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# The ecology and epidemiology of arboviruses in South Africa with reference to their arthropod vectors.

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**1992**

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are floodwater mosquitoes and among them Linthicum et al. (1985) have recently provided evidence that a field population of *Ae. mcintoshi (Ae. lineatopennis)* in Kenya transmitted the virus transovarially. This would enable the virus to survive through dry interepizootic periods probably for several years until climatic conditions, notably rainfall, were suitable to trigger the next epizootic. We have so far been unsuccessful in obtaining evidence to show that a species of floodwater *Aedes* in South Africa can transmit the virus transovarially (McIntosh et al. 1980 and Gargan et al., in press).

The present paper reports the results of further vector competence studies. Experiments with *Ae. juppi* were conducted to investigate possible transovarial transmission and assess the horizontal transmission potential of this species. Horizontal transmission studies were also conducted with 3 floodwater *Aedes* species: *Ae. (Neo.) unidentatus* McIntosh and *Ae. (Aedimorphus) dentatus* (Theobald) from the inland plateau and *Ae. (Aed.) argenteopunctatus* (Theobald) which occurs in the Natal coastal lowlands. (McIntosh 1971, 1975). Additionally there was *Culex poicilipes* which occurs on the Natal coast [illegible]nly *Ae. argenteopunctatus* is an uncommon [illegible]cies. The specimens of this species and *Cx. poicilipes* used for the tests were collected in the Transvaal lowlands because heavy rain had fallen in that area making mosquitoes available. These experiments were designed to identify epizootic vectors and potential reservoir vectors.

## MATERIALS AND METHODS

*Mosquitoes.* From the inland plateau region, *Ae. juppi* was collected at Bethlehem, Orange Free State Province, while *Ae. unidentatus* and *Ae. dentatus* came from Heidelburg in the southern Transvaal. From the eastern Transvaal lowlands, *Ae. argenteopunctatus* and *Cx. poicilipes* were collected at Hazyview.

*Infecting Meals.* The mosquitoes were infected by allowing them to feed on hamsters which were viremic 30 hours after intraperitoneal inoculation of the AN 1830 strain of RVF virus. The virus had been passed 6 times in mice and 3 times in Vero cell cultures. The concentration of virus in the blood of each hamster was determined by titration in Vero cells of a sample collected shortly before feeding. For this, 0.1 ml of blood was removed from the heart and diluted with 0.9 ml of Leibovitz's medium. Streptomycin, neomycin, gentamicin and fetal calf serum were incorporated in this medium. Tenfold serial dilutions were then inoculated into monolayers of Vero cells in 96 well plates. Vero cells respond to RVF virus by visually displaying cytopathy, plaques forming after 3 days. The 50% cytopathic dose endpoints were calculated by the method of Reed and Muench (1938) and expressed as $Log_{10}CPD_{50}$/ml.

Infected insects were held at 25–26°C and 75–80% relative humidity for varying periods before individual specimens *(Ae. juppi)* or groups of them were allowed to feed on hamsters, or single mosquitoes were tested by the *in vitro* "capillary" method to determine if they could transmit virus.

*Transmission attempts.* Transmission of virus to hamsters was established by isolation of virus in infant mice inoculated intracerebrally with extracts of the livers of dead animals.

The capillary method for showing *in vitro* transmission by individual mosquitoes was based on the method of Aitken (1977). With this technique a fine capillary tube containing a mixture of equal parts of fetal and calf serum and 10% sucrose solution was inserted over the proboscis of a mosquito which was then allowed to feed on the mixture. Before feeding the tube contained about 0.004 ml of the mixture which after feeding decreased to about 0.0025 ml. The mosquito injects saliva and also virus if present into the capillary tube while feeding. The remaining 0.0025 ml was added to 0.25 ml of Leibovitz's tissue culture medium and tested for virus by inoculation into cultures of Vero cells. A proportion of these specimens were titrated as already described but these titers were expressed as $Log_{10}CPD_{50}$/mosquito capillary fluid. Development of cytopathy due to the presence of virus indicated transmission had occurred. Only visibly engorged infected mosquitoes were considered in estimating the transmission route which is expressed as the percentage of infected mosquitoes transmitting virus.

*Mosquito infectivity.* The number of mosquitoes infected was determined by testing them individually for virus by inoculation into Vero cell cultures, usually on the same day as their transmission feed. Each mosquito was titurated in 1 ml of Leibovitz's medium, centrifuged at 900 RPM for 5 mins and the supernatant fluid inoculated into established monolayers of Vero cells in 4 wells of a microtiter plate. Titrations were performed on 12 of the mosquitoes by inoculating 10 fold serial dilutions of the supernatant. Titers were expressed as $Log_{10}CPD_{50}$/mosquito. The infection rate was expressed as the percentage of mosquitoes feeding on a viremic hamster which became infected.

*Transovarial transmission.* To investigate transovarial transmission, *Ae. juppi* were inoculated with virus, allowed to feed on susceptible hamsters daily from days 4 to 6 postinoculation

and $F_1$ eggs obtained from days 6 to 20. The subsequently reared $F_1$ adults were fed on hamsters for attempted transmission of virus. In addition, both the $F_1$ female and male mosquitoes were tested for virus by intracerebral inoculation of infant mice.

## RESULTS

The results of the horizontal transmission experiments, given in Table 2, show that all 4 species of mosquitoes were readily infected at the high titers of virus used, and transmitted the virus into capillary tubes. In the case of *Ae. argenteopunctatus* and *Cx. poicilipes* transmission by bite to hamsters was also attempted with groups of 6–18 mosquitoes which was successful. Capillary tube transmission rates were low for *Ae. argenteopunctatus*, moderately high for *Ae. unidentatus* and *Ae. dentatus* and very high for *Cx. poicilipes*. In the case of *Cx. poicilipes*, however, the maximum transmission rate occurred later (30 days) indicating a longer extrinsic incubation period.

The results of the titration of individual mosquitoes and capillary fluids are shown in Table 3. While titers of capillary fluids were similar for *Ae. unidentatus* and *Ae. dentatus* (1.8–2.5 $Log_{10}CPD_{50}$/mosquito), these titers as well as titers for mosquitoes reached a higher level in the case of *Cx. poicilipes* (2.3–3.0 and 3.3–6.3 $Log_{10}CPD_{50}$/mosquito, respectively).

The *Ae. juppi* females had an infection rate of 93% (50/54) when tested 5–15 days after their inoculation with virus (6.5 $log_{10}CPD_{50}$/ml). However, the horizontal transmission rate determined by feeding single infected mosquitoes on single hamsters on day 4 was 0/36. Larval mortality was high when rearing the $F_1$ progeny so that only 461 females were successfully reared and fed on hamsters. There were no transmissions to any of 10 hamsters. Subsequently, 656 females and 246 males were tested for virus in 19 pools with no isolations of virus.

## DISCUSSION

*Aedes juppi* was investigated as a possible overwintering reservoir because 3 isolations of virus had been obtained from a large collection of this species made during an epizootic at Tweespruit in 1975 and the species had been shown to transmit the virus horizontally (McIntosh et al. 1980). The failure of 4 of the 54 mosquitoes tested in the present study to become infected after inoculation was probably due to inadequate inoculation in the case of these 4 mosquitoes. The mosquitoes were fed

Table 2. Results of vector competence tests with Rift Valley fever virus and 4 mosquito species infected orally.

| Species | Titer of infective feed in $Log_{10}CPD_{50}$ PFU/ml | Mosquito infection: Days after infective meal | Mosquito infection: Rate[a] | Transmission: Days after infective meal | Transmission: Test | Transmission: Rate[b] |
|---|---|---|---|---|---|---|
| *Ae. argenteopunctatus* | 7.8–9.8 | 14–26 | 29/38 (76%) | 9;11 | hamster | + ; + |
| | | | | 30 | capillary | 1/7 (14%) |
| *Ae. unidentatus* | 6.8–7.8 | 11 | 12/14 (86%) | 11 | capillary | 7/12 (58%) |
| *Ae. dentatus* | 6.8–7.8 | 11 | 28/31 (90%) | 11 | capillary | 9/28 (32%) |
| | | 17 | 6/8 (75%) | 18 | capillary | 3/6 (50%) |
| *Cx. poicilipes* | 7.8 | 15 | 26/29 (90%) | 15 | capillary | 4/26 (15%) |
| | | | | 30 | capillary | 24/30 (80%) |
| | | | | 15;15 | hamster | + ; + |

[a] = numerator = no. mosquitoes infected; denominator = no. mosquitoes tested.
[b] = numerator = no. mosquitoes transmitting; denominator = no. infected mosquitoes feeding.

Table 3. Titers of Rift Valley fever virus infected mosquitoes and capillary fluids, $Log_{10}CPD_{50}$/mosquito.

| Species | Days post-infection of capillary feed | Mosquitoes | Capillary fluid |
|---|---|---|---|
| *Ae. argenteopunctatus* | 30 | 5.0 5.5 | 0. 2.3 |
| *Ae. unidentatus* | 11 | Not done | 2.0 2.0 2.0 2.3 2.5 2.5 2.5 |
| *Ae. dentatus* | 11 | Not done | 1.8 1.8 1.8 1.8 1.8 1.8 2.3 2.5 2.5 |
| | 18 | Not done | 2.0 2.3 2.5 |
| *Cx. poicilipes* | 15 | 3.3 4.0 4.5 5.0 5.5 | — — — 3.3 2.3 |
| | 30 | 3.3 4.3 4.3 5.3 6.3 | 2.3[a] 2.5[a] 3.0[a] 3.0[a] |

[a] = the mosquitoes corresponding to these capillary fluids were not titrated.

soon after inoculation with virus (4 days) because they were already old insects. As inoculation bypasses the midgut, the establishment of infection in the salivary glands should have occurred within this period so the negative transmission rate indicates that this species has a poor vector potential. This concurs with the transmission rate of one out of 18 mosquitoes determined previously (McIntosh et al. 1980). The difficulty in rearing progeny of *Ae. juppi* meant that only a small sample could be made available for the transovarial transmission test. In other investigations on *Ae. juppi* the same problem prevented the production of much larger numbers of adults in the $F_1$ generation; this was the testing of adults reared from overwintering eggs collected at Tweespruit in 1984 (Gargan et al., in press), when no viruses were recovered from 5,425 mosquitoes tested in 557 pools. All the available evidence therefore suggests that *Ae. juppi* has a low vector potential and that it is probably only a subsidiary epizootic vector not at all involved in vertical transmission of the virus.

The 4 species of mosquitoes orally infected with virus were allowed to feed on hamsters circulating high titers of virus. Similar titers are reached and sometimes exceeded in lambs and calves (McIntosh, 1973b).

In the previous horizontal transmission tests done with several mosquito species (McIntosh et al. 1980) the transmission rates were determined using hamsters whereas in the present work an *in vitro* method was used for most of the tests. This capillary method has been compared with hamster feeding in the case of *Cx. (Cux.) univittatus* Theobald and West Nile and Sindbis viruses when it was found that although hamsters were slightly more sensitive indicators of transmission, the transmission rates obtained with the two methods were not significantly different (Cornel and Jupp, unpublished work). It is likely that the same would apply to various mosquito species and RVF virus in which case the transmission rates shown in Table 1 would also have been a little higher if determined using hamsters. All 4 species listed are potential vectors according to the results in the tests although no isolations of viruses have yet been made from wild-caught mosquitoes of any of them in southern Africa except one isolation from *Ae. dentatus* collected in Zimbabwe (McIntosh 1972). *Aedes argenteopunctatus* must have a low degree of importance as a vector on account of its low transmission rate and uncommon occurrence in the Natal coastal area. The floodwater mosquitoes *Ae. unidentatus* and *Ae. dentatus* on the other hand, can both be prevalent in the highveld region of the inland plateau and this, taken together with their moderately high transmission rates, make them more significant as potential epizootic and possibly reservoir vectors. *Ae. unidentatus* may well be important as a reservoir vector since it is closely related to *Ae. mcintoshi* (reported as *Ae. lineatopennis)* the species shown to transmit transovarially in Kenya (Linthicum et al. 1985). Comparison of the present results with results of a vector competence test done with *Ae. mcintoshi (Ae. lineatopennis)* by McIntosh et al. (1980), shows that *Ae. unidentatus* is a considerably more efficient vector than *Ae. mcintoshi (Ae. lineatopennis)* from the western Transvaal with both a higher susceptibility and transmissibility with RVF virus. *Culex poicilipes* appears to be a more efficient vector than *Cx. zombaensis* which was the major vector during an epizootic at Mtubatuba in the Natal coastal lowlands (McIntosh et al. 1983) but has a similar long extrinsic incubation period necessary for maximum transmission. This mosquito is fairly common in the woodland savannah regions of the Natal coast although not as abundant as *Cx. zombaensis* and could play the role of epizootic vector in this habitat.

## ACKNOWLEDGMENTS

One of us (P. G. Jupp) wishes to acknowledge a grant from the Medical Research Council which contributed towards this work. We also thank the Director-General of National Health for permission to publish.

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# A BIONOMIC STUDY OF ADULT *AEDES* (*NEOMELANICONION*) *CIRCUMLUTEOLUS* IN NORTHERN KWAZULU, SOUTH AFRICA

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ABSTRACT. Sixteen monthly visits were made to the Ndumu game reserve when resting *Aedes circumluteolus* mosquitoes were collected on the ground. The population density reached exceedingly high levels 7 or more days after the Usutu and/or Pongola rivers inundated their flood plains, and was related mainly to river flooding and far less to local rainfall. During dry months male *Ae. circumluteolus* disappeared while females persisted at very low levels. Blood-feeding and ovarian development occurred throughout the year and precipitin tests showed the preferred host was almost exclusively antelope (Bovidae), the dominant large mammal present. It was concluded that *Ae. circumluteolus* is a floodwater mosquito. Owing to the low density of females during dry months and low infection rates with arboviruses it is considered unlikely that *Ae. circumluteolus* would support viral transmission throughout the year. It is more likely that viruses survive in overwintering eggs and are transovarially transmitted.

## INTRODUCTION

The Ndumu game reserve is regarded as situated in the tropical, eastern coastal plain of South Africa. This is based on the 18°C mean midwinter isotherm and the 500 mm isohyet (Poynton 1964). From April 1956 through December 1968 our Unit made a viral survey of the mosquito population. *Aedes (Neomelaniconion) circumluteolus* (Theobald) was shown to be the most prevalent mosquito and over the period yielded 75 isolates which included strains of 11 arboviruses (Worth et al. 1961, McIntosh et al. 1972a). *Aedes circumluteolus* was the most frequently infected species and laboratory tests had shown it capable of transmitting the flaviviruses Wesselsbron (Muspratt et al. 1957) and Spondweni, and the bunyaviruses Pongola and Bunyamwera among the viruses isolated (Arbovirus Unit, unpublished data). It was concluded at that time that *Ae. circumluteolus* was an important vector of arboviruses even if only in an incidental capacity (McIntosh et al. 1972a). Additionally, 2 strains of the phlebovirus Rift Valley fever virus had also been isolated from the species in another part of northern Kwazulu (Kokernot et al. 1957).

A study of the adult bionomics of *Ae. circumluteolus* was carried out at Ndumu from August 1967 through December 1968 to better understand its role as a vector of arboviruses and the results are reported here. Since this study was completed further work in South Africa has further incriminated *Ae. circumluteolus* as a vector of the important Rift Valley fever virus (Jupp et al. 1983, McIntosh et al. 1983). Additionally, Linthicum et al. (1985) in Kenya have obtained evidence that a feral population of another member of the same *Neomelaniconion* subgenus, *Ae. lineatopennis* (Ludlow) (= *Ae. mcintoshi*; Huang 1985), acts as a reservoir vector of Rift Valley fever virus by transovarial transmission.

Because only one isolate of Pongola virus and one of Germiston virus were made from the 41,600 female *Ae. circumluteolus* collected in our bionomic study, we were unable to relate viral infection data to the bionomic data collected. The diminished infectivity could have been caused by a major change in blood-feeding patterns by this species – from cattle to antelope species – as a result of the transformation of the Reserve into a wild life sanctuary which was complete by mid-1967 just before the study began (McIntosh et al. 1972a). Alternatively there could have been a fall off in the number of available susceptible vertebrate hosts.

## STUDY AREA

The geographical position, topography and climate of the Ndumu game reserve has already been described (Worth et al. 1961). The area of northern Natal in which it is located – Tongaland – is now part of the region known as Kwazulu. Nearly half of the 10,000 hectare game reserve lies within the flood plain of the Usutu and Pongola rivers which meet at the north-eastern corner of the reserve. The Usutu River forms the reserve's northern boundary and the border with Mozambique, while the Pongola River runs parallel and close to its eastern boundary. Thirteen sites were chosen for collecting mosquitoes resting in ground vegetation. Ten of these were just within or on the edge of the flood plain of the Usutu river, while 3 sites were much drier and located outside the flood plain. During periodic floodings of the rivers various low lying areas of the reserve suddenly become inundated and the water level in certain pans rose. The mosquito collection sites situated at or near the flood plain were close to the areas

Reprinted from Journal of The American Mosquito Control Association
Vol 3 June 1987 Number 2

where larvae of *Ae. circumluteolus* developed. Each site measured 743 sq. meters and had ground cover shaded by trees throughout the year. At Ndumu rain falls usually in the summer from November through March. The rest of the year is much drier, particularly June through August when there is little or no rain.

## METHODS

*Mosquito collecting:* The portable "hand suction trap" made by us for collecting mosquitoes off ground vegetation was similar to the trap of De Freitas et al. (1966). It consisted of a "Perspex" cylinder 18 cm long and 15 cm in diameter housing a 12 volt, 3 watt, DC electric motor with a 3-bladed rubber propeller. In our design the mosquitoes were aspirated directly through the fan into a 20 $cm^3$ collecting cage. About 10% of the catch was damaged at high mosquito densities. The inlet was covered by wire mesh screen comprised of squares measuring 13 × 13 mm to prevent debris and very large insects being aspirated. The operator of a trap carried a satchel on his back containing the 12 volt motor cycle battery, held the trap by its upwards curving handle in his right hand and a short stick equipped with a metal hook in his left hand. He combed one-half of the area of a collecting site parting the vegetation with his stick while keeping the inlet of the cylinder about 10 cm off the ground. Monthly visits were made to Ndumu from August 1967 through December 1968 except in November 1968 when weather conditions in northern Kwazulu prevented access to the Ndumu area. Each visit usually consisted of 6 collecting days: each morning 2 men collected for half an hour at each of four sites so that each of the sites 1-12 was sampled twice. Site 13, the forest site, was used only from February 1968 and was not sampled as frequently as the other sites.

*Examination of mosquitoes:* Mosquitoes were brought back alive to the field laboratory on the day of collection, killed with hydrogen cyanide and examined microscopically. The specimens of *Ae. circumluteolus* were extracted from each collection, the number of males and females counted and females classified as to their metabolic state. The undamaged females were separated into 3 classes: "gravid," "blood-engorged" and "empty." Mosquitoes with enlarged ovaries and no detectable unassimilated blood were recorded as "gravid," specimens with any detectable blood whether the ovaries were partly developed or not were recorded as "blood-engorged," while all other females were regarded as "empty." This last category included females with an undistended abdomen, those where it was fluid-filled and those where it may have contained fatty material. A small proportion of females were unclassified owing to damage – this number was recorded. Blood-meals from a proportion of the engorged mosquitoes in each collection were stored on filter paper and later tested for vertebrate host origin by the precipitin test according to the method used by Anderson (1967). During the period February through October 1968 mosquitoes other than *Ae. circumluteolus* were also identified, usually from half of the collections.

