The mechanical transmission of (*Cimex lectularius* L.) in South Africa

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Summary

Tests for both hepatitis B surface antigen (HBsAg) and hepatitis e antigen (HBeAg) were carried out on wild-caught and laboratory-colonized bedbugs (*Cimex lectularius* L.), the latter after hepatitis B virus (HBV)-positive blood-meals. Positivity for both antigens was interpreted as an indication of HBV infectivity. Of 22 pools in which were tested 211 bugs collected in the northern Transvaal, 18 were HBsAg-positive and 17 HBeAg-positive, with estimated infection rates of 156,7 and 137,7 per 1000 bugs respectively. Passage of HBV in bugs, allowing an extrinsic incubation period of 57-69 days, resulted in 19 out of 25 bugs being positive for HBsAg after the first passage; only a small number of these were positive for HBeAg. After the second passage all bugs tested were HBsAg-negative, showing that the virus had disappeared. Tests on the salivary glands and carcass of each bug at intervals up to 31 days after an infective meal showed a positivity rate of 98% (HBsAg) and 17% (HBeAg) for carcasses and 20% (HBsAg) and 0% (HBeAg) for salivary glands. Attempts to detect HBV particles in the salivary glands by electron microscopy failed. Bugs were shown to continue to excrete HBsAg in their faeces up to the 42nd day, and both HBsAg and HBeAg together up to the 30th day. HBsAg particles were only detected by electron microscopy in faeces harvested on the 10th day. The results as a whole indicate that no biological multiplication of virus occurs in *C. lectularius* but that mechanical transmission from insects to man could occur by: (*i*) contamination of a person when crushing infective bugs; (*ii*) contamination from infected faeces; and (*iii*) infection by bite due to regurgitation or interrupted feeding.

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Studies already reported from our laboratory have provided a considerable amount of evidence to incriminate the common bedbug, *Cimex lectularius* L., as a vector of human hepatitis B virus (HBV) in South Africa. High positivity rates for hepatitis B surface antigen (HBsAg) were shown in bugs collected from huts located in the northern Transvaal.[1] In addition, the results of laboratory experiments indicated that the bug probably transmits infection mechanically between humans and that the virus does not multiply biologically in the insect.[2,3] Since that work was carried out a radio-immunoassay test for hepatitis e antigen (HBeAg) has also become available. Since data obtained for both HBsAg and HBeAg on a specimen gives a firmer indication that it contains infectious HBV,[4,5] further studies were conducted incorporating both types of test. We thought this should provide stronger evidence as to whether biological or mechanical transmission occurs. The results of this work are reported in this paper.

Further batches of bugs which had been collected in the field at Louis Trichardt in the northern Transvaal and stored at −20°C were chosen for testing because our previous work[1] had shown that the highest infection rate occurred in bugs collected in villages at this particular locality. We also repeated our serial passage of HBV in bugs to see whether the virus disappeared during passage, which would indicate a lack of multiplication. This time we allowed a longer extrinsic incubation period of about 60 days, which is within the range for the duration of HBV incubation in the human host, so as to give the maximum opportunity for viral multiplication to occur. Furthermore, we tested both the salivary glands and the remainder of each insect at intervals after an infective meal to ascertain whether the virus could be replicating in these glands as in the case of an arbovirus. Lastly, we collected and tested faeces from bugs after an infective feed to investigate whether virus was excreted in them making them a source of infection.

Material and methods

Field collection of bugs

Bedbugs were collected from huts in three villages near Louis Trichardt in the northern Transvaal.[1] They were captured alive, killed shortly afterwards by freezing and kept at −20°C except during transportation from Pietersburg to Johannesburg when they were held at 4°C. Before the bugs were processed for virus assay they were identified and separated into pools, usually of 10 insects, according to their stage of development (nymph or adult), their sex in the case of adults, and their state of engorgement. The infection rates per 1 000 bugs were estimated statistically.[6]

Bedbug colony

Bugs from the colony described previously[2] were used for all the laboratory experiments.

Bedbug feeding

For all infective meals bugs were fed through a membrane on defibrinated blood drawn from one particular donor on the day of the feed. The membrane feeding was done as described before[2] and the bugs were then kept in an insectary where the temperature was 25 - 26°C and the relative humidity 75 - 80%.

