solution to obtain a 10 pmol/ml concentration in the hybridization solution. (Example: 30 ml's prehybridization solution add 3 ul of probe 1913)

3. Allow the probe to hybridize for 1-6 hours at 39 degrees C. Longer is acceptable.

4. At the end of the hybridization, pour the hybridization solution from the tube into a 50 ml polypropylene tube. This hybridization solution contains unannnealed DIG- labeled 1913 probe. The entire solution can be reused in future hybridization experiments. Label and store oligo probe solutions at -20 degrees C. DIG-labeled probes stored in this manner are stable for at least 1 year For reuse, thaw and denature by heating to 95 degrees C for 10 minutes.

5. Wash the membrane twice, 5 minutes per wash, in 2 X Wash Solution at room temperature. These washes remove unbound probe, which will lead to high backgrounds if not removed.

6. Wash the membrane twice in 0.5 X Wash Solution at 39 degrees C for 5 minutes.

COLORMETRIC DETECTION WITH NBT AND BCIP:

PRODUCTS REQUIRED: DIG Nucleic Acid Detection Kit (Cat. No. 1175041).

Table 2. Additionally required reagents for DIG Nucleic Acid Detection.

Additionally required reagents	Description
Buffer 1	100 mM maleic acid, 150 mM NaCl; pH 7.5 (20□C adjusted with NaOH, autoclaved.
Buffer 2	1% Blocking reagent diluted in buffer 1 (final concentration = 1% blocking reagent)
Washing Buffer	Buffer 1 plus 0.3% (v/v) Tween-20
Buffer 3	100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$; pH 9.5 (20□C)
Buffer 4 (TE Buffer)	10 mM Tris-HCl, 1 mM EDTA; pH 8.0 (+20 C)
Color-substrate solution (freshly prepared)	200 ul NBT/BCIP stock solution (vial 4) are added to 10 ml of Buffer 3

Procedure: (Perform all incubations at **room temperature**.)

1. After hybridization and post-hybridization washes, equilibrate the membrane in Washing Buffer for 1 minute.

2. Using a freshly washed dish or bag, block the membrane by gently agitating it in Buffer 2 for 30 minutes. Near the end of the blocking period, prepare the antibody solution

3. Dilute the Anti-Digoxigenin-AP (vial 3 in refrigerator) 1:5,000 (after centrifugation) in Buffer 2 for a working concentration of 150 mU/ml. Mix gently by inversion. (Example add 6 ul Anti-Digoxigenin-AP to 30 ml of blocking solution.)

4. Pour off the Buffer 2 and incubate the membrane for 30 minutes in the Antibody Solution prepared in step 3. Agitate the tray gently to ensure that the membrane is always covered.

5. Discard the antibody solution. Wash twice, 15 minutes per wash, in 100 ml's Buffer 1. This wash removes unbound antibody.

6. Added 200 ul of NBT/BCIP solution to 10 ml's of Buffer 3. This will be used in step 8 and needs to be protected from light.

7. Equilibrate the membrane in 20 ml Buffer 3 for 2 minutes.

8. Pour off Buffer 3, and add approximately 10 ml's color substrate solution directly to the membranes surface by pipet. Incubate the membrane in a box in the dark. Do not shake the container while the color is developing. The color precipitate starts to form within a few min, and the reaction is usually complete after 12 hours.

9. Once the desired spots or bands are detected wash the membrane with Buffer 4 to prevent over-development.

10. The membrane can be stored in a ziplock bag containing buffer 4 to hold the color.

11. Take a picture of the membrane using Ektachrome 160 Tungsten film on the tungsten light table.

WORKING STOCK SOLUTIONS:

0.5 M EDTA (pH 8.0)
93.05 g disodium EDTA
500 ml MQ water
adjust pH to 8.0 with NaOH pellets
autoclave to sterilize

1 M Tris (pH 7.4)
60.55 g Tris Base
500 ml's MQ Water
adjust pH to 7.4 with 6 N HCl
autoclaved to sterilize

1 M Tris-HCl, pH 7.0 (GMW 157.56)
15.756 g Tris-HCl
100 ml's MQ water

2.5 M NaOH (40.00 GMW)
50 grams NaOH
500 mls MQ water
autoclave to sterilize

6 M NaCl
175.32 g NaCl
500 ml MQ water
autoclave to sterilize

1% N- Lauroylsarkosine
1 g N-lauroylsarkosine
100 ml's MQ water

20 X SSC
175.3 g NaCl
88.2 g Sodium citrate
1000 ml's MQ water
adjusted pH to 7.0
autoclave to sterilize

10% TWEEN-20
10 ml Tween-20
90 ml's MQ water

0.5 M $MgCl_2$ (FW 203.3g)
10.1 g $MgCl_2$
100 ml's MQ water

10X TBE
108 g Tris Base
55 g Boric Acid
40 ml's 0.5M EDTA (pH8.0)
960 ml's MQ water

HYBRIDIZATION WORKING SOLUTIONS:

Pre-hybridization and [5X SSC; blcoking reagent 1% (w/v); N-lauroylsarkosine 0.1% (w.v); SDS 0.02% (w/v)] **freeze at -20C**

25 mls 20 X SSC
1 gram Blocking reagent
0.2 mls 10% SDS
10 mls 1 M N-lauroylsarkosine
64.8 mls MQ water

Hybridization

prehybridization solution
10 pmol/ml of labeled primer

2 X Wash Solution (2 X SSC, containing 0.1 % SDS)

100 mls 20 X SSC
10 mls 10% SDS
890 mls MQ water

0.5 X Wash Solution (0.5 X SSC containing 0.1 % SDS)

25 mls 20 X SSC
10 mls 10% SDS
965 mls MQ water

COLORMETIC DETECTION SOLUTIONS:

Buffer 1 (0.1 M Malic Acid; 0.15 M NaCl; pH 7.5)
13.4 g Malic Acid (FW 134.6)
25 mls 6 M NaCl
975 mls MQ H2O
Autoclave

Buffer 2 (1% blocking stock in Buffer 1) **Make Fresh**
0.5 grams Blocking stock
50 mls Buffer 1

Buffer 3 (100 mM Tris-HCl; 100 mM NaCl; 50 mM $MgCl_2$; pH 9.5)
50 mls 1 M Tris-HCl
8.4 mls of 6 M NaCl
50 mls 0.5 M $MgCl_2$
391.6 mls MQ water

Buffer 4 (10mM Tris-HCl; 1 mM EDTA; pH 8.0)
5 mls 1 M Tris-HCl
1 ml 0.5 M EDTA
494 ml's MQ water

Washing Buffer:
3 mls 10% Tween-20
97 mls Buffer 1

APPENDIX C

DATA

Table 1: Persistence of ingested HBV in bodies of *An. stephensi* and *Ae. triseriatus*. Engorged mosquitoes ingested between 2-3 ul of infected blood during feeding. Each ul of the HBV blood suspension contained approximately 116-174 copies of HBV DNA.

	PCR	Southern blot
An. stephensi		
3 days	0/3	0/3
7 days	0/3	0/3
14 days	0/3	0/3
Ae. triseriatus		
3 days	0/3	0/3
7 days	0/3	0/3
14 days	0/3	0/3

Table 2: Persistence of ingested HBV in bodies of *An. stephensi* and *Ae. triseriatus*. Engorged mosquitoes ingested between 2-3 ul of infected blood during feeding. Each ul of the HBV blood suspension contained approximately 116-174 copies of HBV DNA.