*Analysis of results, rainfall and river flow:* The monthly population index used for *Ae. circumluteolus* was the number of mosquitoes collected per trap hour expressed as Williams' modification of the geometric mean (Mw) (Haddow 1960). It was calculated from the total number of mosquitoes collected, including those damaged, and the total number of trap hours during each visit to Ndumu. The proportion of each category of females was expressed as the percentage of the total collection of female mosquitoes during each month and the number damaged was not included. Rainfall was recorded at the general store just outside the game reserve. Monthly averages are shown in Fig. 1. Flow rates for the Pongola River were recorded in a gauging station 22.5 km upstream from the gorge at Josini where a dam has subsequently been built. Flow rates for the Usutu were recorded at a station 22.5 km upstream from the confluence with the Pongola River. A flow greater than 2,500 cusecs (70, 8 $m^3$/sec) was regarded as indicative of the river being in flood on the advice of hydrologists in the Department of Water Affairs.

## RESULTS

*Monthly fluctuations in population level of* Ae. circumluteolus*:* Table 1 shows the numbers of male and female mosquitoes collected monthly over the 16 months of the study, together with the corresponding Williams' means. The same figures are expressed graphically in Fig. 1 together with the monthly rainfall. The histogram in Fig. 2 illustrates the relationship between population density and river flooding (Usutu and/or Pongola rivers) in time. The winter and spring months, June to October 1968, viz the period when *Ae. circumluteolus* was nearly inactive is omitted from this figure.

Table 2 illustrates productivity of the 13 trapping sites when expressed in terms of the average catch of all *Ae. circumluteolus* mosquitoes per man hour over the whole study period.

*Seasonal pattern of blood-feeding and egg production:* Figure 3 shows the relative proportions

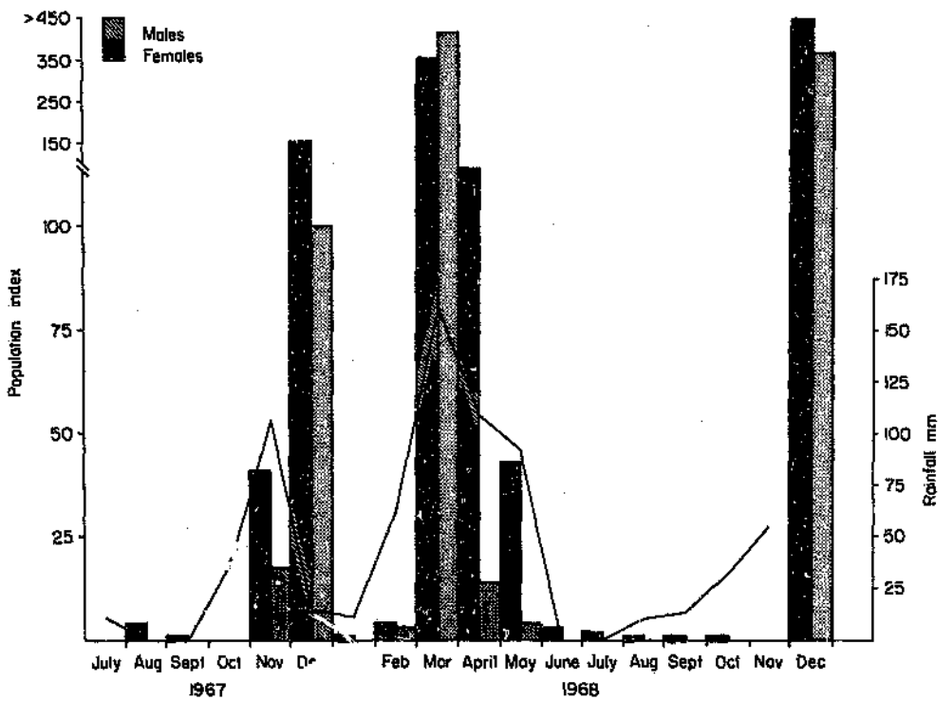



Fig. 1. Monthly fluctuations in rainfall and population level of *Aedes circumluteolus* at Ndumu: mean catch per trap hour expressed as Williams' mean.

Table 1. Monthly fluctuations in population level of *Aedes circumluteolus** at Ndumu, Kwazulu, South Africa.

| | No. trap hrs | Females | | Males | |
|---|---|---|---|---|---|
| | | No. mosqs | Mw/trap† hr | No. mosqs | Mw/trap† hr |
| Aug. 1967 | 16 | 102 | 4 | 3 | <1 |
| Sept. | 24 | 23 | 1 | 1 | <1 |
| Oct. | 24 | 4 | <1 | 0 | 0 |
| Nov. | 24 | 1,290 | 41 | 700 | 17 |
| Dec. | 22 | 7,377 | 164 | 7,230 | 100 |
| Jan. 1968 | 24 | 37 | 1 | 6 | <1 |
| Feb. | 26 | 310 | 4 | 293 | 3 |
| Mar. | 24 | 16,151 | 354 | 28,457 | 416 |
| April | 25 | 3,776 | 114 | 669 | 14 |
| May | 25 | 1,601 | 43 | 200 | 4 |
| June | 25 | 222 | 3 | 13 | <1 |
| July | 16 | 43 | 2 | 0 | 0 |
| Aug. | 13 | 11 | 1 | 0 | 0 |
| Sept. | 25 | 25 | 1 | 11 | <1 |
| Oct. | 21 | 22 | 1 | 0 | 0 |
| Dec. | 25 | 18,971 | 561 | 17,857 | 371 |

*The numbers in this table and in Fig. 1 include the damaged but identifiable mosquitoes.
†Williams' modification of the mean.

of gravid, blood-engorged and empty individuals in each month's catch of *Ae. circumluteolus*. Engorged and empty mosquitoes occurred in every catch and gravid insects as well except in October 1967 and August 1968 when catches were at their lowest (4 and 11 mosquitoes, respectively). In those summer months when the male catch was about the same as the female catch, i.e., December 1967 and February and December 1968, or greater as in March 1968, only 2–9% of female mosquitoes were gravid. During these months the catches were almost certainly composed predominantly of newly emerged mosquitoes which had not yet developed eggs. Such mosquitoes would account for the large proportion of "empty mosquitoes" present during these 4 months, i.e., 57–86% of the total catches.

*Precipitin test:* Blood meals from 1,018 *Ae. circumluteolus* tested by the precipitin test revealed that 96% were of antelope (Bovidae) origin.

*Prevalence of other mosquito species:* *Culex* species were always present in the collections with *Culex (Culex) neavei* Theobald, *Cx. (Cux.) zombaensis* Theobald, *Cx. (Eumelanomyia) insignis* (Carter) and *Cx. (Cux.) antennatus* (Becker) being the commonest species in that order. The population densities of these mosquitoes all fell to low levels during the dry winter months except *Cx. neavei* which usually persisted in significant numbers. During the dry

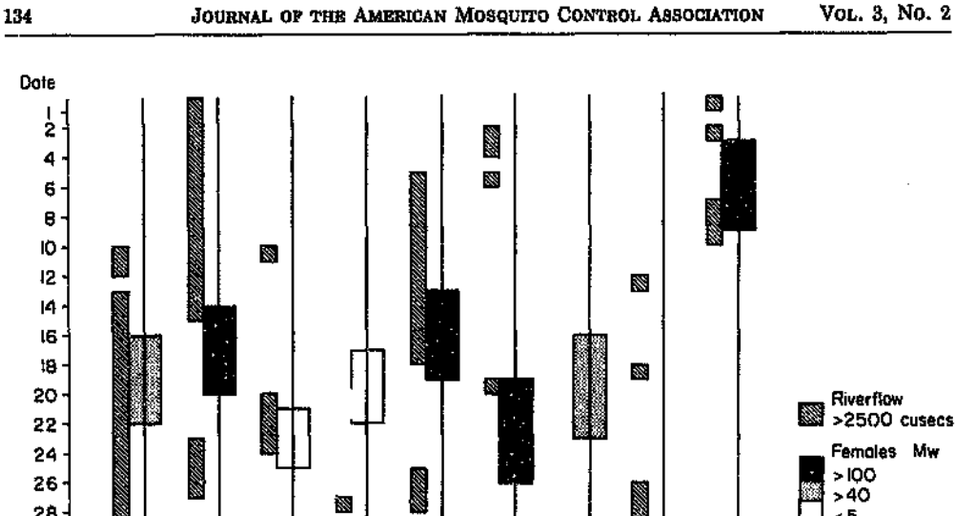



Fig. 2. Density of *Aedes circumluteolus* at Ndumu related to flow of Pongola and/or Usutu rivers: June–October 1968 data are omitted.

Table 2. Average catch of *Aedes circumluteolus** per man hour according to location of sites at Ndumu.

| Site | Location | No. mosquitoes |
|---|---|---|
| 10 | Near floodplain | 738 |
| 4, 5 and 7 | Near floodplain | 426–492 |
| 6, 9 and 11 | Near floodplain | 216–290 |
| 2, 3 and 13 | Intermediate | 134–186 |
| 1, 8 and 12 | Outside floodplain | 71–103 |

* All mosquitoes - males, females and damaged.

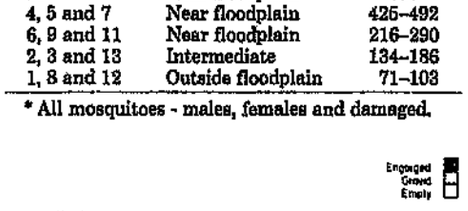



Fig. 3. Seasonal pattern of blood-feeding and egg production of *Aedes circumluteolus* at Ndumu; numbers above columns are total numbers of females collected.

months, June through October 1968, aedine mosquitoes nearly disappeared from the catches except *Ae. (Aedimorphus) albocephalus* (Theobald) and *Ae. circumluteolus*. In March and December 1968 when densities of *Ae. circumluteolus* were at their highest numbers of all other species of mosquitoes collected in the traps were extremely low, possibly because they had been displaced from their usual resting places.

## DISCUSSION

The only local rainfall at Ndumu which could have had any significant influence on the hatching of the eggs of *Ae. circumluteolus* and their subsequent development into adults is rain which was concentrated on one day or several consecutive days prior to a collecting trip carried out during the warmer months (mean monthly temperature 22.2–27.8°C). Such rainfalls only occurred on October 24–27, 1967 (34.5 mm), November 17–22 (81.5 mm), March 7, 1968 (133 mm) and April 19 (62 mm). Although Fig. 1 shows there is some relationship between monthly rainfall and population indices of females and males, the relationship is by no means consistent, suggesting there must be another cause for some of the population peaks. Study of Fig. 2 reveals that high population levels were mainly related to the degree of previous river flooding expressed in terms of river flow. During the period of the study the Usutu River was more frequently in flood than the Pongola River. As shown in Table 2 resting sites where the highest densities of *Ae. circumluteolus* were collected were those closest to areas in the reserve most frequently flooded by the Usutu River (sites 4–7 and 9–11). Sites with intermediate catches (2, 3 and 13) were further away from such inundated areas, while sites 1, 8 and 12 located in dry areas of the reserve gave the

smallest catches. Each monthly collecting trip shown in Fig. 2 is examined with regard to its relationship to rainfall and river flooding.

The modest population level which occurred in November 1967 can only have been caused by a combination of the previous 34.5 mm rain of October 24–27 and river flooding which began in November 6 days before the start of mosquito collecting but which continued throughout the 6 day trip. However, in the laboratory we have found that at an air temperature of 25°C this mosquito requires a minimum of 7 days from egg hatching to adult emergence. Nieschulz et al. (1934) also reported a similar developmental time from observation on *Ae. circumluteolus* in the field. This means that mosquitoes present in the catches on the first day or second day of this trip probably originated from larval sites provided by local rain rather than river flooding, while the latter could have accounted for mosquitoes collected over the remaining 4 days. The high density of December 1967 is readily explained by river flooding which commenced 14 days before the start of collecting. The 82 mm rain recorded from November 17 to 22 may have also contributed to this mosquito population. Low populations in January and February 1968 followed months of low rainfall; there was no river flooding in February while flooding in January occurred too late. The population peak in March with more males than females, was clearly due to extensive river flooding which started 8 days before the start of collecting and the 133 mm of local rain which fell in a single day, March 7. On this trip the number of mosquitoes taken increased daily from the first until the last collecting days indicating emergence was occurring over the collecting period. In April the density of males dropped while females remained at a fairly high level, indicating that little emergence was still taking place. As there was limited river flooding in April it is most likely that the majority of the females taken in the catches were survivors from mosquitoes which had emerged the previous month. The modest density in May is explained by the absence of river flooding since April 19. The 62 mm recorded on this date, a month before collecting began, may have produced a limited number of mosquitoes of which a proportion were still alive. River flooding which started on November 27 is the only explanation for the December 1968 population level which had the highest density of females and second highest density of males in the study.

On 3 occasions larvae of *Ae. circumluteolus* were collected in inundated areas adjacent to pans within the flood plain of the Usutu River. Several attempts to find larvae in temporary rain water pools away from the flood plain were all unsuccessful. This also indicates that river flooding rather than local rain *per se* is important to cause the hatching of dormant eggs.

*Aedes circumluteolus* therefore fits the category of a typical floodwater *Aedes*, whose eggs hatch mainly because of riverine flooding in the Tongaland area of northern Kwazulu, with subsequent peaks in adult population density. This compares with other floodwater mosquitoes, such as *Ae. (Ochlerotatus) juppi* McIntosh and *Ae. (Neomelaniconion) unidentatus* McIntosh occurring on the South African Highveld (T. P. Gargan, R. J. Novak and P. G. Jupp, unpublished data) and *Ae. (Aedimorphus) vexans* and *Ae. (Ochlerotatus) sticticus*, Meigen occurring in the Columbia River Valley in North America (Gjullin et al. 1950). Our unit has not encountered *Ae. circumluteolus* in other parts of northern Kwazulu at such high levels as those reached at Ndumu. However, it has been collected at river margins or marshes after rain in smaller numbers. It appears that local rainfall needs to be heavy and concentrated to cause similar surface flooding to that associated with riverine flooding. The observations of Nieschultz et al. (1934) are in accord with this.

During the dry winter and spring months (June to October) the mosquito dropped to very low levels but never completely disappeared. Male mosquitoes were either absent or fewer than females. It appears that during this dry period a very small proportion of the total population is surviving for long periods; the females collected from August to October usually appeared old. Theoretically if virus infection rates in *Ae. circumluteolus* were high enough the small residual winter-spring population could include infected mosquitoes which would carry a virus through the dry season at Ndumu. However, the infection rates in summer populations have always been low. (Worth et al. 1961, McIntosh et al. 1972a). It seems more likely that these viruses overwinter in the egg stage and survive by transovarial transmission, particularly in view of the work done by Linthicum et al. (1985).

The 2 months when gravid mosquitoes did not feature in the collections are not thought to indicate a cessation of ovarian development, especially as blood-engorged mosquitoes were collected on both occasions. Rather they were probably due to the small size of the sample of mosquitoes available for examination. The results indicate that blood-feeding and ovarian development occur at Ndumu throughout the year and that there is no reduction of these activities during the winter. The almost exclusive preference for antelope blood shown by *Ae. circumluteolus* at Ndumu in 1967–68 differs from the preferences shown in a study prior to 1964 (Paterson et al. 1964). In that study the

mosquito was shown to have a wider host range, feeding on man and his domestic animals besides antelope. However, the new host preference probably reflects the change which took place in the Ndumu reserve by mid-1967 when the natives and their animals were moved out (McIntosh et al. 1972a).

As concerns the other main species of mosquito collected in the study, it appears that *Ae. albocephalus* is the only other ground-breeding *Aedes* in which small numbers of adults occur throughout the winter in the reserve. The paucity of *Cx. (Cux) poicilipes* (Theobald) in these resting collections as compared to the frequency at which it has been sampled in bait traps at Ndumu (McIntosh et al. 1972b) indicates this species may rest elsewhere, possibly above ground level.

Since the study was conducted, the Pongola River has been dammed. This has prevented it from flooding as frequently as before including where it passes through the Ndumu game reserve. This development appears to have caused a reduction in population densities of *Ae. circumluteolus* in the reserve and presumably in the whole of Tongaland judging from the infrequency at which the species has been collected during malarial vector surveillance and during collections for arboviral vector research.

## ACKNOWLEDGMENTS

We are most grateful to the Director and Staff of the Natal Parks, Game and Fish Preservation Board for the facilities granted us in the Ndumu reserve and for their co-operation throughout the project. We are also indebted to the Departments of Water Affairs of South Africa and Mozambique respectively for records of river flow.

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No. 70A

# MOSQUITO VECTORS

P.G. Jupp

## Introduction

Among the pathogens that cause disease in livestock in southern Africa, only a few viruses are transmitted by mosquitoes (Table 1). These and other arthropod-borne viruses (arboviruses) characteristically multiply in both the vertebrate host and the mosquito vector. Successful transmission is dependent upon the virus producing a viraemia in the vertebrate, while the mosquito which feeds on the vertebrate must develop a salivary gland infection followed by excretion of virus in the saliva. The arboviruses belong to several families and genera and their taxonomic categories and host relations are discussed elsewhere (see Chapter on Arboviruses).

Of the mosquito-borne viruses that occur in south.. .. Africa, Rift Valley fever (RVF) virus is periodically responsible for serious disease in sheep and cattle in the subcontinent, while Wesselsbron (WSL) virus causes a milder disease, affecting sheep in particular. West Nile (WN) and Banzi (BAN) viruses, however, have only been experimentally incriminated as causes of congenital abnormalities in lambs (See Chapter on Viral teratology in ruminants) in South Africa $10$. In France, WN virus has been documented as the cause of encephalomyelitis in horses $22, 23$.

Other mosquito-borne viruses of the families Togaviridae and Flaviviridae that do not occur in southern Africa, but which cause

25X

important diseases mainly in equines and humans outside Africa include the alphaviruses of eastern, western and Venezuelan encephalomyelitides and the flaviviruses of Murray Valley, St. Louis and Japanese B encephalitides (see Chapters on the specific diseases). It is possible that Akabane virus, which is a bunyavirus of the family Bunyaviridae, is also present in southern Africa, although this still remains to be demonstrated. This virus has been isolated from *Culicoides* spp. and from mosquitoes in Japan, suggesting it is also mosquito-borne $81$. Akabane virus has been incriminated as the cause of abortion and congenital abnormalities in livestock $51$ (see Chapter on Viral teratology in ruminants).

Rift Valley fever, WSL and WN viruses are also important in causing human disease, while two other mosquito-borne viruses, namely chikungunya (CHIK) and Sindbis (SIN) viruses, only cause disease in humans. Their epidemiology and association with human disease have been summarized recently by McIntosh $62$.

Apart from virus isolation from wild-caught mosquitoes, the lines of evidence incriminating a mosquito species as a vector are its susceptibility to infection with, and its ability to transmit, the virus concerned (vector competence) plus its relative density and ecological characteristics. Vector competence is assessed quantitatively in laboratory tests while data on density and ecology are obtained by field observations. Relevant aspects of ecology are feeding behaviour, including host preferences, larval habitat associations and biology of the egg stage.