Virological techniques

Donor blood. On the day of each infective feed a serum specimen was also obtained from the donor, upon which several tests were done to confirm that she remained a highly infectious HBV carrier. Each specimen was tested for HBsAg, HBeAg and antibody to HBeAg by radio-immunoassay (RIA) (Ausria II, procedure B and Abbott-HBeTM, Abbott Laboratories) and the results were interpreted according to the manufacturer's instructions. In addition, the level of hepatitis B DNA polymerase was determined.[7]

Bugs and their salivary glands. In laboratory experiments bugs were killed for antigen assay at varying intervals after the infective blood-meal by placing them in a −70°C freezer, where they were stored until processed. Bugs from either the field or the laboratory were ground in bovine phosphate albumin (BPA) (volume 1,0 ml for pools of 10 nymphs or adults from the field and 1,0 ml for single 5th-instar nymphs or adults from the laboratory colony). The homogenates were centrifuged at 3 000 rpm for 30 minutes and the supernatants frozen immediately at −20°C. Later they were thawed and tested for HBsAg and HBeAg by RIA. In experiment 9 the salivary glands were removed from each adult bug; these were processed and tested separately from the remainder of the insect (the carcass), which was treated in the same way as an intact adult bug. Both salivary glands were carefully removed from each bug in a drop of BPA under a dissecting microscope; one gland was tested by RIA for HBsAg while the other was either tested for HBeAg by RIA or examined under the electron microscope. For RIA a salivary gland was placed in 0,2 ml BPA in a glass storage vial and macerated using fine forceps under the microscope, after which it was centrifuged at 1 500 rpm for 5 minutes and stored at −20°C until tested. For electron microscopy a gland was placed on a glass slide in a drop of anti-HBsAg-positive serum diluted 1:10 with sterile water and allowed to dry (the object of the anti-HBsAg serum is to agglutinate any viral particles, thus increasing the chances of finding them with the microscope). The dry material on the slide was then rehydrated by adding a drop of distilled water. To a drop of this solution of lysed cells was added a drop of 3% phosphotungstic acid, pH 6,0, and a drop of this mixture was applied to a carbon-coated grid. Excess fluid was removed with filter paper and the dry grid examined in a Philips electron microscope operated at 80 kV.

Bug faeces. Faeces harvested from infected bugs were tested by RIA for HBsAG and HBeAg. The bugs were kept in cannisters containing two pieces of filter paper upon which they rested and defaecated. Filter papers were removed at intervals after the infective feed (see Table IV) and replaced with fresh papers. In experiments 7 and 8, faeces on each paper were extracted with 0,03 m phosphate buffer, pH 7,0, at 4°C. The extracts were lyophilized, dissolved in BPA and tested by RIA. In experiment 9 the faeces were extracted with sterile water instead of buffer. Several extracts were prepared from each paper, some of which were rehydrated in 0,5 ml aliquots of BPA and tested by RIA while others were placed on a glass slide, rehydrated in sterile water and allowed to dry before being examined for particles of HBV by negative-contrast electron microscopy as already described.

Serial passage experiment. The main purpose of one experiment (No. 7) was to passage HBV in bugs. This was done in essentially the same manner as the earlier passage experiment.[3] Fourth-instar nymphs were fed on blood from the carrier donor (see Table II). On the 27th day after this feed when the bugs had moulted to 5th-instar nymphs 3 bugs were killed, stored in a −70°C freezer and subsequently tested singly for HBsAg and HBeAg. On the 63rd day 427 bugs were killed and stored but another 25 were kept alive until the 64th-69th day, when they were tested for HBsAg and in some cases for HBeAg as well. The 427 bugs were ground in BPA, 61 bugs per 2 ml and centrifuged. The supernatant was then added to defibrinated blood in the proportion of 1:2 to provide the second blood-meal. A second fresh batch of 4th-instar nymphs was fed on this second serial blood-meal, a sample of which was tested for HBsAg. On the 9th day 4 of these bugs, now 5th-instars, were killed and on the 57th day another 15. All were then tested for HBsAg. The results for RIA of HBsAg and HBeAg were expressed as P/N values, i.e. the number of ^{125}I counts for the sample divided by the number of counts for the negative control.

Results

Infectivity of field-collected bugs

The proportions of the pools of unengorged and engorged bugs, adults or nymphs, positive for each antigen are shown in Table I. As can be seen, most or all pools were positive, with a total of 18 out of 22 positive for HBsAg and 17 out of 22 for HBeAg. The infection rate for HBsAg was 156,7/1 000 and that for HBeAg 137,7/1 000, which is extremely high.

Serial passage of HBV in bugs

Table II gives the result of this experiment. After the first passage only 19 out of 25 insects were HBsAg-positive. Of these 19 not all were HBeAg-positive, and bugs with the lower P/N values for HBsAg were negative for HBeAg. After a second passage, also with long incubation, the bugs were all HBsAg-negative, indicating the complete disappearance of HBV.