Bodies	PCR	Southern blot
An. Stephensi		
0 hours	4/4	4/4
6 hours	5/5	5/5
12 hours	3/5	5/5
24 hours	7/10	7/10
48 hours	4/10*	4/10*
72 hours	0/10	0/10
Ae. triseriatus		
0 hours	2/2	2/2
6 hours	0/2	0/2
12 hours	2/2	2/2**
24 hours	2/2	2/2
48 hours	2/2	2/2***
72 hours	2/2	2/2

* one mosquitoes legs were positive by both PCR and Southern blot
**one mosquitoes body, saliva and legs all positive by Southern blot
***one mosquitoes legs were positive by Southern blot

Table 3. Persistence of ingested HBV in feces of *An. stephensi* and *Ae. triseriatus*. Detection of the HBV genome in the feces of *An. stephensi* and *Ae. triseriatus*. Number of positive samples over total number of samples collected.

	PCR	Southern blot
An. stephensi		
3 days	6/22	10/22
7 days	0/10	0/10
14 days	0/10	0/10
Ae. triseriatus		
3 days	1/30	1/30
7 days	3/13	4/13
14 days	0/11	0/11

Table 4. Persistence of ingested HBV in feces of *An. stephensi* and *Ae. triseriatus*. Detection of the HBV genome in the feces of *An. stephensi* and *Ae. triseriatus*. Number of positive samples over total number of samples collected.

	PCR	Southern blot
An. stephensi		
0 hours	6/18	8/18
6 hours	0/9	0/9
12 hours	0/4	1/4
24 hours	1/6	1/6
48 hours	6/6	6/6
72 hours	5/7	6/7
Ae. triseriatus		
0 hours	0/18	0/18
6 hours	no feces	no feces
12 hours	0/3	0/3
24 hours	1/6	1/6
48 hours	0/12	0/12
72 hours	1/9	1/9

Table 5: Transmission potential of *An. stephensi* during refeeding. An. stephensi fed an HBV infected blood meal and then held 72 hours and allowed to oviposit. A second non-infectious blood meal was provided for feeding. Engorged mosquitoes, feeding membrane, blood and fecal filter paper were collected immediately after feeding.

Sample	PCR	Southern blot
mosquito bodies	0/4	0/4
blood	Neg	Neg
membrane	Neg	Neg
fecal filter paper	0/2	0/2

Table 6. Persistence of ingested HBV in *C. lectularius* bodies. Detection of the HBV virus genome in C. lectularius bodies. Engorged Cimex ingested 3-5 ul of infected blood (1485 pg/ml) during feeding. Each ul of HBV blood contained approximately 720-1000 copies of HBV DNA.

Sample	PCR	Southern Blot
Day 1	1/1	1/1
Day 7	0/5	3/5
Day 14	5/5	5/5
Day 21	5/5	5/5*
Day 28	5/5	5/5*
Day 35	3/3	3/3

*one bed bugs legs were positive by Southern blot on each of these days

Table 7. Persistence of inoculated HBV in *C. lectularius* bodies. Detection of the HBV virus genome in *C. lectularius* bodies. Bed bugs were inoculated with 0.5 ul of HBV suspension that theoretically contained 45 viral particles.

Bodies	PCR	Southern blot
Day 7	0/5	4/5*
Day 14	0/5	4/5
Day 21	0/5	2/5

*2 bed bug's legs were positive by Southern blot at this time point

Table 8. Persistence of ingested HBV in *C. lectularius* feces. Detection of the HBV genome in the feces. Number of positive samples over total number of samples collected.

Collection Day	PCR	Southern blot
Immediately after feeding	0/2	0/2
Day 1	0/18	1/18
Day 7	9/23	12/23
Day 14	4/6	5/6
Day 21	4/14	4/4
Day 28	2/2	2/2
Day 35	1/1	1/1

Table 9. Trans-stadial transmission of ingested HBV. Larva were allowed to molt following an HBV infected blood meal. They were collected 14 days post infected feeding for testing. All exuviae were collected and divided into 2 samples.