Climatic factors influence arboviruses both directly and indirectly. Temperature conditions directly affect the multiplication of the virus in the mosquito vector, low temperatures slowing down viral replication and lowering vector competence $28, 83$. However, more importantly, climatic

factors operate through their influence on the geographical distribution and prevalence of the viral hosts, both mosquito and vertebrate. In the case of mosquitoes, high rainfall and temperatures can lead to higher population densities of species belonging to all genera, particularly *Aedes*. Climate therefore has a considerable effect on the epidemiology of arbovirus infections.

Apart from some work in the temperate, moist highlands and subtropical, moist lowlands of Zimbabwe, the areas of southern Africa lying outside South Africa have largely only been studied for the presence of mosquito-borne viruses by means of serology without virus isolation from wild-caught mosquitoes being attempted. There is considerable arbovirus activity in the tropical region of Mozambique and the Zambezi valley, and in the subtropical, moist northern belt that extends westwards from Zimbabwe and embraces the Caprivi strip, the Okavango swamps in northern Botswana, and Etosha pan in north-eastern Namibia $11, 46, 50$.

Arbovirus activity in South Africa is more or less confined to the subtropical and tropical Transvaal Lowveld, the Natal coastal lowlands, and to the temperate, high inland plateau of the Karoo and Highveld. These regions all fall in the summer rainfall area. The inland plateau has virtually no rain from May to November, while in the winter, from June to August, night temperatures are often near freezing and periodically fall below this. In the Transvaal Lowveld and Natal lowlands, winters are dry but mild.

Records of rainfall in the summer rainfall region of South Africa since 1910 reveal a 20 year oscillation between decades of above and below average rainfall $88$. The 1950s was a decade of high average rainfall, the 1960s below average and the 1970s again above average. During the period 1957-1979, the major outbreaks of WN, CHIK and RVF infections in

25×10

humans and domestic animals have occurred mainly during periods of above average rainfall. One can therefore expect that the 1980s should be a period of low arboviral activity and the 1990s one of high activity.

During times of heavy rain certain parts of the inland plateau, notably the panveld area of the western Orange Free State and north-eastern Cape Province, may become so saturated that large pans and areas of pasture are flooded. Such accumulations of water may remain standing on farms for several months, particularly if there is a granite underlay as in the case of the Ghaapse Plateau. Under these conditions large populations of floodwater *Aedes* mosquitoes are produced initially, which are subsequently replaced by equally large populations of *Culex* mosquitoes. At such times epidemics of RVF may occur, but the extremely high densities of mosquitoes and other biting flies are also detrimental to livestock in other ways. These include reduced milk production by dairy cows and the development of a poor general state of health in sheep and cattle. Sheep may even die from loss of blood as a result of the continual biting by vast numbers of insects. This kind of situation is probably the main reason in South Africa for mosquito control being necessary. However, effective large-scale mosquito control on farms would be difficult and very expensive to carry out.

Virus isolations from mosquitoes

Table 2 lists the six mosquito species which yielded multiple isolations of RVF virus when collected on farms during RVF epidemics among sheep and cattle. The largest number of isolations on the inland plateau came from *Culex theileri* (plate 1) and in the Natal lowlands from *Cx. zombaensis* (plate 2). Fewer isolations were made from aedine mosquitoes because collecting only began once an epidemic was already

well under way, by which time the aedine populations were waning. All the *Aedes* spp. given in Table 2 are floodwater mosquitoes belonging to the subgenera *Neomelaniconion (Neo.)* and *Ochlerotatus (Och)*. *Aedes (Neo.) mcintoshi* and *Ae. (Och.) juppi* are illustrated in plates 3 and 4.

As shown in Table 3, 33 isolations of WSL virus have been made from mosquitoes collected in South Africa and 15 from mosquitoes collected in Zimbabwe. Apart from two, all these isolations were made from floodwater aedine mosquitoes. However, RVF virus isolations were obtained from numerous *Culex* as well as *Aedes* spp. Like RVF virus (Table 2), the most important *Aedes* spp. involved with WSL virus were *Ae. (Neo.) circumluteolus* (Natal lowlands), *Ae. (Och.) caballus* s.l. (inland plateau) and *Ae. (Neo.) mcintoshi* (Zimbabwe highlands).

From 1956 to 1980, 139 isolations of WN virus were made from mosquitoes in South Africa. Of these, 136 came from mosquitoes collected on the inland plateau and three from the Natal coastal lowlands. On the plateau, 128 of the isolations were from *Culex univittatus* which occurs throughout this region \$67\$. There was a large epidemic of WN fever in the Karoo and northern Cape in 1974, when the infection rate of the *Cx. univittatus* population reached 39 infected mosquitoes per 1000 at Upington \$74\$. The low incidence of WN virus in the Natal coastal lowlands is apparently due to the poor susceptibility of *Culex neavei* to infection \$40\$. *Culex neavei* replaces *Cx. univittatus* allopatrically in the Natal lowlands, where it is apparently the main vector \$26, 67, 69, 90\$.

Banzi virus has been isolated almost exclusively from *Culex (Eumelanomyia) rubinotus* at Harare in Zimbabwe and at Port Shepstone and Mtubatuba-Empangeni on the Natal coast. Infection rates in this species have often been high \$75\$.

Vector competence

Values for the 50 per cent infection threshold and the transmission rate determined in horizontal biological transmission tests for six species of mosquito with RVF virus are given in Table 2. Data indicating the approximate length of the extrinsic incubation period for each species are also shown. On the basis of these results the order of vectorial importance is *Cx. theileri*, *Ae. circumluteolus*, *Cx. zombaensis*, *Ae. mcintoshi* and *Ae. juppi*.

Table 4 gives the results for preliminary tests on three further mosquito species, all of which had high infection rates after feeds of high titre of virus, and which subsequently transmitted the virus. Comparison of the results for *Ae. unidentatus* (Table 4) with those for *Ae. mcintoshi* (summarized in Table 2) shows that *Ae. unidentatus* is a more efficient vector.

Field studies during RVF epidemics have shown only low infection rates in the mosquito vectors, often no more than one infected mosquito per 1000 $59, 73$. In addition, high vector competence evidently requires high levels of viraemia in the vertebrate host $35, 68, 73$. Despite this, a striking feature of RVF epidemics among sheep on the inland plateau of South Africa is the rapidity with which infection spreads through flocks, causing heavy losses. To account for the high level of infection, biological infection by mosquitoes is almost certainly augmented by mechanical transmission by various biting flies. In mechanical transmission there is no viral replication in the vector, only a passive transfer of virus. Experimental mechanical transmission of RVF virus has been accomplished by various haematophagous Diptera: *Glossina morsitans*, *Stomoxys calcitrans*, *Lutzomyia longipalpis*, *Culicoides variipennis*, and several species of *Aedes* and *Culex* $43, 18$. In interrupted

transmission tests from viraemic to susceptible hamsters by the bite of *Ae. aegypti formosus* $43$, transmission was achieved by groups of mosquitoes for up to 25 minutes after the infective feed but not after 5,5 hours. After shorter intervals of three to five minutes, single mosquitoes were also shown to transmit the virus on three occasions.

The results of earlier vector competence tests with WSL virus are shown in Table 5. They indicate that *Cx. theileri* can be excluded as a vector, while *Ae. circumluteolus, Ae. caballus s.l.* and *Cx. zombaensis* are potential vectors.

Laboratory evaluation of all the common mosquito species occurring in the Highveld as vectors of WN virus showed that *Culex univittatus* is by far the most efficient vector, with a low 50 per cent infection threshold of about 2,3 log $LD_{50}$/ml and a high transmission rate of 97 per cent $28, 30$.

Owing to the high infection rates of BAN virus in natural populations of *Cx. rubinotus* at Port Shepstone, it was possible to conduct a transmission experiment in the field in which individual hamsters were exposed to 16 groups of between four and 19 wild-caught mosquitoes. Banzi virus isolates were made from the mosquito groups that had fed on 11 out of 16 hamsters, which suggested a high degree of vector efficiency $65$.

Ecology of mosquito vectors

*Culex (Cux.) theileri*

*Culex* spp. deposit their egg rafts on the water surface, where, depending on the temperature, they hatch within two or three days. These eggs cannot resist drying, hence the preferred types of larval habitat are permanent or semi-permanent ground pools, although temporary pools are sometimes used. *Culex theileri* is the most abundant and consistently

25×

prevalent mosquito throughout the inland plateau region of South Africa, but it is usually rare on the Natal coast and in the Transvaal Lowveld $36, 41, 73, 89$. This species feeds on goats, cattle, sheep and humans, mainly at ground level during the first three hours after sunset. It also feeds to a limited extent in the tree canopy as it is moderately ornithophilic $1, 27, 36, 41$. The larvae are found in a wide range of ground pools varying from clear vleis with emergent vegetation to pools highly polluted with organic waste from cattle $20, 24$.

*Culex (Cux.) zombaensis*

This species is common in the northern Natal coastal plain $14, 69, 70, 76$, but rarer in the southern part $37$. It is mainly a ground frequenting species which feeds nocturnally on cattle, sheep, goats, antelope and humans $70, 76, 82$. *Culex zombaensis* seems more selective in its choice of larval habitat than *Cx. theileri* and is probably more confined to cleaner water with emergent vegetation, such as river margins and permanent pans.

*Culex (Cux.) univittatus*

This mosquito occurs throughout the Highveld and Karoo regions and is usually one of the most abundant *Culex* spp. taken in catches $36, 41, 89$. It is predominantly ornithophilic but sometimes feeds on humans and other mammals $1, 27, 36. 41$. It feeds nocturnally, particularly in the tree canopy but also at ground level. While this species is only slightly anthropophilic it has been shown to feed on humans indoors to some extent (endophagy) $27$. Epidemics of WN are probably partly a result of increased feeding on humans when particularly high densities of the vector occur, as they did at Upington in 1974 $74$. Precipitin tests on

25×10

blood-meals of *Cx. univittatus* collected on a cattle farm near Johannesburg showed that 37 per cent of them were of mammalian origin, and 55 per cent of these mammalian meals were from cattle.

*Culex univittatus* will utilize temporary, semi-permanent and permanent ground pools as larval habitats, although it prefers sites with clear water and emergent vegetation $20, 24$. Reed beds at the margins of the Orange river were probably utilized at Upington in 1974 and 1976 when densities of *Cx. univittatus* were high there. It appears to be important for *Cx. univittatus* adults and larvae to enter a state of quiescence to overwinter, although the available evidence indicates that adults pass the winter in a nulliparous rather than a parous condition $25, 29$.

*Culex (Eum.) rubinotus*

*Culex rubinotus* can be collected in reasonable numbers only within or near its preferred habitat. This is dense marshland vegetation, always associated with permanent water such as lagoons, rivers or streams, and dams or lakes. Rank reeds, papyrus or grass are always present but trees are either few or absent. Although this type of habitat occurs on the inland plateau, it is particularly common on the Natal coast, and large populations of the mosquito have been sampled at Mtubatuba and Empangeni $75$. The mosquito probably seeks out this dense vegetation because of its sensitivity to wind and low humidity. Large numbers have also been taken in mature sugar cane fields when these adjoined the natural habitat $39$.

*Culex rubinotus* feeds primarily on rodents, to a moderate degree on domestic ungulates and rarely on humans $39$. The feeding rate is high on hamsters in mosquito traps placed on the ground. Female mosquitoes confine their activity almost entirely to ground level and rarely enter traps in the tree canopy $39$.

25×

Larvae of *Cx. rubinotus* have been collected in shallow water in reed beds $39$. Hopkins $20$ recorded the larval habitats as being papyrus swamps and weedy or *Pistia*-covered borrow-pits.

*Aedes (Neo.) circumluteolus*

This is the predominant member of the subgenus *Neomelaniconion* to be found in the tropical coastal lowlands from northern Natal in South Africa northwards into Mozambique. In Tongaland in northern Natal it is often one of the most prevalent of all mosquitoes during the summer $12, 38, 58, 69, 70, 90$. It has been collected in much smaller numbers in other parts of northern Natal at river margins or in marshes after rain, although it can apparently become quite prevalent at Lake St Lucia $13$. This is a floodwater species which oviposits at the edge of rivers and pans within the flood plain. Aedine eggs are resistant to dessication as long as a high level of humidity is maintained within their microhabitat under the soil where, like *Ae. juppi*, they are probably deposited within the top 50 mm $15$. They do not hatch until they are inundated at the next river flooding. Local rainfall apparently has to be heavy and concentrated to cause surface flooding similar to that associated with river flooding $38$.

A study at Ndumu in Natal showed that during the dry winter and spring months (June to October) *Ae. circumluteolus* declined to very low numbers, but it never completely disappeared. Blood-feeding and ovarian development occurred throughout the year $38$. *Aedes circumluteolus* is a ground feeder feeding during the day and also at night from about half an hour after sunset. It feeds avidly on cattle, sheep, antelopes and humans $38, 70, 82$.

25×

*Aedes (Neo.) unidentatus* and *Aedes (Neo.) mcintoshi*

In 1971 McIntosh revised the subgenus *Neomelaniconion* in southern Africa $58$. He named two new species, *Ae. unidentatus* and *Ae. luridus*, making a total of eight species including *Ae. circumluteolus* and *Ae. lineatopennis*. Prior to this revision *Ae. unidentatus* may have been called *Ae. lineatopennis* in Johannesburg and at Lake Chrissie for example $36$, while *Ae. luridus* may have been confused with *Ae. albothorax*. In 1985 Huang described a new species, *Ae. (Neo.) mcintoshi*, and showed that African mosquitoes previously reported as *Ae. lineatopennis* really belonged to this species $21$. *Aedes lineatopennis* itself was shown to be confined to the Philippines. Thus, reports of *Ae. lineatopennis* in the literature after 1971 refer to *Ae. mcintoshi* but before 1971 could have been either *Ae. unidentatus* or *Ae. mcintoshi*.

*Aedes unidentatus* is probably more common in the moister eastern part of the Highveld and the middle veld of the Orange Free State where it is probably the numerically dominant species of the subgenus $58$. *Aedes mcintoshi* is a more widely distributed species adapted to regions with markedly different climates: it occurs in the Karoo, Highveld, Transvaal lowveld, Natal coastal lowlands of South Africa and the Zimbabwe highlands. In the northern part of the Natal lowlands this species appears to be dominated by *Ae. circumluteolus*. Its prevalence relative to *Ae. unidentatus* and *Ae. luridus* in the Highveld and Karoo respectively is not yet properly known because of insufficient sampling. Large numbers of *Ae. mcintoshi* have recently been collected at Villiers in the north-eastern Highveld, where it was about as common as *Ae. unidentatus*. In southern Africa the distribution of *Ae. mcintoshi* indicates that it is probably best adapted to regions of intermediate temperature.

*Aedes unidentatus* feeds both by day and night, with peak biting commencing about half an hour after sunset in Johannesburg. It feeds voraciously on goats, cattle, sheep and humans $1, 36, 58$.
*Aedes mcintoshi* has the same host preferences although its biting cycle still needs to be determined in South Africa.

Larvae of *Ae. unidentatus* have been collected near Johannesburg in temporary rainwater pools in flooded pasture $24$. Both *Ae. unidentatus* and *Ae. mcintoshi* larvae occur in large numbers in flooded pans and, to a lesser extent, in vleis in South Africa $4$. Recent studies on the ecology of *Ae. mcintoshi* near Nairobi in the Kenya highlands have indicated a behaviour pattern similar to that of south African populations. Larvae have been collected in very large numbers in dambos (pans) on bushed grassland $53$. Adult females feed avidly on cattle and humans, with peaks just before sunset and just after sunrise $54, 56$. The dispersal distance from a dambo was found to be 0,07 and 0,15 km for males and females respectively, and the daily survival rate 0,83 and 0,85 $52$.

*Aedes (Och.) juppi* and *Aedes (Och.) caballus*

McIntosh made a taxonomic reassessment of *Aedes (Ochlerotatus) caballus* and found that mosquitoes that had been identified as this species before 1973 could have been either *Ae. caballus s.s.* or a new species *Ae. juppi* or a mixture of both these species $60$. From the limited number of locality records available for these two species $60$ it appears that *Ae. juppi* is adapted to temperate conditions and is endemic to the temperate regions of southern Africa. It probably outnumbers *Ae. caballus* in the Highveld of the Transvaal and northern Orange Free State, and seems to be the only species

in the western and coastal regions of the Cape Province. It is sympatric with *Ae. caballus* over a large area, including the Karoo and southern Orange Free State, but the relative proportions of the two species require further elucidation. *Aedes caballus* is apparently adapted to higher temperatures and a lower rainfall than *Ae. juppi* and is absent from the coastal regions of Natal and Mozambique.

*Aedes juppi* and *Ae. caballus* have very similar ecologies. Both feed avidly in the light and just before and after sunrise on goats, sheep, cattle and humans $33, 41, 73$. Larvae of *Ae. caballus s.l.* have been recorded from small or large temporary pools or pans $79$. More recent studies have shown that the eggs of both species are laid in sedge or grassland within low-lying depressions (pans) and hatch to produce enormous numbers of larvae when these pans fill after heavy rainfall $15, 32$. Hence larvae of the subgenus *Ochlerotatus* are found in similar habitats to those of the subgenus *Neomelaniconion*, and often species belonging to both subgenera are collected together at the same aquatic site on the inland plateau.

Viral maintenance and occurrence of epidemics (Figs. 1 and 2)

Many years of work in southern Africa and in other African countries have failed to provide evidence supporting a continuous cryptic vertebrate-mosquito virus transmission cycle that serves to maintain RVF virus during the inter-epidemic period. Evidence has, however, been obtained in southern Africa and Egypt that transmission by various mosquito species, notably *Culex* spp., occurs during epidemics in sheep and cattle $16, 19, 73, 76$. Virus isolation attempts over 20 years from some 40 000 mosquitoes trapped in RVF areas in Natal during the inter-epidemic period failed to incriminate mosquitoes $2$.

More recently, Linthicum *et al.* $55$ isolated RVF virus from mosquitoes collected in Kenya during an inter-epidemic period. These included eight isolations from adult *Ae. mcintoshi* (reported as *Ae. linestopennis*) that had been collected as larvae or pupae and reared to adulthood in the laboratory. These immatures came from newly flooded dambos in bushed grassland near Nairobi. The only other RVF virus isolations from aedine mosquitoes were one from wild-caught *Ae. cumminsi* adults and another from wild-caught *Ae. mcintoshi* adults. This important finding provides evidence that RVF virus can be maintained throughout inter-epidemic periods in a state of dormancy in the eggs of *Ae. mcintoshi* and can then be transmitted transovarially to the next generation of mosquitoes. Linthicum *et al.* $55$ concluded that *Ae. mcintoshi* is important in the generation of epidemics by the primary seeding of virus in cattle and sheep. Subsequently, other mosquitoes, *Culex* and *Anopheles* spp., colonizing temporary and permanent water in the epidemic areas, are able to propagate the virus in a mosquito-sheep/cattle transmission cycle to maintain an epidemic $55$.

In South Africa, *Cx. theileri* and *Cx. zombaensis* are the important species on the inland plateau and Natal coastal lowlands respectively. There is strong evidence that these species transmit RVF virus biologically between sheep and cattle, and that mechanical transmission by several mosquito species and other biting flies is responsible for the maintenance of the epidemic. Humans are also thought to become infected on occasions through transmission by *Cx. theileri* and *Cx. zombaensis* although some human infections during epidemics are undoubtedly caused by contagion during the slaughtering of infected animals.