Infectivity of adult bugs and their salivary glands

The proportion of bug carcasses and their corresponding salivary glands positive for HBsAg and HBeAg in batches of bugs killed at intervals up to 31 days after the infective meal is shown in Table III. In the case of three groups of these insects one salivary gland of the pair was used for electron microscopy instead of RIA for HBeAg, but no virus particles were detected in the slides examined representing 31 insects. All except 1

TABLE I. RESULTS OF HBsAg AND HBeAg TESTS ON BEDBUGS COLLECTED AT MODENA VILLAGE IN THE LOUIS TRICHARDT AREA, NORTHERN TRANSVAAL

	No. of bugs collected	No. of pools	HBsAg-pos.† (No. of pools)	HBeAg-pos.‡ (No. of pools)
Unengorged adult*	64	7	5	5
Engorged adult	36	4	4	4
Unengorged nymph	29	3	3	3
Engorged nymph	82	8	6	5
Total	211	22	18	17

*Male and female adult bugs were tested in separate pools but the two sexes gave similar results.
†Infection rate 156,7/1 000 bugs, estimated statistically (94 and 245 bugs from two other villages, La Juma and Kranspoort respectively, were negative for both antigens).
‡Infection rate 137,7/1 000.

TABLE II. SERIAL PASSAGE OF HBV IN BEDBUGS*

Passage level	1st passage			2nd passage (day 63)	
P/N value of blood-meals	77,9†			30,5	
Days after meal	27		64-69	9	57
P/N values (individual bugs)	**84,2 (2,9)**	**34,6**	**5,4**	**1,0 - 1,1‡ (4 bugs)**	**1,1 - 1,4‡ (15 bugs)**
	27,2 (3,4)	**32,9**	**4,1 (1,1‡)**		
	1,3† (0,9)	**17,4**	**3,6 (1,2‡)**		
		14,9 (2,6)	**2,9**		
		14,5	**2,3**		
		12,9 (1,5‡)	**2,3**		
		12,4 (3,7)	**2,0‡(1,2‡)**		
		9,8 (1,7‡)	**1,4‡**		
		8,1 (1,7‡)	**1,4‡**		
		7,4	**1,4‡**		
		6,8 (1,4‡)	**1,3‡**		
		6,3	**1,3**		
		6,1			

*P/N values by RIA for HBsAg and HbeAg (in parentheses) in blood-meals and bedbug samples (experiment 7).
†Donor blood had the following other characteristics: HBeAg P/N = 13,5; anti-HBeAg-negative; hepatitis B DNA polymerase = $0,19 \times 10^{-1}$ pmol/h/ml on bleeding.
‡Negative P/N values.

TABLE III. TESTS FOR HBsAg and HBeAg IN SALIVARY GLANDS AND CARCASS AND ATTEMPTS TO VISUALIZE HBV PARTICLES IN SALIVARY GLANDS IN INDIVIDUAL ADULT BEDBUGS (EXPERIMENT 9)

Days after infective meal*	No. of bugs HBsAg-pos.		No. of bugs HBeAg-pos.		Electron microscopy of salivary gland
	Carcass	Salivary gland	Carcass	Salivary gland	
14	**10/10**	**3/10**	**2/10**	**0/10**	**N/D**
15	**10/10**	**4/10**	**6/10**	**ND**	**0/10**
21	**10/10**	**1/10**	**1/10**	**0/10**	**ND**
22	**13/13**	**5/13**	**0/13**	**ND**	**0/13**
30	**10/11**	**0/11**	**1/11**	**0/11**	**ND**
31	**10/10**	**0/10**	**1/10**	**ND**	**0/10**
Total	**63/64 (98%)**	**13/64 (20%)**	**11/64 (17%)**	**0/31**	**0/33**

*Donor blood had the following characteristics: HBsAg P/N = 76,0; HBeAg P/N = 16,5; hepatitis B DNA polymerase = $4,4 \times 10^{-2}$ pmol/h/ml.
ND = not done.

carcass were HBsAg-positive and 17% were HBeAg-positive. Although 20% of the salivary glands were HBsAg-positive, none was HBeAg-positive.

Infectivity of bug faeces

The results of tests on the bug faeces are given in Table IV. The insects continued to excrete HBsAg up to the 42nd day after the infective blood-meal (experiment 9) and both antigens together up to the 30th day (experiment 7). This result of experiment 7 means that the faeces could have remained positive for both antigens for up to 27 days after defaecation in the insectary at 26°C and 75% relative humidity. Other faeces harvested in experiment 9 but not included in Table IV were deliberately stored in the insectary after harvesting, and these remained HBsAg-positive for at least 40 days but HBeAg-positive for only 4 - 6 days after defaecation. Attempts to visualize HBV particles with the electron microscope in faeces deposited in experiment 9 were negative except on day 10, when a small 22 nm HBsAg particle was detected (Fig. 1).