Stage Tested	PCR	Southern blot
2nd instar	1/1	1/1
3rd instar	4/4	4/4
4th instar	8/8	8/8
5th instar	1/1	1/1
adult	8/8	8/8
exuviae	POS	POS

Table 10. Persistence of inoculated HBV in *R. prolixus*. Detection of the HBV virus genome in *R. prolixus* samples. *R. prolixus* were inoculated with 1.0 ul of HBV suspension that theoretically contained 90 viral particles

Bodies	PCR	Southern blot
Day 7	0/5	3/5*+
Day 14	0/5	0/5
Day 21	0/5	0/5
Day 28	0/5	0/5
Day 35	0/3	0/3

* 2 kissing bug's saliva was positive by Southern blot

+ 1 kissing bug's legs were positive by Southern blot

APPENDIX D

Human and Animal Use

UNIVERSITY COMMITTEE ON RESEARCH INVOLVING HUMAN OR ANIMAL SUBJECTS

The Graduate School
Michigan State University
118 Linton Hall

University and federal policies and procedures require that **all** research involving human or animal subjects receive prior approval from the appropriate review board. (See Faculty Handbook, p. 116-117 and the Academic Programs book, p. 60.)

HUMAN SUBJECTS

Does the thesis or dissertation you are submitting include research involving human subjects or materials of human origin? (Research involving human subjects includes surveys and telephone interviews used for research; materials of human origin include human blood and /or tissue.)

Yes ☒ No ☐

If yes, indicate UCRIHS log number for the approved protocol and attach the UCRIHS approval letter for that protocol to this form.

UCRIHS Log Number: IRB# 96-039

ANIMAL SUBJECTS

Does the thesis or dissertation you are proposing to submit include research involving vertebrate animals in any way?

Yes ☒ No ☐

If yes, and an animal use form was submitted to the All-University Committee on Animal Use and Care (AUCAUC), please list the approval number below and attach a copy of the AUCAUC approval letter to this form.

AUF Number: 05/96-053-00

If yes, but your project did not need an animal use form, provide a copy of the letter from the AUCAUC which cites the relevant exclusionary policy.

Jamie A. Blow
Student's Name (print)

Jamie A. Blow
Student's Signature

Dr. Edward D. Walker
Major Professor's Name (print)

Edward D. Walker
Major Professor's Signature

APPENDIX E

Entomology Vouch Specimens

Record of Deposition of Voucher Specimens*

The specimens listed on the following sheet(s) have been deposited in the named museum(s) as samples of those species or other taxa which were used in this research. Voucher recognition labels bearing the Voucher No. have been attached or included in fluid-preserved specimens.

Voucher No.: 1998-3

Title of thesis or dissertation (or other research projects):

Persistence of the Hepatitis B viral genome in selected hematophagous insects.

Museum(s) where deposited and abbreviations for table on following sheets:

Entomology Museum, Michigan State University (MSU)

Other Museums:

Investigator's Name (s) (typed)
Jamie A. Blow

Date July 21, 1998

*Reference: Yoshimoto, C. M. 1978. Voucher Specimens for Entomology in North America. Bull. Entomol. Soc. Amer. 24:141-42.

Deposit as follows:

Original: Include as Appendix 1 in ribbon copy of thesis or dissertation.
Copies: Included as Appendix 1 in copies of thesis or dissertation.
Museum(s) files.
Research project files.

This form is available from and the Voucher No. is assigned by the Curator, Michigan State University Entomology Museum.

Voucher Specimen Data

Page 1 of 1 Pages

Species or other taxon	Label data for specimens collected or used and deposited	Number of: Eggs	Larvae	Nymphs	Pupae	Adults ♀	Adults ♂	Other	Museum where deposited
Aedes triseriatus	MSU colony, June 1998					6	5		MSU
Anopheles stephensi	MSU colony, June 1998					8	7		MSU
Culex quinquefasciatus	USAMRID colony, April 1998					7	1		MSU
Cimex lectularius	MSU colony, June 1998			2		25			MSU
Rhodnius prolixus	MSU colony, June 1998					17			MSU

(Use additional sheets if necessary)

Investigator's Name(s) (typed)

Jamie A. Blow

Date July 21, 1998

Voucher No. 1998-3

Received the above listed specimens for deposit in the Michigan State University Entomology Museum.

Curator Date 21 July 1998