In South Africa, transovarial transmission tests and testing of mosquitoes reared from field-collected eggs have been undertaken to try to incriminate various floodwater *Aedes* spp. as reservoir vectors of RVF virus $15, 35, 73$. Such studies have so far been directed mainly at *Aedes juppi* and have indicated that this species has a low vector potential, that it is only a subsidiary epidemic vector, and that it is not involved in vertical transmission of the virus.

The paucity of isolations from floodwater *Aedes* during epidemics is partly because sampling of the mosquito populations for virus assay invariably began late in the course of an epidemic. Despite this, isolations have been made from *Ae. mcintoshi* and *Ae. juppi* on the inland plateau and from *Ae. circumluteolus* on the Natal coastal lowlands. If one or more of these species should be responsible for transovarial transmission, such infected mosquitoes probably occur only in a few endemic foci where RVF virus erupts to trigger a new epidemic in sheep and cattle when climatic conditions are favourable. Figure 1 summarizes the known epidemic cycle and the hypothetical maintenance cycle for RVF virus in South Africa. Theoretically it might also be possible for some infected *Culex* spp. to overwinter and initiate transmission the next spring, especially as small numbers of *Cx. theileri* adults have been collected in the winter in Johannesburg $25, 29$.

Antibody surveys in humans and livestock have shown that WSL virus is more common in the moist tropical part of southern Africa--northern coastal Natal, northern Kwazulu, Mozambique and Namibia--than on the inland plateau, the southern Natal lowlands and the Cape Province of South Africa $46, 47, 64, 77, 85$.

On the temperate South African plateau the *Ae. caballus-juppi* and *Ae. mcintoshi-luridus-unidentatus* group of mosquitoes are probably

vectors. Along the tropical, eastern coastal plain of northern Natal and Mozambique, *Ae. circumluteolus* is the most likely vector. Because the tropical areas of northern Natal and Mozambique receive a higher rainfall, and are therefore moister than the drier temperate areas of South Africa, *Aedes* spp. are probably active there over longer periods. This would account for the higher level of WSL virus activity in the tropical areas \$61\$.

The only evidence for the possible existence of a wild vertebrate host for WSL virus is the single isolation of virus from the gerbil (*Desmodillus auriculatus*) \$6\$. However, the absence of *Culex* vectors suggests that viral maintenance would be through transovarial transmission from one generation of floodwater *Aedes* to the next by vertical transmission, the virus surviving dry periods in the eggs of these mosquitoes. Domestic ungulates are important incidental hosts with which the vector could probably maintain the virus for quite long periods in the absence of a primary vertebrate maintenance host. Livestock, particularly adult sheep and cattle, are often inapparently infected. Humans can be infected either by contagion through handling infected carcases, or by mosquito bite (Fig. 2).

During each summer on the inland plateau of South Africa, WN virus and SIN viruses are maintained in a feral cycle between *Cx. univittatus* and various avian species. The same mosquito, *Cx. univittatus*, also transfers infection to man \$28, 34, 36, 71, 72, 74\$. An outbreak of WN fever in the human population will depend upon an epidemic situation first developing in the avian population. The exposure of sentinel pigeons at Bethulie indicated the occurrence of such epidemics in April 1970 and April, 1971 \$66\$.

Serological surveys for neutralizing antibodies in livestock have shown a prevalence of 16 per cent for WN in sheep and cattle on the Highveld generally, and rates of from 21-47 per cent in older cattle, oxen and horses in Johannesburg $49, 64$. It appears, therefore, that livestock also become infected with WN virus in the field, although calves show only a trace of viraemia after inoculation with virus $3$. Such infections might be expected sometimes to lead to the development of abnormalities in the foetuses of pregnant animals $10$.

*Culex rubinotus* maintains BAN virus among wild rodents. That rodents are indeed the feral vertebrate maintenance hosts is clear from aspects of the studies referred to above. Additionally, there is the isolation of virus from, and the presence of antibodies in, *Mastomys natalensis* $63$, plus the viraemic response of wild rodents to inoculation with the H336 strain of the virus $57$. The occurrence of *Cx. rubinotus* in other parts of Africa, with associated viral isolations, suggests that it is probably the vector of BAN virus over a large part of the continent $17, 78$.

Serological surveys in the Natal coastal lowlands showed that neutralizing antibodies were present in cattle at rates of from two to 16 per cent and in sheep at rates of from zero to seven per cent $47$. This evidence, together with the moderate feeding of *Cx. rubinotus* on domestic ungulates, suggests that pregnant cattle, for example, could become infected, particularly if they were to browse near the marshy reed beds where *Cx. rubinotus* is prevalent. It would seem worthwhile to examine foetal material arising from natural abortions on the Natal coast for the presence of the virus and developmental abnormalities. Whether bovine infections ever attain the level of an epidemic is not known. Banzi virus has been isolated from humans and is known to cause at least mild

illness \$87\$, but the extent to which it causes morbidity in man is uncertain.

Mosquito control

From the point of view of controlling RVF, prior immunization of livestock would be more cost-effective than vector control. However, for other reasons already mentioned, it may on occasion be advisable to institute some mosquito control measures to reduce the degree of exposure of stock to mosquito bites. Since the peak period for biting is from just before sunset until sunrise, and because mosquitoes are always active at or close to their breeding sites, stock should, if possible, be moved away from pans and vleis to higher ground before nightfall. In the case of dairy farms, the milking sheds should be made mosquito proof, and perhaps *Culicoides*-proof, and monthly sprays of their interior walls should be carried out with a residual insecticide other than an organochlorine. Organophosphate insecticides such as malathion, fenthion (Baytex), fenitrothion (Sumithion), or carbamates such as carbaryl (Sevin) and propoxur (Arprocarb), can be used at rates of 1,5-2,0g/$m^2$ \$84\$.

Control of adult mosquitoes outdoors would probably be neither effective nor economic. Outside control could, however, be directed at larvae in the main breeding pans, vleis and dams. Larvicides such as Timephos (Abate) can be incorporated into sand granules or pellets and sprinkled into the breeding place. In the case of pans, a dry pan could be seeded with such pellets just before a flood is expected so that the insecticide is released into the water as the aedine eggs hatch. An alternative larvicide is the bacterium, *Bacillus thuringiensis israeliensis*, whose spores can be incorporated into pellets. This may become practicable if its present high cost is

reduced. The emphasis should be on a control programme with measures that are selective and integrated.

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Table 1 Mosquito-borne viruses causing disease in livestock and/or humans in southern Africa

| | Virus | Mosquito vector | Wild vertebrate host | Hosts showing disease | |
|---|---|---|---|---|---|
| | | | | Humans | Livestock |
| Togaviridae | | | | | |
| Alphavirus | Chikungunya | Ae. (Dic.) furcifer | Primates | + | - |
| | Sindbis | Cx. (Cux.) univittatus | Birds | + | - |
| Flaviviridae | | | | | |
| Flavivirus | Banzi | Cx. (Eum.) rubinotus | Rodents | + | +[a] |
| | Wesselsbron | Ae. (Nec.) circumluteolus | | | |
| | | Ae. (Nec.) mcintoshi | Rodents? | + | + |
| | | Ae. (Och.) caballus/juppi | | | |
| | West Nile | Cx. (Cux.) univittatus | Birds | + | +[a] |
| Bunyaviridae | | | | | |
| Phlebovirus | Rift Valley fever | Cx. (Cux.) theileri | Antelope? | + | + |
| | | Cx. (Cux.) zombaensis | | | |
| | | Ae. (Nec.) circumluteolus | | | |
| | | Ae. (Nec.) mcintoshi | | | |
| | | Ae. (Och.) caballus/juppi | | | |

[a] Disease produced after inoculation with virus in laboratory

Table 2 Southern African mosquito species yielding multiple isolations of Rift Valley fever virus from 1953-81; number of isolations and results of transmission experiments

| Region | Species | No. isolations | 50% infection threshold[a] $Log_{10}LD_{50}/m\ell$ | Transmission rate[b] (days post-infection) | References |
|---|---|---|---|---|---|
| South Africa: | | | | | |
| Natal coastal lowlands | Cx. (Cux.) zombaensis | 7 | c. 7,5 | 3%, 41%, 42%, (14, 22, 29) | 44, 76 |
| | Ae. (Nec.) circumluteolus | 3 | c. 7,5 | 32-38% (16-31) | 42, 76 |
| South Africa: | | | | | |
| Inland plateau | Cx. (Cux.) theileri | 22 | c. 5,7 | 13-35% (15-22) | 16, 31, 59, 68, 73 |
| | Ae. (Och.) juppi | 3 | 8,7 | 6% (17) | 73 |
| | Ae. (Och.) caballus s.l. | 6 | 8,0[c] | 0%[c] (12, 13) | 16, 73 |
| | Ae. (Nec.) mcintoshi[c] | 2 | 8,1 | 17% (10, 17) | 73 |
| Zimbabwe highlands | Ae. (Nec.) mcintoshi[d] | 2 | | | 59 |

a. The titre of virus needed to infect 50% of the mosquitoes

b. The proportion of infected mosquitoes transmitting virus to hamsters

c. Experiments done with Ae. caballus s.s.

d. Reported as Ae. lineatopennis

Table 3 Number of isolations of Wesselsbron virus from mosquitoes collected in southern Africa

| Region | Species | No. isolations | References |
|---|---|---|---|
| South Africa: | | | |
| Natal | Ae. (Nep.) circumluteolus | 13 | 5, 61, 86, 90 |
| coastal | Ae. (Adm.) minutus | 1 | 90 |
| lowlands | Ae. (Adm.) veeniae | 1 | 9 |
| | Mansonia (Mns.) uniformis | 1 | 48, 90 |
| | Cx. (Cux.) neavei[a] | 1 | 90 |
| South Africa: | | | |
| Inland | Ae. (Neo.) mcintoshi[b]/albothorax[c] | 3 | 48 |
| plateau | Ae. (Och.) caballus s.l.[d] | 13 | 48 |
| Zimbabwe highlands | Ae. (Neo.) mcintoshi[b] | 15 | 7, 59, 61 |

a. Reported as Cx. (Cux.) univittatus

b. Reported as Ae. (Neo.) lineatopennis

c. Probably Ae. (Neo.) luridus

d. Could be Ae. caballus s.s. or Ae. juppi

Table 4 Results of vector competence tests with Rift Valley fever virus and three mosquito species; infective feeds on viraemic hamsters but transmission by the in vitro capillary method $35$

| Species | Titre of infective feed in $Log_{10}CPD_{50}$ PFU/mℓ[c] | Mosquito infection | | Transmission | |
|---|---|---|---|---|---|
| | | Days after infective meal | Rate[a] | Days after infective meal | Rate[b] |
| Ae. (Neo.) unidentatus | 6,8 - 7,8 | 11 | 12/14 (86%) | 11 | 7/12 (58%) |
| Ae. (Adm.) dentatus | 6,8 - 7,8 | 11 | 28/31 (90%) | 11 | 9/28 (32%) |
| | | 17 | 6/8 (75%) | 18 | 3/6 (50%) |
| Cx. (Cux.) poicilipes | 7,8 | 15 | 26/29 (90%) | 15 | 4/26 (15%) |
| | | | | 30 | 24/30 (80%) |

a. numerator = no. mosquitoes infected; denominator = no. mosquitoes tested

b. numerator = no. mosquitoes transmitting; denominator = no. infected mosquitoes feeding

c. Cytopathic dose (CPD)

Plaque forming units (PFU)

Table 5 Results of vector competence tests with various mosquito species and Wesselsbron virus

| Species | Viraemic infecting host | Blood-meal titre $\log_{10}LD_{50}/ml$ | Infection rate | Susceptible host/s | Transmission attempts | Reference |
|---|---|---|---|---|---|---|
| Ae. (Nec.) circumluteolus | Artificial | 7,2 | 7/9 | Mice | 16/18[a] | 80 |
| Ae. (Och.) caballus s.l. | Mosqs. collected in epidemic | | NT | Lambs | 1/1[a] | 45 |
| Ae. (Och.) caballus s.l. | Mosqs. collected in epidemic | | NT | Mice | 1/31[a] | 45 |
| Ae. (Och.) caballus s.l. | Lambs | NT | NT | Mice | 2/2[a] | 45 |
| Cx. (Cux.) zombaensis | Hamsters | 8,4 | 13/26 | Hamsters | 1/2[b] | 8 |
| Cx. (Cux.) theileri | Hamsters | 6,7-7,2 | 21/52 | Mice | 0/15 | 8 |

a. Numerator = no. successful transmissions; denominator = no. attempts with groups of mosquitoes.

b. Transmission rates

NT Not tested

Legends Mosquito vectors (m/s 70A)

Fig. 1 *Culex theileri*

Fig. 2 *Aedes (Neo.) mcintoshi*

Fig. 3 *Aedes (Och.) juppi*

Fig. 4 Transmission cycles of Rift Valley fever; maintenance of virus through the dry winter is hypothetically by means of vertical (transovarial) transmission by *Aedes* spp. or by overwintering adult *Culex* mosquitoes

Fig. 5 Transmission cycles of Wesselsbron virus; maintenance of virus through the dry winter is hypothetically by means of vertical (transovarial) transmission by *Aedes* (*Neomelaniconion*) or *Aedes* (*Ochlerotatus*) mosquito species

# POTENTIAL FOR DENGUE IN SOUTH AFRICA: MOSQUITO ECOLOGY WITH PARTICULAR REFERENCE TO *AEDES AEGYPTI*

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ABSTRACT. Observations on prevalence, geographical distribution, utilization of artificial larval habitats and anthropophilism were made on diurnal mosquitoes at selected localities along the coast of Natal and inland in the Transvaal to identify potential vectors of dengue in South Africa. Larval collections made in artificial containers on the ground, the exposure of bamboo pots as ovitraps in trees and collection of mosquitoes biting man showed the following species as the most likely candidates for vectors: *Aedes aegypti, Ae. demeilloni, Ae. simpsoni, Ae. strelitziae, Ae. furcifer, Ae. cordellieri* and *Eretmapodites quinquevittatus*. The bamboo pots showed that *Ae. aegypti* and *Ae. simpsoni* were the most widespread species, occurring at 11 of 12 localities. *Aedes aegypti* was the most prevalent species with mean pot index of 60.3 ± 9.8% (SE) and abundance index of 0.43 ± 0.15 (SE). *Aedes aegypti* was frequently present as larvae in artificial containers at indices of 11–83% (mean 56.8 ± 5.6%, SE) and was the most anthropophilic species with average biting rates of 10–29 per man-hour at 7 localities. Although *Ae. aegypti* was abundant in the pots at Ndumu (northern Natal) and at Skukuza (eastern Transvaal), the local populations were poorly anthropophilic at these localities. At some localities, populations of *Ae. demeilloni, Ae. simpsoni* and *Ae. strelitziae* had average biting rates of 5.4–9.6 per man-hour. *Aedes furcifer* was collected for the first time at Durban, extending its distribution southward to latitude 29°53′S.

## INTRODUCTION

Investigations of dengue vectors in countries outside South Africa have only incriminated aedine mosquitoes, mostly of the subgenus *Stegomyia*. The rural endemic vector in Asia is *Aedes* (*Stg.*) *albopictus* (Skuse), while the urban vector in Asia, West Africa and tropical America is *Ae.* (*Stg.*) *aegypti* (Linn.) (Gubler 1988). In West Africa, other *Stegomyia* species apparently play the role of enzootic sylvan vector, together with 2 species of the subgenus *Diceromyia*, i.e., *Ae. furcifer* (Edwards) and *Ae. taylori* Edwards (Cordellier et al. 1983, Roche et al. 1983). *Aedes aegypti* is widespread in the tropical and subtropical areas in eastern South Africa (Muspratt 1956), while *Ae. furcifer* is limited to the tropical areas of northern Natal and the northeastern Transvaal (Jupp 1980).

Our Arbovirus Unit is engaged in a project designed to investigate tree hole and container breeding *Aedes* species, principally members of the subgenera *Stegomyia* and *Diceromyia*, in eastern South Africa. The objective is to assess these mosquitoes as potential vectors of dengue viruses which could participate in epidemic transmission should dengue be reintroduced into the country. The project has several aspects which have already been defined in a publication on the taxonomic status of *Ae. aegypti* populations (Jupp et al., in ms.). This study is concerned with the ecology of potential vectors. Collecting localities were chosen along the Natal coast and at Skukuza in the eastern Transvaal as representing points where a dengue outbreak might begin. Furthermore, Ndumu (Natal) and the Magaliesberg and Mica (Transvaal) were chosen primarily for investigations of non-anthropophilic populations of *Ae. aegypti*, while Tzaneen (northern Transvaal) was selected for its anthropophilic population; these *Ae. aegypti* collections were relevant to the taxonomic study (Jupp et al., in ms.). Observations were made on prevalence, geographical distribution, utilization of artificial containers as larval habitats and diurnal biting activity on human bait to determine to what extent different species possessed ecological attributes which made them likely dengue vectors.

## MATERIALS AND METHODS

*Ovitraps in trees:* Bamboo pots simulating tree holes were exposed as ovitraps to collect tree hole breeding mosquitoes. Eggs, larvae and pupae were obtained from the various localities mapped in Fig. 1 by exposing bamboo pots for extended periods ranging from 38 to 153 days between November 7, 1989, and May 17, 1990. Eggs were also obtained by exposing black plastic bottles of water containing wooden paddles for oviposition (Jupp and McIntosh 1990) on a farm in the Magaliesberg mountains and at Mica. The ovitraps were suspended in shady trees at heights ranging from 1 to 4 m above ground level. At the end of the exposure period, ovitraps were returned to the laboratory where the eggs were hatched by flooding with tap water and adults reared out. The bamboo pot index for each species is defined as the percentage of

pots found to contain that species. The abundance index is the number of mosquitoes at each locality divided by the product of the number of pots and number of days they were exposed.

*Artificial containers:* Mosquito larvae were sampled from water-retaining artificial containers in motor car scrap yards, municipal rubbish dumps and other peridomestic sites at various localities in Natal and in the Transvaal (Fig. 1) from November 5, 1988, to April 18, 1989. The following containers had culicine larvae and pupae: used tire casings (most commonly), metal drums, floor pans and tire wells in scrapped cars, paint cans, beverage cans and beverage cartons. Samples were taken from tires containing water and leaf litter by dipping a ladle repeatedly into the water until at least 50 larvae and/or pupae had been obtained, or at least 3 times until it was evident that no culicines were present. The other, more accessible containers were scrutinized for immature culicines. Larvae were reared out in the laboratory and identified. Container indices were recorded as the percentage of containers found to contain *Ae. aegypti.*

*Human-baited collections:* These collections were carried out during the daytime because *Ae. aegypti* and other species of *Stegomyia* are diurnal biters. Volunteers used test tubes to collect adult female mosquitoes which probed and engorged on their bare legs and feet. The biting rates thus obtained were based on 2–5 volunteers collecting in the open at suitable sites, usually near artificial containers in the urban localities and the bamboo pot sites at Palm Beach, Armadale, Ndumu and Tzaneen. Collections were made over the months of January to April 1989, November 1989 to February 1990 and January to February 1991, usually during 2 periods of the day, 0530–0930 h and 1500–1900 h. No collections were undertaken during the crepuscular periods or at night. Collecting generally took place on days when the prevailing climatic conditions were warm, humid, partly cloudy and calm; mosquitoes remained active during slightly windy conditions. Mosquitoes were separated according to species, and biting rates were calculated by combining data from several collections per locality and dividing by the total number of man-hours invested (the collection effort). Observations were made on various aspects of feeding behavior.