Discussion

All the studies so far reported on the infection of bedbugs (both the common *C. lectularius*[1-3,8] and the tropical bedbug *C. hemipterus*[9-11]) with HBV have relied entirely on tests for the

TABLE IV. TESTS FOR HBsAg and HBeAg AND ATTEMPTED VISUALIZATION OF RELATED PARTICLES IN BUG FAECES STORED AT −70°C ON HARVESTING AFTER INFECTIVE FEEDS IN THREE EXPERIMENTS

Experiment No.	Bug instar	No. of bugs giving faeces	Days after infective meal	HBsAg	HBeAg	Electron microscopy
7	5th instars	500	30	+	+	ND
	5th instars	500	64	-	ND	ND
8*	Adults	283	10	+	+	ND
9	Adults	40	15	+	+	ND
	Adults	*c.*80	6	+	+	-
			10	+	+	HBsAg
			15-38†	+	-	-
			42	+	-	ND
			49	-	-	ND

*Donor blood in experiment 8 had the following characteristics: HBsAg P/N = 66.5; HBeAg P/N = 20.1; anti-HBeAg-negative; and hepatitis B DNA polymerase = 1.53 x 10^{-1} pmol/h/ml.
†On day 32 bugs were refed on uninfected blood.
ND = not done; + = positive; - = negative.

Fig. 1. Spherical 22 nm particles from bedbug faeces negatively stained with phosphotungstic acid at pH 6,0 (x 180 000).

presence of HBsAg as an indication of infectivity and probable presence of HBV. However, a low HBsAg titre in human serum is usually considered to indicate low infectivity and absence of HBV.[12] Because of this the present series of studies was carried out, using tests for both HBsAg and HBeAg because the presence of both these antigens, with HBsAg in high titre, indicates infectious HBV.[4,5,12] In this way it has been possible for us to reach a firmer conclusion as to the mechanical nature of the transmission of HBV by bugs. The overall evidence from the present experiments taken together with the result of the previous transmission experiments in our laboratory[2] suggests that only mechanical transmission occurs between bugs and man and that it can occur in several ways.

The lengthy extrinsic incubation period allowed for bugs in the serial passage of HBV lead to its disappearance in some bugs after one passage and in all bugs after the second passage. This is in contrast to our previous experiment,[3] when HBsAg disappeared from the bugs only after three short passages. A longer viral incubation period of the duration which may occur in man[13] therefore failed to enable biological replication of the virus to occur in the insects.

The high positivity rate for both HBsAg and HBeAg determined in the wild-caught bugs from Louis Trichardt indicates that such bugs would probably release highly infectious material if they were crushed by a person. Human infection could probably result by this method through the contamination of skin lesions or mucosal surfaces. The presence of both antigens was also shown in the carcasses of laboratory-infected bugs (Table III), although HBeAg was present only in 17% of the sample.

The presence of HBsAg in 20% of the salivary glands of the laboratory infected bugs (Table III) but the complete absence of HBeAg indicates that HBV is not multiplying in these glands as it should if it were a true arbovirus. Furthermore, if HBV were replicating, virus particles (i.e. Dane particles as well as the smaller 22 nm particles) should have been present in sufficient quantity to be visualized under the electron microscope. Nothing was detected, so virus particles — perhaps only the 22 nm particles — must have occurred at concentrations below 10^6/ml.

Taylor and Morrison[14] showed that faeces from *C. lectularius* remained HBsAg-positive for up to 35 days after an infective feed. In our experiments we detected both HBsAg and HBeAg in such faeces at intervals up to the 30th day after the feed, i.e. up to 23 days after defaecation. Hence it appears that bug faeces contain HBV and that they could serve as a means of spreading HBV to man by contamination, as suggested by Ogston and London,[15] who showed that HBsAg was excreted in the faeces of *C. hemipterus*. Infection of a susceptible person by means of the faeces could occur by scratching of skin lesions or mucosal surfaces or by inhalation of faecal dust. Our results as a whole indicate that the amount of virus excreted in the faeces decreases with time; since HBsAg particles were detected by electron microscopy in faeces collected on the 10th day after the infective meal, it would appear that the maximum level of HBV was reached at that time with more than 10^6 particles per millilitre present.

In conclusion, the results of our study indicate that no biological multiplication of HBV occurs in *C. lectularius* and that transmission from insects to man is entirely mechanical, taking place by three probable routes: (*i*) contamination of a person who crushes infective bugs; (*ii*) contamination from infected faeces, especially by scratching of bites; and (*iii*) infection by bite, in which infective material is probably regurgitated from the gut or because of interrupted feeding. Regurgitation has been observed in *Rhodnius prolixus*[16] and probably occurs in bedbugs. Interrupted feeding has been observed in *C. lectularius*,[17] and it is likely that a bug might start to feed on one person but complete its meal on a second person sleeping nearby.

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