*Mosquito identification:* The mosquito nomenclature follows that of Knight and Stone (1977) and Ward (1984) except where more recent taxonomic revisions have occurred. These relate to the transfer of certain *Ae.* (*Aedimorphus*) species into the subgenus *Albuginosus* of *Aedes* (Reinert 1986) and the description of *Ae.* (*Diceromyia*) *cordellieri* Huang in the *Ae. furcifer* group (Huang 1986a).

## RESULTS

*Ovitraps in trees:* The results of the survey for tree hole breeding mosquitoes by means of bamboo pots are given in Table 1. Twenty-eight species in 3 genera were sampled at 8 localities in Natal and 4 localities in the Transvaal (Fig. 1). *Toxorhynchites brevipalpus* Theobald and *Ae. aegypti* utilized the pots most frequently at the Natal localities (mean bamboo pot indices > 50%). The most abundant species in Natal were, in order, *Ae. dendrophilus* Edwards, *Ae. capensis* Edwards and *Ae. aegypti*, while the most widespread were, in order, *Tx. brevipalpus* and *Ae. simpsoni* (Theobald) followed by *Ae. aegypti*, *Ae. dendrophilus* and *Ae. capensis*. At the Transvaal localities *Ae. aegypti* had the highest pot index and was the most abundant and widespread species.

Overall, *Ae. aegypti* and *Ae. simpsoni* occurred at 11 of the 12 South African localities. *Aedes aegypti* was found most often in the bamboo pots (mean pot index = 60.3 ± 9.8%, SE) and was the most abundant species (mean abundance index = 0.43 ± 0.15, SE) except for *Ae. dendrophilus* (mean abundance index = 0.47 ± 0.26).

In the subgenus *Diceromyia*, *Ae. cordellieri* occurred in bamboo pots at Ndumu in northern Natal and, to a small extent, at Skukuza in the eastern Transvaal. *Aedes furcifer* utilized the bamboo pots in Durban, Ndumu and Skukuza with pot indices of 20, 71 and 47%, respectively. The abundances of the 2 species were low in all cases.

*Artificial containers:* Container indices were determined for *Ae. aegypti* (Table 2) because this was by far the most prevalent species occurring as larvae in artificial containers on the ground and because of the known importance of the species as a vector of dengue and other viruses outside South Africa. Nine of the 14 localities where containers were sampled had container indices greater than 50%, with the 3 highest occurring at Port Shepstone (83%), Empangeni (78%) and Durban (76%). Other species quite frequently encountered in these containers were *Ae. simpsoni*, *Eretmapodites quinquevittatus* Theobald, *Tx. brevipalpus* and *Culex quinquefasciatus* Say. The first 3 of these species were also found to be common in containers sampled in Eshowe in Natal, although *Ae. aegypti* was uncommon when that locality was sampled in November 1988.

*Water-retaining plants:* Larvae and pupae of *Ae. simpsoni* and *Ae. strelitziae* Muspratt were

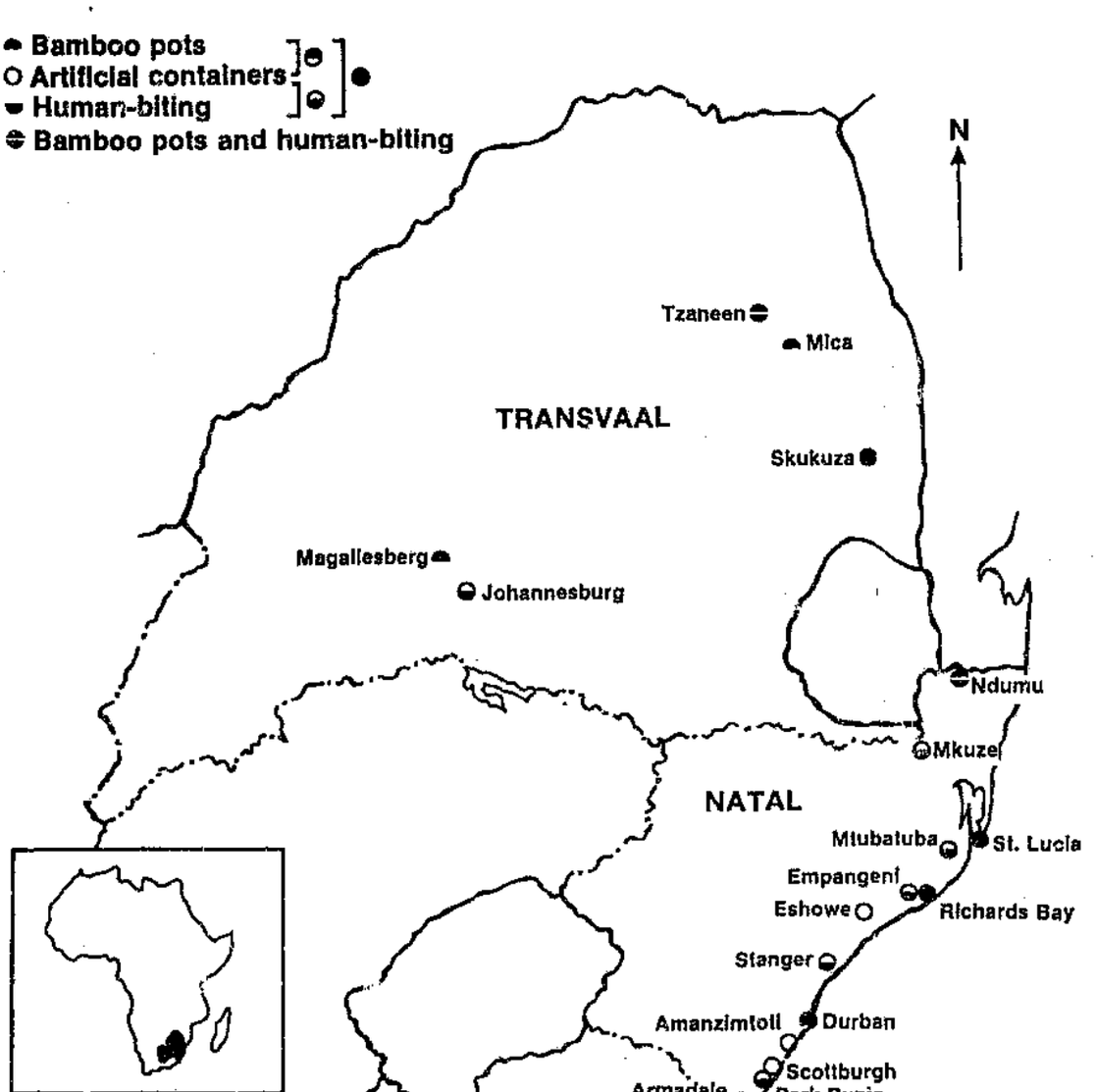



Fig. 1. South African localities where mosquitoes were sampled in bamboo pot ovitraps in trees, in artificial containers on the ground and by diurnal human baited collections.

collected from the leaf axils of *Strelitzia nicolai* Regel and Koern (Strelitziaceae) at Palm Beach and the immatures of *Ae. demeilloni* Edwards, *Ae. simpsoni* and *Er. subsimplicipes* Edwards from *Dracaena hookeriana* Koch (Agavaceae) at Oslo Beach. Large stands of *Strelitzia nicolai* and *Dracaena hookeriana* were also found in undisturbed, indigenous, coastal forest on the farm, Armadale, about 14 km north of Port Shepstone. *Eretmapodites quinquevittatus* larvae were collected from the dried, opened husks of the fruit of the black or spineless monkey orange tree, *Strychnos madagascariensis* Poiret (Loganiaceae), at St. Lucia Estuary.

*Human-baited collections:* Biting rates, as a measure of anthropophily, were significant for *Ae. aegypti* at 7 of the urban localities (10–29 per man-hour: Table 2), although the Johannesburg sample is probably not from a perennial population. Biting rates were generally low at localities in Natal further north than Empangeni, correlating with generally low abundances (Table 1). Two adult collections at a Durban site, spaced one month apart in early 1991, gave

Table 1. Survey with bamboo pots for mosquitoes at various localities; bamboo pot indices (% of positive pots); and, in parentheses, abundance indices (no. mosquitoes/no. pots/no. days exposed).

| | P. Edw. | Palm B. | Oslo B. | Armadale | Durban | R. Bay | St. Lucia | Ndumu | Magal. | Skukuza | Mica | Tzaneen |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| No. of pots | 5 | 10 | 15 | 6 | 15 | 14 | 23 | 44 | 22 | 23 | 26 | 15 |
| No. of days exposed | 57 | 57 | 57 | 57 | 57 | 57 | 56 | 64 | 112 | 153 | 31 | 38 |
| *Ae. (Aëd.) capensis* | 100 (1.16) | 80 (1.68) | 0 | 67 (1.07) | 0 | 0 | 57 (0.25) | 30 (0.09) | 0 | 0 | 0 | 0 |
| *hauorthi* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 9 (0.01) | 0 | 0 |
| *kennethi* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 34 (0.05) | 0 | 0 | 0 | 0 |
| *marshalli* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 36 (0.01) | 57 (0.016) | 0 | 40 (0.08) |
| *Ae. (Dic.) adersi* | 0 | 0 | 0 | 0 | 7 (<0.01) | 64 (0.17) | 0 | 0 | 0 | 0 | 0 | 0 |
| *cordellieri* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 29 (0.02) | 0 | 5 (<0.01) | 0 | 0 |
| *furcifer* | 0 | 0 | 0 | 0 | 20 (0.02) | 0 | 0 | 71 (0.04) | 0 | 47 (0.02) | 0 | 0 |
| *furcifer* group | | | | | | | | 32 (0.05) | | 39 (0.01) | | |
| *Ae. (Fin.) fulgens* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 7 (0.02) | 0 | 17 (<0.01) | 6 | 13 (0.03) |
| *Ae. (Psa.) natalensis* | 0 | 0 | 7 (0.05) | 0 | 0 | 0 | 35 (0.06) | 0 | 0 | 0 | 0 | 0 |
| *Ae. (Stg.) aegypti* | 40 (0.10) | 100 (1.17) | 93 (0.85) | 0 | 53 (0.14) | 50 (0.07) | 9 (<0.01) | 73 (0.35) | 45 (0.01) | 100 (0.55) | 67 | 93 (1.39) |
| *contiguus* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 50 (0.05) | 0 | 0 | 0 |
| *demeilloni* | 20 (0.02) | 30 (0.02) | 7 (<0.01) | 33 (0.04) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *dendrophilus* | 100 (1.53) | 100 (1.30) | 20 (0.05) | 100 (2.74) | 0 | 0 | 4 (<0.01) | 16 (0.02) | 0 | 7 (<0.01) | 0 | 0 |
| *heischi* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 46 (0.10) | 0 | 0 | 0 | 7 (0.01) |
| *ledgeri* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 11 (0.01) | 0 | 87 (0.36) | 44 | 47 (0.73) |
| *metallicus* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 41 (0.08) | 0 | 100 (0.28) | 39 | 47 (0.14) |
| *simpsoni* | 20 (0.02) | 70 (0.46) | 33 (0.15) | 17 (0.01) | 13 (0.01) | 7 (0.02) | 4 (<0.01) | 68 (0.14) | 5 (0.03) | 70 (0.15) | 0 | 33 (0.08) |
| *soleatus* | 0 | 0 | 0 | 0 | 0 | 0 | 87 (0.74) | 11 (0.01) | 0 | 0 | 0 | 0 |
| *strelitziae* | 0 | 30 (0.03) | 13 (0.01) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *unilineatus* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 5 (<0.01) | 0 | 13 (<0.01) | 6 | 0 |
| *Cx. (Cux.) pipiens* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 14 | 0 | 0 | 0 |
| *zombaensis* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 (<0.01) | 5 | 0 | 0 | 0 |
| *Cx. (Cui.) cinerellus* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 20 (0.01) | 0 | 0 | 0 | 0 |
| *cinereus* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 23 (0.05) | 0 | 0 | 0 | 0 |
| *nebulosus* | 0 | 0 | 13 (0.02) | 0 | 7 (0.03) | 0 | 9 (0.03) | 20 (0.04) | 0 | 4 (<0.01) | 0 | 7 |
| *Cx. (Eum.) horridus* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 16 (0.01) | 0 | 48 (0.21) | 0 | 7 |
| *Cx. (Lut.) tigripes* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 5 | 0 | 0 | 0 |
| *Tx. (Tox.) brevipalpus* | 100 | 88 | 27 | 60 | 40 | 43 | 61 | 66 | 0 | 0 | 0 | 0 |

Table 2. *Aedes aegypti* collections at various localities: artificial container indices for larvae and average biting rates in human-baited collections.

| Locality* | Larval collections | | Adult collections | |
|---|---|---|---|---|
| | No. containers examined | Container index (%) | Man-hours | Biting rate (per man-hour) |
| *Natal* | | | | |
| Port Edward | 17 | 47 | — | — |
| Oslo Beach | — | — | 2.0 | 0 |
| Palm Beach | NF** | — | 31.2 | 11.2 |
| Port Shepstone | 18 | 83 | — | — |
| Park Rynie | 37 | 54 | 4.0 | 18.0 |
| Scottburgh | 19 | 11 | — | — |
| Durban | 45 | 76 | 9.0 | 29.0 |
| Stanger | 13 | 54 | 5.0 | 12.4 |
| Empangeni | 18 | 78 | 4.5 | 18.0 |
| Richards Bay | 53 | 62 | 23.5 | 1.5 |
| Mtubatuba | 21 | 33 | 5.5 | 2.9 |
| St. Lucia | 9 | 56 | 8.0 | 0.8 |
| Mkuze | 10 | 70 | 5.5 | 3.5 |
| Ndumu | NF | — | 5.0 | 0.2 |
| *Transvaal* | | | | |
| Johannesburg | 33 | 45 | 2.0 | 13.0 |
| Skukuza | 10 | 70 | 1.8 | 0.5 |
| Tzaneen | — | — | 1.0 | 10.0 |

* All localities are urban except for Ndumu, which is rural; all sites in the urban localities are peridomestic except at Johannesburg, where it is domestic.

** NF = no artificial containers found.

biting rates of 15 and 89 per man-hour, respectively. Both collections had taken place during the midday period, defined for our purposes as 1000 -1500 h. Rainfall totals over 30 days prior to each collection were 43 mm for the first and 263 mm for the second. The Ndumu and Skukuza populations, known and assumed to be non-anthropophilic, respectively, had biting rates lower than 1 per man-hour even though they were prevalent in the bamboo pots (Table 1).

Male mosquitoes subsequently captured and identified as *Ae. aegypti* were often observed to swarm in small numbers (rarely greater than 10) about the bait, a man's exposed leg and foot. Within minutes of swarm-formation, the conspecific females arrived at the bait. They were immediately approached by the males and copulation ensued in the air. Mating mosquitoes either remained *in copula* in flight or landed on the ground or on the bait to mate. The females then approached the bait to feed. Female *Ae. aegypti* were often slow to alight before feeding, particularly if a slight breeze was blowing. Males also alighted on the bait but apparently only to rest, because as soon as females approached, the males would fly up to meet them. Occasionally, females arrived and fed on the bait without the presence of males in the vicinity. Similar behavior patterns were observed for *Ae. demeilloni* and *Ae. strelitziae*, whereas males of *Ae. simpsoni* and *Er. quinquevittatus* commonly occurred at and rested on the bait. At Palm Beach, where no obvious concentration of larval habitats other than water-retaining plants occurred, the diurnal, anthropophilic mosquitoes were only collected in small numbers at any one site. It was necessary to move regularly over small distances to find fairly large numbers of these species biting.

Four *Stegomyia* species other than *Ae. aegypti* were taken in the human-baited collections. Their average biting rates are shown in Table 3 together with the rate for *Er. quinquevittatus*. *Aedes demeilloni* was found to bite readily during the midday period. The biting rates measured in 1991 were 13 per man-hour at Armadale (collection effort = 4.2 man-hours between 1000 and 1215 h) and 38.8 per man-hour at Palm Beach (collection effort = 1.7 man-hours between 1155 and 1250 h). *Aedes simpsoni* was only encountered at high biting rates in Johannesburg in the late afternoon. *Aedes strelitziae* was the most common anthropophilic species at Armadale, with biting rates ranging from 8.7 to 16.3 per man-hour, the higher obtained during 1000–1215 h (collection effort = 4.2 man-hours). *Eretmapodites quinquevittatus* was not commonly collected as adults except for a site in Richards Bay where the biting rate in December 1989 was

Table 3. Daytime human-b ited collections at various localities; average biting rates of the most prevalent species other than *Aedes aegypti.*

| Species | Biting rate (per man-hour) | Locality |
|---|---|---|
| *Ae.* (*Stg.*) *demeilloni* | 5.4 | Palm Beach |
| | 5.5 | Armadale* |
| *dendrophilus* | 0.1 | St. Lucia Estuary |
| *simpsoni* | 1.2 | Palm Beach |
| | 2.0 | Oslo Beach |
| | 0.5 | Park Rynie |
| | 0.2 | Richards Bay |
| | 0.3 | St. Lucia Estuary |
| | 7.5 | Johannesburg |
| | 0.5 | Skukuza |
| *strelitziae* | 2.5 | Palm Beach |
| | 9.6 | Armadale* |
| | 0.4 | Richards Bay** |
| *Er. quinquevittatus* | 0.7 | Palm Beach |
| | 0.6 | Stanger |
| | 2.3 | Richards Bay |
| | 0.2 | Mtubatuba |
| | 0.9 | St. Lucia Estuary |

* Collection effort = 15 man-hours.
** Collection effort = 14 man-hours.

10 per man-hour (collection effort = 2 man-hours in the late afternoon).

No evidence of the exotic species, *Ae. albopictus,* was found in the course of this survey, despite the recent discovery of immatures in tire casings imported from Southeast Asia and landed in Cape Town (Hunt et al. 1990).

## DISCUSSION

Our study has concentrated on particular areas of South Africa. First, those where dengue is likely to be successfully introduced into the country via the shipping and tourist industries and, second, where the most probable vectors, the *Stegomyia* and *Diceromyia* species, are to be found. The characteristics of potential vectors are their appropriate distribution, abundance and anthropophilic behavior. If a species is a known vector of dengue virus outside South Africa, this is also an important consideration. *Aedes aegypti* and *Ae. furcifer* are dengue vectors elsewhere (Gubler 1988, Roche et al. 1983). *Aedes cordellieri* must also be considered, even though its potential for dengue transmission is unknown. A dengue 2 epizootic in the Ivory Coast and Upper Volta was associated with *Ae. furcifer* and *Ae. taylori* (Cordellier et al. 1983). More recently Huang (1986a) has described *Ae. cordellieri,* a third member of the *Ae. furcifer* group from the same region, and it is possible that this species may have played a role in the epizootic. *Aedes demeilloni, Ae. simpsoni, Ae. strelitziae* and *Er. quinquevittatus* are potential vectors of dengue because of their domestic or peridomestic occurrence. Each species will be discussed in the above order.

*Aedes aegypti:* The adult mosquito can survive under widely variable climatic conditions, and anthropophilic populations occur in the southeastern Cape Province at Grahamstown (Edwards 1941; Jupp, unpublished data) and East London (Mattingly 1953; Kemp, unpublished data), both above 33°S latitude. Furthermore, the species is represented by established populations at a variety of altitudes ranging from near sea level to 1,800 m (Johannesburg) in South Africa, mainly associated with wooded savannah (bushveld) and forest (coastal scrub, riverine and montane) or with the urban environment (Muspratt 1956). This species has become adapted to a wide range of larval habitats ranging from natural tree holes and leaf axils to artificial containers in the domestic and peridomestic environments (Hopkins 1952, Horsfall 1955).

The southern Natal coast has been urbanized extensively to cater for the tourist industry, and the endemic populations of *Ae. aegypti* appear to have adapted to utilize the peridomestic environment. These local populations were shown to be highly anthropophilic (Table 2) and could constitute a serious health hazard should they be found to be efficient vectors of dengue. In northern Natal the urban areas are fewer and less developed except for Richards Bay, an international harbor. High densities of anthropophilic *Ae. aegypti* were not as evident in our collections at these localities, although container indices (Table 2) were significant at the collection sites which consisted mainly of tire dumps. The collections at Ndumu, a relatively undeveloped game reserve on the Mozambique border, are considered to represent a non-anthropophilic population of *Ae. aegypti.* Artificial containers were not present at this locality. An extensive series of human-baited collections in the reserve (McIntosh et al. 1972) failed to demonstrate *Ae. aegypti* in gallery forest dominated by *Ficus sycamorus* Linn. (Moraceae). This was confirmed by the present adult collections at the same site, even though ovitraps yielded abundant eggs of *Ae. aegypti* (Table 1).

The Transvaal collections include anthropophilic populations of *Ae. aegypti* from Johannesburg in the south and Tzaneen in the north. The Johannesburg population, however, is probably not an established one, i.e., does not overwinter there. At least some of the tires which formed the larval habitats were imported via Durban, and it is probable that *Ae. aegypti* eggs were introduced with the tires. The relevant site was

on the grounds of a small business concern which reprocessed the rubber from tires into commercial goods, e.g., sandals. This illustrates the unusual mode of range extension in the form of eggs being dispersed by the used tire industry, not only intercontinentally as in the case of *Ae. albopictus*, but from the subtropical region where the species is endemic to the temperate region which can support the species during the summer in South Africa.

Egg collections were made at a farm in the Magaliesberg mountains, Skukuza in the Kruger National Park and a game farm near Mica to sample what were considered to be non-anthropophilic populations due to their feral nature. Limited human-baited collections at the Magaliesberg locality proved negative for *Ae. aegypti* and more extensive collections at Skukuza yielded few adults, in spite of the presence of the species in ovitraps at both localities. Skukuza, paradoxically, is an urban locality in a game reserve and consists of a large staff village associated with an industrial site (workshops and light industry) and tourist camp. The Mica locality in the wooded savanna region of the northeastern Transvaal yielded collections of *Ae. aegypti* in ovitraps. Human baited collections were not attempted, as an earlier study (Jupp and McIntosh 1990) described the non-anthropophilic nature of the population.

The strong diurnal pattern in the biting activity of *Ae. aegypti* is well known. However, the possibility of biting peaks just after sunrise and just before sunset was indicated by the work of Reed and colleagues in 1901 (see Horsfall 1955) and that of Trpis et al. (1973). This was the basis for the timing of our human-baited collections. There is also strong evidence that major biting peaks exist in the hour before midday (Teesdale 1959) and from 1200 to 1400 h and during the crepuscular periods (Lumsden 1957). Biting activity during the daytime hours outside of our standard periods was demonstrated in the present study: the Durban and Palm Beach populations of *Ae. aegypti* were collected from 1000 to 1500 h at very high biting rates.

Twenty-four-hour human-baited collections yielded biting rates for *Ae. aegypti* of up to 13.4 per man-hour during the 1952–53 epidemic of chikungunya fever in Tanzania (Lumsden 1955). Trpis et al. (1973) made extensive recordings of landing rates on man for the species in an automobile dump in Dar-es-Salaam, Tanzania. The landing rate is an approximation of biting rate, and if one extracts their data for periods in the day corresponding to the times when our collections were made, a rate of 9.9 per man-hour is obtained. The biting rates for the anthropophilic populations of *Ae. aegypti* in South Africa are thus comparable to those reported from East Africa. The occurrence of a highly anthropophilic, peridomestic population in the metropolis of Durban is potentially very important in terms of epidemiology.

*Aedes furcifer:* Our study was not designed to sample adults of this species, which is primarily nocturnal with a crepuscular activity peak just after sunset (Port and Wilkes 1979). The *Ae. furcifer* group was previously thought to be confined to the tropical regions with the southernmost distribution being in the region of Lake St. Lucia (28°S latitude) on the 18°C midwinter isotherm (Jupp and McIntosh 1988). *Aedes furcifer sensu strictu* was found in the ovitraps placed in a horticultural nursery in the suburb of Congella in Durban at 29°53′S latitude (Table 1). However, the abundance of the mosquito in the bamboo pots was low and the epidemiological threat may consequently be slight. Sizable populations only occur after heavy summer rainfall in the often drier wooded savanna of northern Natal and the northeastern Transvaal (Jupp and McIntosh 1988). Because it has been established as a vector of dengue in West Africa (Cordellier et al. 1983), it must be regarded as a potential vector in the tropical regions of South Africa.

*Aedes cordellieri:* As for the previous species, experimental design precluded the collection of adults of the nocturnally active *Ae. cordellieri*. It was present during the rural epidemic at Mica (McIntosh et al. 1977), being identified then as *Ae. taylori*. It constituted about 1% of the *Ae. furcifer/cordellieri* total based on the proportion of males collected (Jupp and McIntosh 1990). This species was represented in the ovitraps in the tropical region in association with *Ae. furcifer* (Table 1). The abundance in the bamboo pots appeared to be too low for the species to be considered epidemiologically important, even though it must be considered a potential vector of dengue due to its taxonomic and behavioral similarity to *Ae. furcifer*.

*Aedes demeilloni:* This species is limited to the eastern coastal plain largely due to special habitat requirements. The leaf axils and apical crown of the plant, *Dracaena hookeriana*, form the natural breeding places (Muspratt 1956). Even though *Ae. demeilloni* was found in bamboo pots and sometimes in artificial containers on the south coast of Natal, these populations appear to be dependent on the presence of large stands of *Dracaena* spp. *Aedes demeilloni* is a highly anthropophilic species, as the 1991 collection at Palm Beach shows. Muspratt (1956) reports biting rates of 30–50 per man-hour further south at Port St. Johns on the Transkei coast. It must be considered a potential vector,

as its restricted distribution coincides with the densely populated Natal coast.

*Aedes simpsoni:* Its similarity to *Ae. aegypti* in distribution and ecology strongly suggests that this species is a potential vector. *Aedes simpsoni* is common throughout the tropical and subtropical regions of South Africa (Table 1) and is highly prevalent throughout its range, utilizing natural and artificial larval niches in a variety of environments including the feral (tree holes), agricultural (commercial banana plantations) and domestic (Muspratt 1956, Huang 1986b). It is diurnally active (Muspratt 1956) and bites man readily (Table 3). North and East African records of its biting rate in human-baited collections and its vector competence are open to question after the taxonomic revision of the *Ae. simpsoni* group by Huang (1986a, 1986b). *Aedes simpsoni* was described as a major vector of yellow fever in Nigeria (Lee and Moore 1972), in Uganda (Gillett 1969) and in East Africa (Hamon et al. 1971), but Huang (1986b) suggests that this work relates to *Ae. bromeliae*; apparently *Ae. simpsoni* is confined to southern Africa.

*Aedes strelitziae:* The preferred oviposition site of this species is in the leaf axils of *Strelitzia nicolai*, a common plant on the Natal coast and in montane and riverine forests further inland (Muspratt 1956). This plant is much more prevalent than *Dracaena hookeriana*. Only in a few localities and at low abundance did *Ae. strelitziae* oviposit in bamboo pots (Table 1). Artificial containers were also used to some extent but only when associated with large stands of *Strelitzia nicolai*. This dependence on a particular plant introduces restrictions on geographic distribution, but *Ae. strelitziae* still exists in close proximity to large human populations on the Natal seaboard and is highly anthropophilic (Table 3).

*Eretmapodites quinquevittatus:* This species appeared to be the only member of the genus to occur in abundance in the study area. The oviposition requirements almost always involved small collections of rainwater in discarded tins and cardboard cartons (milk and local beer). Muspratt (1955) confirms these breeding requirements and indicates that natural collections of rainwater in fallen leaves, snail shells and leaf axils are also utilized. The facility with which the species utilizes such small receptacles is apparently augmented by its larval behavior, including the ability for facultative cannibalism and larval activity out of the water (Hopkins 1952), both of which were observed in our laboratory.

Some inconsistencies with respect to larval requirements and behavior exist in the literature. Lounibos (1980) found that *Er. subsimplicipes* is common in and *Er. quinquevittatus* absent from the fruit husks of the Loganiaceae in Kenya (Lounibos 1978, 1980). We found just the opposite with apparently monospecific collections of *Er. quinquevittatus* occurring in fruit husks of *Strychnos madagascariensis* at St. Lucia Estuary. With respect to larval predation, Hartberg and Gerberg (1971) report no evidence of cannibalism for the species in the laboratory, although Haddow (1946) describes larvae from Uganda as predatory. Lounibos (1980) suggests that, unlike *Er. silvestris* Ingram and de Meillon and *Er. subsimplicipes*, it is not able to develop beyond the third instar on a predatory diet alone.

In Natal, *Er. quinquevittatus* has become adapted to both natural and artificial small receptacles for its larval habitat. Furthermore, it is closely associated with man in the domestic environment, has a diurnal biting-activity pattern and is significantly anthropophilic. Consequently, it must be regarded as a potential vector of dengue.

It may be concluded that of the 7 species identified as potential vectors of dengue in South Africa, five are potentially epidemic vectors. The 5 species fall into 2 groups according to our ecological criteria for selection. The most important group comprises *Ae. aegypti*, *Ae. simpsoni* and *Er. quinquevittatus* because of the prevalence of these species and their close association with man in the urban environment. The second group consists of *Ae. demeilloni* and *Ae. strelitziae* which are regarded as less important because of their dependence on specific plants for their larval habitats, which limits their distribution. The 2 remaining species, *Ae. furcifer* and *Ae. cordellieri*, are essentially rural species limited to the tropical region. Therefore, although they are both probably competent vectors, they are unlikely to become involved in epidemic transmission.

## ACKNOWLEDGMENTS

One of us (P.G.J.) acknowledges grants toward this project from the Poliomyelitis Research Foundation and the South African Medical Research Council. We are grateful to the Directors of the National Parks Board, the Kwa Zulu Bureau of Natural Resources and the Natal Parks Board for permission to work in areas under their jurisdiction. We thank staff of the Arboviru. Unit for technical assistance. Our gratitude for hospitality and field collaboration is extended to: J. Henderson of "Armadale," Port Shepstone; P. Volschenk and T. B. Wessels of the Department of Community Services,

Richards Bay; J. Grunenberg of Durban; J. Hiemstra of "Hope," Mica; O. Human of "Kinderstrand," Glenmore Beach; L. E. O. Braak of the Kruger National Park; B. M. McIntosh of Oslo Beach; D. Eckard and G. Kloke of the National Institute for Tropical Diseases (NITD), Eshowe; D. Theron and G. van Eeden, NITD, Tzaneen; G. Wiltshire of the Ndumu Game Reserve; and B. Sharp and D. le Sueur of the Research Institute for Diseases in a Tropical Environment, Congella.

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# THE POTENTIAL FOR DENGUE IN SOUTH AFRICA: MORPHOLOGY AND THE TAXONOMIC STATUS OF *AEDES AEGYPTI* POPULATIONS

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**ABSTRACT.** Some 1,881 *Aedes aegypti* (L.) progeny were reared as sibling samples taken from 196 families representing populations from 18 localities in South Africa, including anthropophilic and non-anthropophilic populations. The number of white scales on tergite I ($T_1$) and in the basal band on tergite II ($T_2$) were counted. Study of family samples showed that 60.2% of families were heterogeneous, containing both the type and *formosus* forms. Hence the division into nominate (type) and *formosus* subspecies is considered invalid. Multivariate statistical analysis of variance in the population samples in respect of $T_1$ and $T_2$ together showed that each population was significantly different from all the others. However, statistical analysis of $T_1$ and $T_2$ alone showed that although some populations differed significantly, there was no consistent difference between anthropophilic and non-anthropophilic populations. It is concluded that in South Africa *Ae. aegypti* is a single polymorphic species displaying plasticity in its man-biting behavior.

## INTRODUCTION

Dengue fever occurred in epidemic form in South Africa in Durban in 1926–1927 (Edington 1927). Since then it has not been reported until 1985 when dengue 1 virus was isolated from one person and dengue antibodies detected in two other people, all of whom were Durban residents recently returned from trips to India (Blackburn and Rawat 1987, Blackburn et al. 1987). This development and the recent outbreaks of dengue in nearby countries, including Moçambique (Anonymous 1985), the Seychelles (Metselaar et al. 1980), and Kenya (Johnson et al. 1982) has caused concern that the virus might be reintroduced into South Africa. Because of this, it was decided that a study should be undertaken of *Aedes aegypti* (Linnaeus), and other mosquitoes, particularly *Aedes* (*Stegomyia*) species, occurring mainly along the eastern coast (Natal) to evaluate their candidature as potential vectors which could participate in epidemic transmission. The overall study has several aspects. The first is a morphological study of *Ae. aegypti* populations to elucidate their taxonomic status, which is reported in this paper. Other aspects to be reported will deal with isozymes and the taxonomy of *Ae. aegypti* the ecology of *Ae. aegypti* and other mosquitoes, and the results of experiments undertaken to assess the vector competence of *Ae. aegypti* and selected mosquitoes with dengue viruses.

Populations of *Ae. aegypti* may vary in their morphology, ecology, physiology and genetics (e.g., Machado-Allison and Craig 1972, McClelland 1974, Trpis and Hausermann 1975, Tabachnick et al. 1979). Domestic and sylvan forms of the mosquito have been recorded in coastal Kenya (Van Someren et al. 1955, Trpis and Hausermann 1975) and apparently in inland Uganda (Haddow 1945). The usually paler domestic form breeds in domestic containers and exists in close association with man, while the usually darker sylvan form breeds in tree holes in rural areas away from houses. In 1957, Mattingly divided the species into two subspecies *viz. Ae. aegypti aegypti*, the type form with pale scaling on the first abdominal tergite and/or a distinctly paler or browner body than the African subspecies *Ae. aegypti formosus* (Walker). *Aedes aegypti formosus*, which never has any pale scales on the first tergite, has a markedly blackish appearance and is confined to Africa

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south of the Sahara. *Aedes aegypti* var. *queenslandensis* (Theobald) has increased white scaling on the abdominal tergites beyond the first tergite and/or has a lighter mesonotal color. Van de Hey et al. (1978) and Tabachnick et al. (1979), respectively, reported extensive morphological and genetic studies and agreed with Mattingly (1957) that *Ae. aegypti* was a polytypic species. In contrast, McClelland (1974), who studied the morphology of populations worldwide, concluded that there were probably two species or incipient species, *viz. Ae. aegypti* and *Ae. formosus*. In these studies these workers did not analyze morphological variation within families to look for intraspecific variation, i.e., polymorphism.

In 1960, McClelland obtained both the type and *formosus* forms in the progeny from matings between *formosus* phenotypes, although he appears to have pooled several families of progeny together upon which to make his morphological analysis. Similar heterogeneous progeny resulted from matings between phenotypes of the type form. Hartberg (1969) appears to be the only worker who has reared separate families from field-collected females, but his important results were only published in a mimeographed document by the World Health Organization. He also found variation in abdominal tergal scale pattern within single families from eggs laid by single *formosus* or type form females.

In South Africa, little has been reported on *Ae. aegypti*. Earlier work by Muspratt (1956) indicated the presence of populations in South Africa with differing ecologies. More recent work by our unit at two rural localities, Ndumu in northern Natal-Kwa Zulu (McIntosh et al. 1972, Kemp and Jupp 1991) and Mica in the northeastern Transvaal (Jupp and McIntosh 1990) has shown the presence there of non-anthropophilic (non-man-biting) populations. At both these localities, mosquito collecting over several years has shown that although *Ae. aegypti* is abundant in these areas as judged by its prevalence in tree holes and bamboo pots, the mosquito is rarely attracted to man and taken off human bait. We have also just completed field studies at Skukuza and in the Magaliesburg Mountains, both inland in Transvaal (Kemp and Jupp 1991), where similar but more limited observations indicated that the same situation probably prevails at these further two rural localities. We have therefore designated these sylvan populations "probably non-anthropophilic" (Fig. 1). In this same project (Kemp and Jupp 1991), collections off human bait have, however, shown that anthropophilic populations are present at domestic or peridomestic sites along the whole Natal coast line and at Grahamstown near the coast in the eastern Cape Province (Fig. 1).

We report here a quantitative examination of the scaling of the first two segments of the abdominal tergite in families reared from anthropophilic and non-anthropophilic population samples of *Ae. aegypti* collected at various localities in South Africa. We wished to determine whether distinct type (nominate) and *formosus* forms were present in South Africa, and if they were present, whether they bred true and could be related to geographically and ecologically different populations of *Ae. aegypti*.

## MATERIALS AND METHODS

Populations of *Ae. aegypti* were sampled at 18 localities: one in the Cape Province, 12 in Natal Province and five in the Transvaal Province (Table 1). The localities are shown on the map (Fig. 1), which also indicates where anthropophilic and non-anthropophilic populations occurred. Mosquitoes were collected either as eggs deposited in bamboo pots exposed in woodland, as larvae removed from artificial containers or as adults taken in landing/biting catches. Eggs and larvae were reared into adults, and the female mosquitoes thus obtained or females collected directly in the field were fed on hamsters in the laboratory. The gravid females were placed singly in tubes containing moist cotton wool and filter paper for oviposition. Subsequently, the different egg batches were reared separately and a group of 10 or more adult female siblings representing each family was pinned. These specimens were examined under a binocular dissecting microscope at 60–80 times magni-

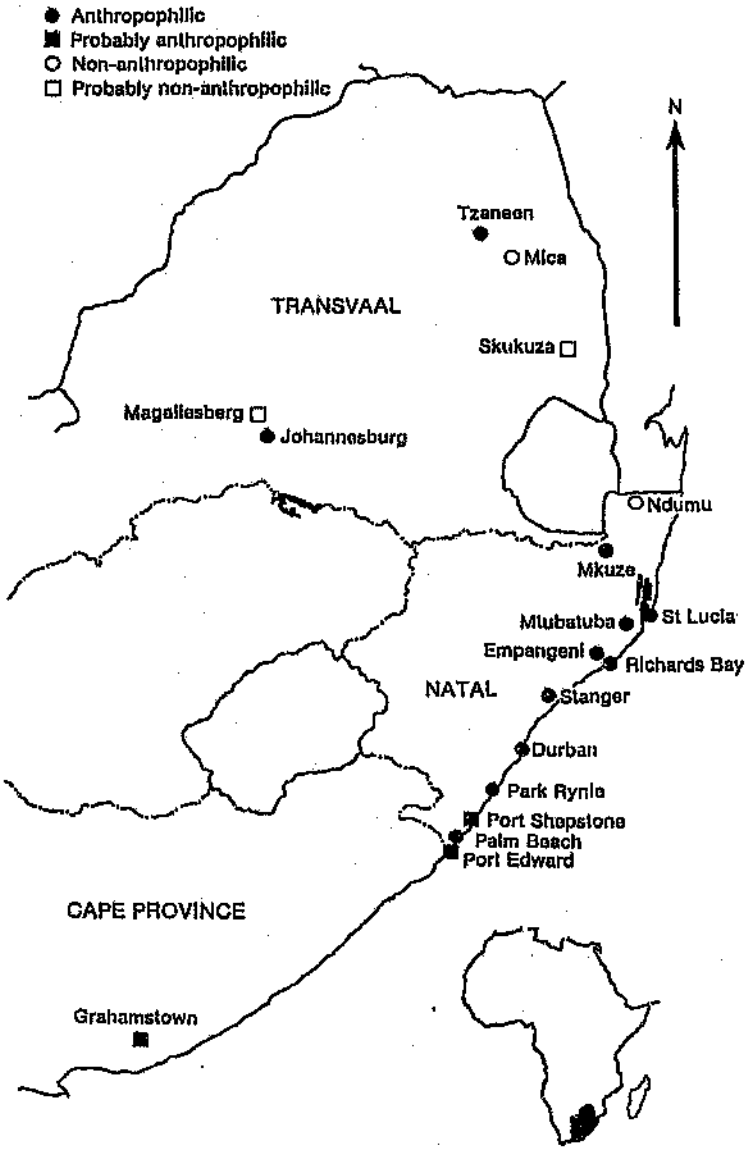



Fig. 1. Localities in South Africa where *Ae. aegypti* populations were sampled.

fication, using a blue filter on incident light. The number of white scales on the first abdominal tergite ($T_1$) and the number in the basal band on the second tergite ($T_2$) were counted. The second tergite was examined in addition to the first because, like McClelland (1960), we had noticed variation; the degree of white scaling at its base varied from complete absence to a well marked band.

The 95% confidence limits were calculated for the mean number of white scales on $T_1$ and $T_2$, respectively, for each of the 18 populations. The data were subjected to further statistical analysis using the general linear models procedure of a SAS statistical package implemented on an IBM mainframe computer. The means of both tergal characters ($T_1$ and $T_2$) together were compared on the 18 population samples by multivariate analysis of variance using the following four statis-

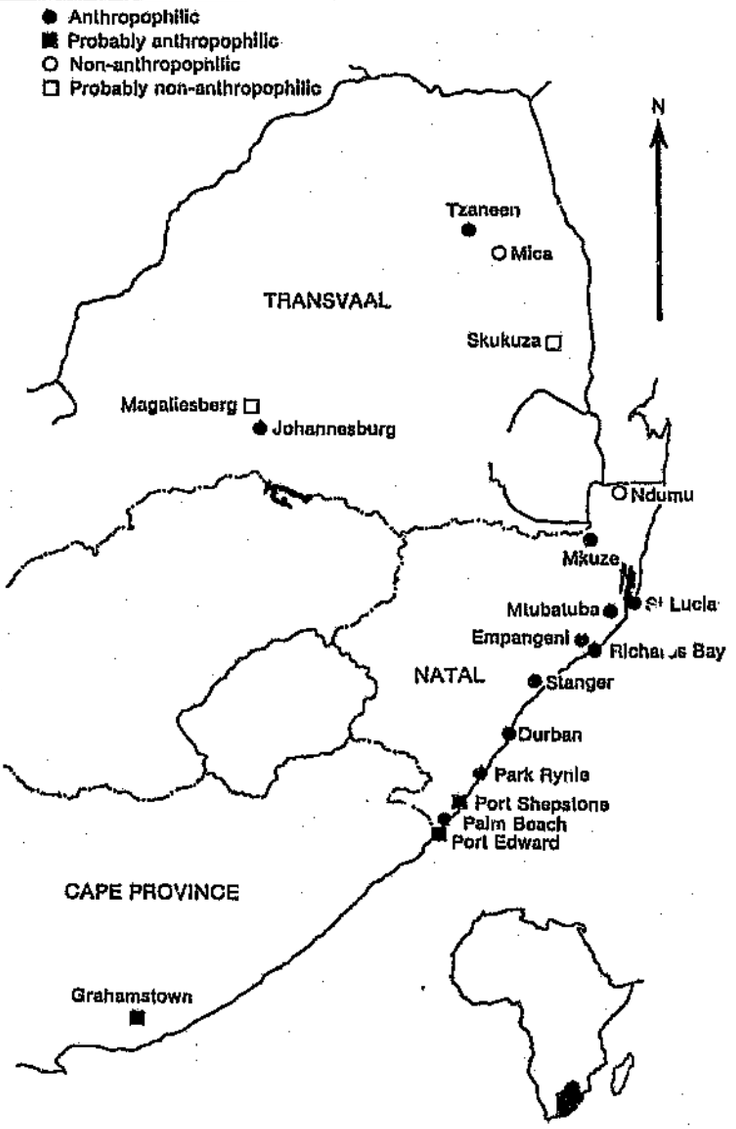



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**Table 1.** Localities in South Africa where *Ae. aegypti* populations were sampled.

| Locality of origin | Stage collected |
|---|---|
| CAPE | |
| Grahamstown | Larvae |
| NATAL | |
| Port Edward | Larvae |
| Palm Beach | Engorged females |
| Port Shepstone | Larvae |
| Park Rynie | Engorged females |
| Durban | Engorged females |
| Stanger | Engorged females |
| Empangeni | Engorged females |
| Richards Bay | Engorged females |
| Mtubatuba | Engorged females |
| St Lucia Estuary | Larvae |
| Mkuze | Larvae |
| Ndumu | Eggs (bamboo pots) |
| TRANSVAAL | |
| Johannesburg | Engorged females |
| Magaliesberg | Eggs (pots) |
| Skukuza | Larvae |
| Mica | Eggs and larvae (pots) |
| Tzaneen | Engorged females |

tics: Wilks' Lambda (Wilks 1932), Pillai's Trace (Pillai 1954), Hotelling-Lawley Trace (Hotelling 1951) and Roy's Greatest Root (Roy 1957). Furthermore, the population means for $T_1$ and $T_2$, respectively, were grouped using the Waller-Duncan K-Ratio t-test (Waller and Duncan 1969) to find which means differed significantly. Conclusions could then be drawn as to the morphological similarity and variability between the 18 populations.

If a mosquito specimen possessed an entirely black $T_1$, it was classified as *formosus* form, while specimens with one or more white scales on this tergite were classified as the nominate or type form. The nature of $T_2$ was not taken into account when assigning a form classification to a specimen. In this manner the sibling groups representing the different families were classified as "homogeneous type form," or "homogeneous *formosus* form" or "heterogeneous with both forms." In a homogeneous family every specimen in the sample belonged to the same form, whereas a heterogeneous family consisted of a mixture of both forms in which one or more specimens did not match the remainder.

## RESULTS

Figure 2 gives the results for three of the families examined. It shows $T_1$ and $T_2$ of three field-collected adult females (mothers) taken biting at Park Rynie in Natal, together with the tergites of 10 of the siblings in their respective $F_1$ families. It can be seen that although the mothers were *formosus* (1 M and 2 M) and type form (8 M) phenotypes, both forms appeared in their progeny with a wide range in the degree of white scaling on both tergites. Some of the specimens from some of the other localities displayed a heavier degree of white scaling than shown in Fig. 2. The types of families identified in the samples of $F_1$ families representing each locality are shown in Table 2. Out of 195 families, only three families (1.5%) were *formosus* form, 75 (38.3%) were type form, and 118 (60.2%) were heterogeneous. When families were classified in a similar way according to the presence of white scaling on $T_2$, it was found that, out of all the families, 55.6% were heterogeneous and 44.4% were homogeneous for presence of white scaling.

In Table 3, all the specimens representing a locality, usually belonging to 10–13 families, were treated as one sample. For each of the 18 samples, the range of values for $T_1$ and $T_2$ is given together with the respective means and standard deviations. All the ranges of values overlap for both $T_1$ and $T_2$. These results were used to calculate the $(1-\alpha)$ 100% large sample confidence limits, were $\alpha = 0.05$, for $T_1$ and $T_2$, respectively. Figure 3 gives these limits as histograms and shows that the confidence limits for $T_1$ and $T_2$ on the various populations differ in length and position, although some populations were very close to one another. When, however, the means of the populations were compared by the Waller-Duncan test in respect of $T_1$ alone, it was found that none of the four non-anthropophilic or probable non-anthropophilic populations, Ndumu, Mica, Magaliesberg and Skukuza, differed consistently from the remain-

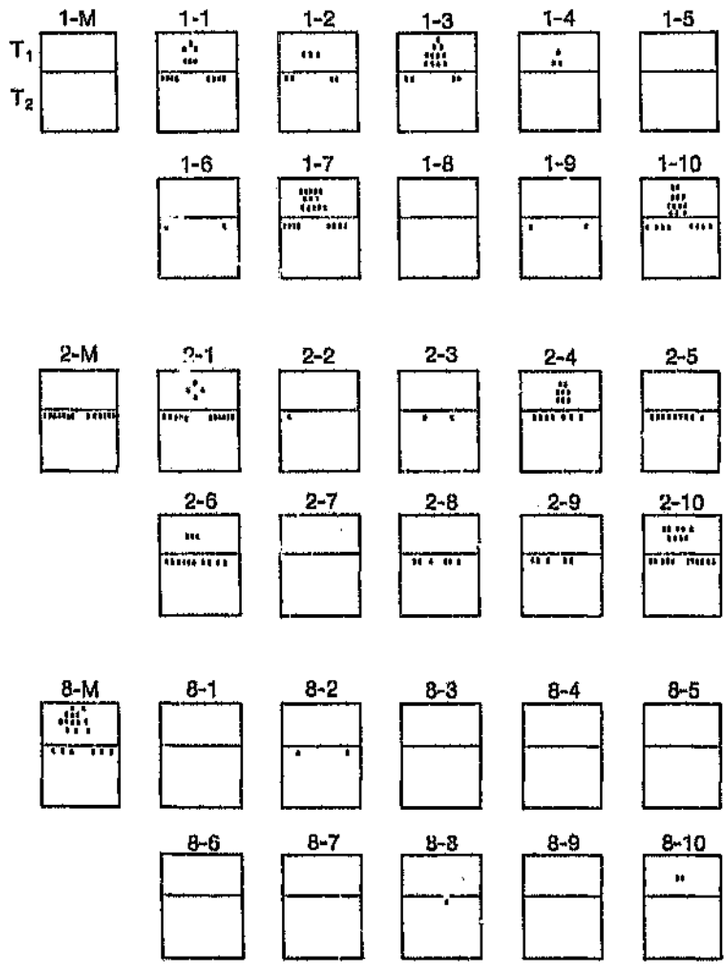



Fig. 2. Distribution of white scales on the first ($T_1$) and second ($T_2$) tergites in three *Ae. aegypti* mothers (M) and samples of their respective $F_1$ progeny.

ing anthropophilic populations $P > 0.05$). The same analysis done for $T_2$ alone indicated some similarity between these four populations in their having more white scales than most anthropophilic populations ($P < 0.0001$). Ndumu differed from all other populations except Mica. Mica differed from all others except Ndumu and Skukuza. Skukuza was similar to Mica and the anthropophilic populations of Port Edward and Johannesburg. Magaliesberg was similar to Richards Bay, Empangeni, Palm Beach and Mkuzi, all anthropophilic populations. Grahamstown differed from all other populations by having a considerable number of white scales.

When both sample means ($T_1$ and $T_2$) were compared together by multivariate analysis, all 18 populations were found to be significantly different from one another ($P < 0.0001$) using the four chosen statistics.

## DISCUSSION

Hartberg et al. (1986) showed that abdominal tergal scale pattern in *Ae. aegypti* appeared to be controlled by one major poly-

Table 2. Classification of $F_1$ families of *Ae. aegypti* from various localities into those which are homogeneous for either the typical or *formosus* forms and those which are heterogeneous.

| Locality | Total | No. families | | |
|---|---|---|---|---|
| | | Homogeneous: typical form | Homogeneous: *formosus* form | Heterogeneous: both forms |
| Grahamstown | 11 | 5 | 0 | 6 |
| Port Edward | 11 | 4 | 0 | 7 |
| Palm Beach | 12 | 3 | 0 | 9 |
| Port Shepstone | 12 | 4 | 0 | 8 |
| Park Rynie | 12 | 0 | 1 | 11 |
| Durban | 10 | 5 | 0 | 5 |
| Stanger | 10 | 4 | 0 | 6 |
| Empangeni | 9 | 4 | 0 | 5 |
| Richards Bay | 11 | 5 | 0 | 6 |
| Mtubatuba | 8 | 3 | 0 | 5 |
| St Lucia Estuary | 11 | 9 | 0 | 2 |
| Mkuze | 10 | 1 | 0 | 9 |
| Ndumu | 11 | 4 | 0 | 7 |
| Johannesburg | 11 | 4 | 0 | 7 |
| Magaliesberg | 15 | 3 | 2 | 10 |
| Skukuza | 11 | 8 | 0 | 3 |
| Mica | 13 | 4 | 0 | 9 |
| Tzaneen | 8 | 5 | 0 | 3 |
| | 196 | 75 | 3 | 118 |
| | | 38.3% | 1.5% | 60.2% |

genic system with modifiers. Hence he considered that it would be better to use differences in ecology, physiology and behavior when attempting to divide *Ae. aegypti* into different species or subspecies. He concluded that while abdominal tergal scale pattern may be useful for distinguishing populations, it may not be valid as the exclusive taxonomic basis for classification. In earlier work, Hartberg (1969) showed that the offspring of 35 female *formosus* and typical forms collected at Dar-es-Salaam, Tanzania, nearly all failed to breed true, indicating that the populations were simply polymorphic for abdominal tergal scaling. In the present study, about two-thirds of the 196 families examined from 18 localities in South Africa similarly failed to breed true, but gave offspring that were heterogeneous for the presence or absence of white scales on the first tergite. This finding shows that the basis for separating *Ae. aegypti* into nominate and *formosus* subspecies proposed by Mattingly (1957) should be regarded as invalid. It would be interesting to study the variety *queenslandensis* in a similar way.

Using two characters, the degree of scaling on both the first and second tergites, we found that all 18 populations of *Ae. aegypti* differed significantly regardless of their degree of anthropophilism or geographic location. This supports the view that *Ae. aegypti* is a single polymorphic species in South Africa with a variable preference for man. Other studies by us using isozyme electrophoresis to analyze genetic difference between South African populations of *Ae. aegypti* (Kemp and Jupp, unpublished observations) also failed to reveal any evidence for the existence of more than

Table 3. Number of white scales on first tergite ($T_1$) and in basal band on second tergite ($T_2$) in $F_1$ female mosquitoes from various localities.

| Locality | n | $T_1$ Range | $T_1$ x | $T_1$ s | $T_2$ Range | $T_2$ x | $T_2$ s |
|---|---|---|---|---|---|---|---|
| Grahamstown | 104 | 0–45 | 7.58 | 9.54 | 4–35 | 18.60 | 6.01 |
| Port Edward | 112 | 0–40 | 5.63 | 7.51 | 0–18 | 7.54 | 4.50 |
| Palm Beach | 110 | 0–30 | 5.73 | 7.06 | 0–25 | 4.55 | 4.63 |
| Port Shepstone | 104 | 0–34 | 5.98 | 7.38 | 0–11 | 3.13 | 2.96 |
| Park Rynie | 110 | 0–24 | 1.95 | 4.38 | 0–15 | 4.15 | 4.44 |
| Durban | 102 | 0–35 | 8.77 | 9.95 | 0–22 | 5.75 | 5.44 |
| Stanger | 100 | 0–60 | 13.08 | 15.11 | 0–25 | 6.01 | 5.34 |
| Empangeni | 94 | 0–30 | 6.62 | 7.12 | 0–23 | 4.84 | 5.02 |
| Richards Bay | 109 | 0–40 | 7.15 | 7.47 | 0–16 | 5.40 | 4.33 |
| Mtubatuba | 81 | 0–24 | 5.80 | 5.47 | 0–17 | 7.09 | 4.66 |
| St Lucia Estuary | 100 | 0–40 | 11.91 | 8.89 | 0–19 | 3.88 | 4.02 |
| Mkuze | 96 | 0–15 | 2.76 | 3.37 | 0–21 | 4.40 | 4.00 |
| Ndumu | 125 | 0–19 | 4.50 | 4.07 | 0–27 | 9.72 | 5.88 |
| Johannesburg | 104 | 0–26 | 5.02 | 6.54 | 0–22 | 7.41 | 5.10 |
| Magaliesberg | 129 | 0–25 | 3.42 | 5.98 | 0–20 | 4.37 | 3.97 |
| Skukuza | 108 | 0–31 | 8.81 | 7.39 | 0–26 | 8.39 | 6.41 |
| Mica | 130 | 0–30 | 5.38 | 5.83 | 0–20 | 9.29 | 4.96 |
| Tzaneen | 63 | 0–30 | 8.05 | 8.34 | 0–16 | 5.68 | 4.95 |
| | 1,881 | | | | | | |

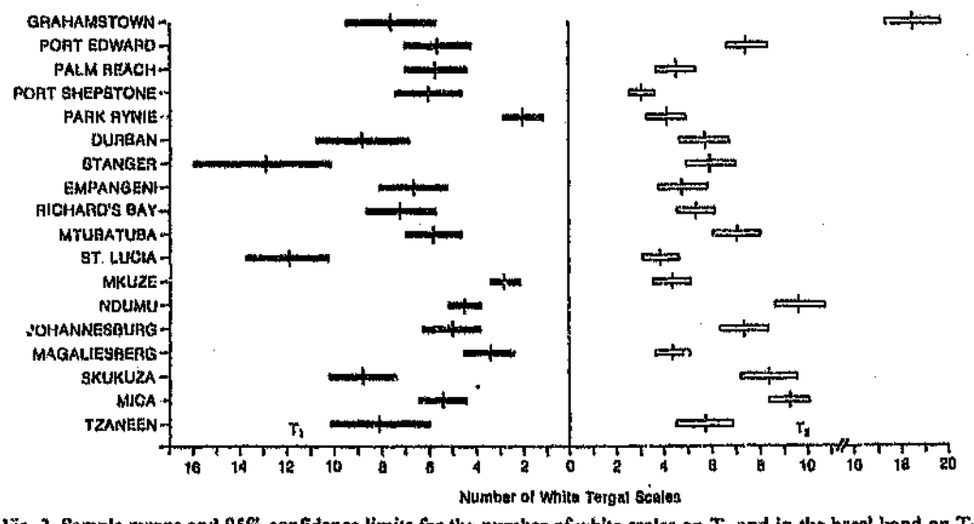



Fig. 3. Sample means and 95% confidence limits for the number of white scales on $T_1$ and in the basal band on $T_2$ in *Ae. aegypti* populations. Fourteen populations were anthropophilic, two non-anthropophilic (Ndumu and Mica) and two were probably non-anthropophilic (Skukuza and Magaliesberg).

one species. Some of the populations were significantly different in respect of the $T_1$ value alone by the Waller Duncan test, although all the ranges of values for $T_1$ overlapped. The ranges of values also overlapped for $T_2$ in all of the populations, although some samples also differed statistically in respect of this character. Nevertheless, our results failed to show that non-anthropophilic populations invariably exhibited a larger number of white scales on $T_2$ than anthropophilic populations. Apart from the work done on *Ae. aegypti* from East African countries, morphological variation has been recorded for Nigeria (Summers-Connal 1927), the Philippines (Mogi et al. 1984) and northern Thailand (Mogi et al. 1989). The presence of such variation needs to be analyzed within families.

It is concluded that in South Africa, *Ae. aegypti* is a single polymorphic species showing a considerable amount of variation in the degree of white scaling on the first two abdominal tergites. The presence of some rural sylvan non-anthropophilic populations in our country (Kemp and Jupp 1991) might, however, indicate incipient speciation.

## ACKNOWLEDGMENTS

We wish to thank the Directors of the KwaZulu Bureau of Natural Resources, the Natal Parks Board and the National Parks Board as well as the head of the Department of Community Services of Richards Bay Municipality for permission to collect mosquitoes in areas under their jurisdiction. Special thanks are due to Mr. Graham Wiltshire (Ndumu reserve), Mr. Tommy Wessels (Richards Bay) and Dr. Leo Braak (Kruger Park) for their kind assistance. Mr. Edward Chauke and Mr. Obed Matlala rendered technical assistance in the laboratory, which is gratefully acknowledged. Finally, one of us (P.G. Jupp) is indebted to the South African Medical Research Council for a grant toward the project.

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# MOSQUITO VECTORS OF DENGUE IN SOUTH AFRICA

by

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presented by B.H. Kay

Although epidemic dengue (DEN) has been known for a long time in Asia and the Caribbean-American region, it has only been reported from Africa much more recently (Gubler, 1988). Not until the 1960's and early 1970's was DEN illness recognised in Nigeria in West Africa, and only in the 1980's were epidemics first documented in East Africa. Epidemics of DEN 2 occurred in the Sudan and Somalia in 1984, in Kenya in 1982 and in the Seychelles in 1976/77. There was an outbreak of DEN 3 in Mozambique in 1984. Durban, the main port on the eastern coast of South Africa experienced DEN in 1897, 1901 and 1926/27. The latter was a large epidemic of DEN (Edington, 1927) later shown retrospectively to have been due to DEN 1 virus. The annual rainfall during 1926/27 was unusually high i.e. 5669mm and 8839mm compared to the normal 1008mm which must have contributed to the outbreak. In 1985 and 1987 several people who had returned home to Durban after visits to India suffered DEN 1 infections (Blackburn and Rawat, 1987; Blackburn *et al.*, 1987). Because of this and the recent outbreaks in nearby countries, it was decided to determine which mosquito species were the potential vectors of DEN 1 and DEN 2 in South Africa.

Only *Aedes* mosquitoes, almost all belonging to the subgenera *Stegomyia* and *Diceromyia* have been found to be vectors in other countries. These are mosquitoes breeding in artificial containers, such as tyres and tin cans, or in natural receptacles such as tree holes or leaf axils. Our investigations were therefore concentrated on mosquitoes in these subgenera in the subtropical regions of Natal and the Transvaal and the tropical region in Northern Natal-Kwazulu. These, the likely regions into which DEN virus could be imported to cause a possible outbreak, are shown in the



25×10

map in fig 1. The principal localities where we collected along the Natal coast and inland in the eastern Transvaal lowveld are also given.

A mosquito would possess a number of particular attributes to qualify as a potentially efficient DEN epidemic vector. Such a mosquito species would be widespread in the tropical and/or subtropical regions, it would be abundant, occur either domestically or peri-domestically, bite man and be a competent laboratory vector with high infection and transmission rates. Our field studies produced a "short list" of 6 species which qualified as potential vectors on grounds of their distribution, abundance, anthropophilism and domesticity (Kemp & Jupp, 1991). *Aedes aegypti*, *Ae. simpsoni* and *Eretmapodites quinquevittatus* were recorded in all the 3 important regions: Natal subtropical, Natal tropical and Transvaal subtropical. *Aedes strelitziae* and *Ae. demeilloni* were limited to the Natal coast, mainly the subtropical region as they depend on the plants *Strelitzia nicolai* and *Dracaena hookeriana* for their larval habitats. *Aedes furcifer* is predominantly a rural species confined to the thornveld savannah of the tropical region of Natal and subtropical north-eastern Transvaal. However, a few specimens were collected in urban Durban indicating that this species may occur much further south than was originally thought.

Vector competence tests were undertaken with an *in vitro* system on field collected mosquitoes or their $F_1$ or $F_2$ progeny except in the case of *Ae. furcifer* where a long established laboratory colony was usually employed. The mosquito species under assessment was first allowed to feed on an infective blood-meal consisting of a defibrinated blood-virus mixture. The engorged mosquitoes were then incubated at 30-31°C for 17-18 days until their transmission feed. For this feed each mosquito was allowed to feed from a capillary tube charged with a mixture of 10% sucrose and foetal calf serum using the method of Aitken (1977). If it transmits, the insect secretes virus into the capillary tube. After a feed the balance of the fluid remaining in the tube was discharged into Bovine Phosphate Albumen which was assayed for virus. This was done by intrathoracic inoculation of a group of *Ae. aegypti* laboratory mosquitoes employing the technique of Rosen and Gubler (1974). After 8 days incubation at 30°C, the heads of 4 of these *Ae. aegypti* were squashed

individually and tested for antigen by the indirect fluorescent antibody test (IFAT). If the capillary tube turned out negative, the original mosquito which had fed in the capillary tube was then tested. The information thus obtained permitted the calculation of a head squash infection rate (HSIR) and a transmission rate which was defined as the percentage of the infected mosquitoes feeding which transmitted virus. A vector competence index (VCI) was also calculated which was the product of the decimal fractions of HSIR and transmission rate. The maximum possible VCI is 1.0. The entire procedure was usually repeated twice on different samples of mosquitoes from each locality.

The results of the vector competence experiments for all the mosquitoes except *Ae. aegypti* are shown in Table 1. Results for DEN 1 virus and DEN 2 virus were generally similar. Three of the species, *Ae. demeilloni*, *Ae. simpsoni* and *Er. quinquevittatus* proved entirely refractory to both types of DEN virus so that transmission tests were not done in these cases. *Ae. strelitziae* gave a mean HSIR of 33% for DEN 1 and 56% for DEN 2. None of the mosquitoes of this species infected with DEN 1 transmitted virus but 2 out of 7 (29%) mosquitoes infected with DEN 2 transmitted virus, giving a VCI of 0.16. In the case of *Ae. furcifer*, the mean HSIR's were 3% and 11% for DEN 1 and 2 respectively. In the case of DEN 2, 2 infected mosquitoes refed in the capillary feeding, one of which transmitted virus, so that the VCI was 0.06.

The results for populations of *Ae. aegypti* from 5 different localities are shown in Table 2 and were rather similar with both viruses. All populations transmitted both serotypes. The vector competence indices were highest for Durban with both viruses, while indices for the other species were considerably lower.

Using the Chi-squared test, the HSIR of each population was compared with each of the other populations in turn. With DEN 1, Palm Beach and Skukuza differed from the other 3 populations, but not from each other reflecting their low HSIR's. With DEN 2, Palm Beach differed from Durban and Ndumu only. The same statistical analysis was conducted on the 5 populations with respect to the vector competence index. In the case of DEN 1, the Durban population had a significantly higher VCI

than all the other populations while Skukuza had a significantly lower VCI than all populations except Palm Beach. With DEN 2, only Palm Beach differed from Durban. Transmission rates were compared using the Fisher's exact test and no statistically significant differ .ices were found between the different populations.

## CONCLUSIONS

1. The primary potential vector for an epidemic of DEN 1 or 2 in South Africa would be *Ae. aegypti* and the secondary such vector would be *Ae. strelitziae*.
2. *Ae. furcifer* would seem only a poor potential vector confined to the rural areas of northern Natal-Kwazulu and the eastern Transvaal.
3. Durban *Ae. aegypti* was the most efficient vector out of the *Ae. aegypti* populations tested from 5 localities.
4. The likelihood of a DEN outbreak occurring in Durban or elsewhere in South Africa has increased in recent years for 3 reasons:-
   a) The virus has been more active than ever before in our neighbouring East African states and was also imported into Durban in 1985 and 1987.
   b) The lifting of sanctions against South Africa is resulting in increased traffic by sea and air between South Africa and Asia enhancing the chances of the virus being imported.
   c) Increased urbanization in our tropical and subtropical regions is providing more breeding places for *Ae. aegypti*

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Table 1 Vector competence of 5 mosquito species after an infective blood-meal containing 6.1-8.4 $\log_{10}$ $MID_{50}$/ml of DEN 1 or DEN 2 virus.

| Species | Head squash infection rate (%)* | | Transmission rate (%)+ | | Vector competence index# | |
|---|---|---|---|---|---|---|
| | DEN 1 | DEN 2 | DEN 1 | DEN 2 | DEN 1 | DEN 2 |
| *Ae. (Stg.) demeilloni* | 0/48 | 0/27 | - | - | | |
| *Ae. (Stg.) simpsoni* | 0/35 | 0/39 | - | - | | |
| *Ae. (Stg.) strelitziae* | 7/21(33) | 10/18(56) | 0/5 | 2/7(29) | 0 | 0.16 |
| *Ae. (Dic.) furcifer* | 1/37(3) | 6/53(11) | 0/1 | 1/2(50) | 0 | 0.06 |
| *Er. quinquevittatus* | 0/49 | 0/31 | - | - | | |

*Numerator, number infected; denominator, number tested.
+Numerator, number transmitting; denominator, number infected mosquitoes that fed, each mosquito fed from a separate single capillary tube.
#defined in text

Table 2 Vector competence of different populations of *Ae. aegypti* after an infective blood-meal containing 6.1-8.4 $\log_{10}$/ml of DEN 1 or DEN 2 virus.

| Locality | Head squash infection rate (%) | | Transmission rate (%) | | Vector competence index | |
|---|---|---|---|---|---|---|
| | DEN 1 | DEN 2 | DEN 1 | DEN 2 | DEN 1 | DEN 2 |
| Palm Beach | 15/72(21) | 20/107(19) | 6/9(67) | 6/7(86) | 0.15 | 0.18 |
| Durban | 28/52(54) | 24/60(40) | 20/24(83) | 15/20(75) | 0.47 | 0.34 |
| Richards Bay | 29/76(38) | 21/72(29) | 10/15(67) | 11/14(79) | 0.25 | 0.23 |
| Ndumu | 32/88(36) | 29/82(35) | 6/9(67) | 12/17(71) | 0.28 | 0.22 |
| Skukuza | 5/47(11) | 20/71(28) | 4/4(100) | 14/18(78) | 0.13 | 0.21 |

Table 3 Interpopulation statistical comparisons by $\chi$-squared test of vector competence in *Ae. aegypti* showing difference (+) or no difference (-) for DEN 1 and DEN 2 viruses respectively.

| | ality | Durban | Richards Bay | Ndumu | Skukuza |
|---|---|---|---|---|---|
| Head squash infection rate | Palm Beach | +#,+* | +,- | +,+ | -,- |
| | Durban | | -,- | -,- | +,- |
| | Richards Bay | | | -,- | +,- |
| | Ndumu | | | | +,- |
| Vector competence index | Palm Beach | +,+ | -,- | +,- | +,- |
| | Durban | | +,- | +,- | +,- |
| | Richards Bay | | | -,- | +,- |
| | Ndumu | | | | +,- |

# DEN 1
* DEN 2

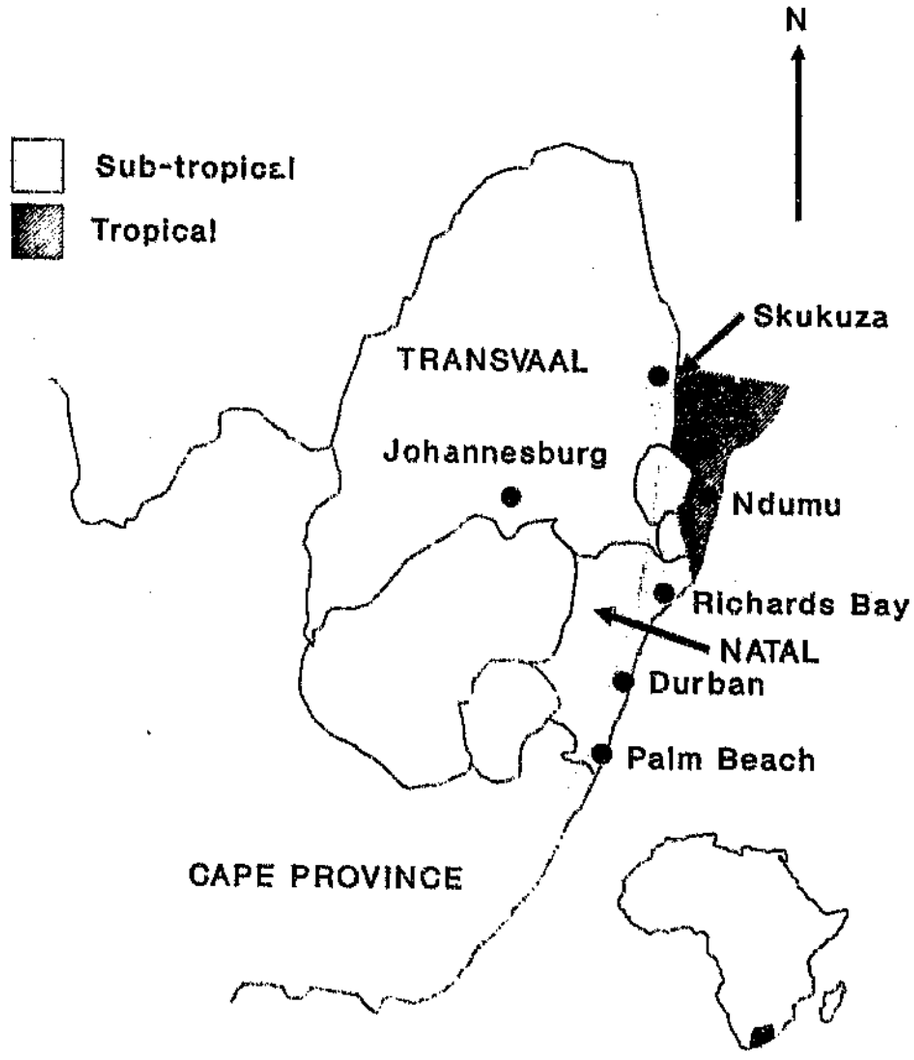



Fig 1. Map of Natal and the Transvaal showing the principal localities of mosquito collection.

# *AEDES ALBOPICTUS* AND OTHER MOSQUITOES IMPORTED IN TIRES INTO DURBAN, SOUTH AFRICA

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ABSTRACT. The results of surveillance for immature stages of mosquitoes in samples of wet tires in 5 consignments imported into Durban from Japan is reported. Three of these consignments contained tires with immatures of *Aedes aegypti*, *Ae. albopictus* and *Uranotaenia n. novobscura*. The proportions of all the wet tires calculated to contain mosquitoes were 189/1,488 (13%), 20/813 (2.5%) and 13/1,032 (1.3%) which emphasizes the need for effective mosquito control measures to prevent *Ae. albopictus* escaping from such tires and establishing itself in Durban.

Figures for 1978–86 revealed that South Africa was the fifth largest importer of used tires from Japan with 1,065,211 tires, i.e., 118,356 per annum (Reiter and Sprenger 1987). According to A. S. Fullard of the South African Tyre Dealers Association (personal communication), the number has now increased with about 154,553 tires being the 1992 import quota. Immature stages of the Asian mosquito *Aedes albopictus* (Skuse) have been collected on 4 occasions in tires landed in Cape Town from Japan. These were dead pupae and pupal exuviae in November 1989, and live larvae on 3 occasions in 1990 (Cornel and Hunt 1991). Some populations of *Ae. albopictus* might survive the cold winter in Cape Town's Mediterranean climate, since populations of the mosquito in northern Asia can apparently survive severe winter conditions (Reiter and Darsie 1984, Hawley et al. 1989). However, in South Africa it is considered much more likely that this predominantly tropical species (Hawley 1988) would establish itself in Durban with its subtropical climate should it be introduced into this city. For this reason we are undertaking surveillance for *Ae. albopictus* and other exotic mosquitoes in Durban at the premises of companies which import second-hand tires from the Far East. Durban is situated on the east coast of South Africa in the Province of Natal and is our busiest port.

The first aspect of this surveillance began in late November 1991, when we initiated the sampling of each consignment of wet tires landed. Two inspectors from the Port Health Office search for mosquito larvae and pupae in a consignment with wet tires for at least 1 hour. Any live immatures are brought to the laboratory, where they are reared to adults, while larval and pupal pelts are stored in alcohol to facilitate identification. Identifications have been made using the keys for mosquitoes of Japan and Korea by Tanaka et al. (1979). So far 5 consignments of wet motor car tires, landed in containers between November 21, 1991 and January 16, 1992, have been sampled and live immature mosquitoes were collected in 3 of them.

Table 1 gives details of the 5 shipments, all from Japan. Seventeen 2nd or 3rd instar larvae and 2 pupae were collected. All were reared to adults except 7 specimens in collection No. 2; these were one *Aedes aegypti* (Linn.) which died on reaching the 4th instar and six 2nd instar larvae which died before they became identifiable. The other species were *Uranotaenia n. novobscura* Barraud and *Ae. albopictus*. During the time the health inspectors could spend sampling wet tires, only a relatively small proportion (4–13%) of all the wet tires in a consignment could be examined. However, the proportions of all the wet tires estimated to contain larvae or pupae were calculated from the proportion of tires found to contain immature stages in the samples examined. The figures shown in Table 1 indicate that a considerable number of larvae-bearing tires must have escaped detection, e.g., 182 (189 minus 7) in collection No. 2.

In view of the above, it seems possible that some *Ae. albopictus* could have established founder populations in the vicinity of the tire importers' premises. The second aspect of our surveillance is aimed at the detection of such *Ae. albopictus* populations. This project is in progress and involves the exposure of bamboo pots as ovipositional sites within the grounds of the 2 largest importers of second-hand tires from the Far East in Durban. *Uranotaenia n. novobscura* is not medically important but exotic *Ae. aegypti*, and more particularly *Ae. albopictus*, are potential dengue vectors. The latter and possibly the former, would be expected to have a higher vector potential for dengue viruses than the indigenous mosquito species along the Natal coast. It is therefore important that control measures are effected to prevent the escape of exotic mosquitoes from the imported tires lest such mosquitoes become established in Durban. A similar system of surveillance is planned for the other main centers in South Africa where

Table 1. Second-hand tire casings landed in Durban, South Africa, from Japan: results of sampling for immature mosquitoes.

| Collection no. | Place of origin | Date sampled* | No. tires in consignment | Estimated no. tires wet (%) | Proportion of tires examined found with mosquitoes (%) | No. of all wet tires estimated to contain mosquitoes | Mosquito identity |
|---|---|---|---|---|---|---|---|
| 1 | Osaka | Nov. 21, 1991 | 1,551 | 310 (20) | 0/40 | — | — |
| 2 | Yokohama | Nov. 21, 1991 | 1,860 | 1,488 (80) | 7/55 (12.7) | 189 | 1 *Ae. aegypti*<br>8 *Ur. novobscura*<br>6 unidentifiable |
| 3 | Tokyo | Dec. 20, 1991 | 1,626 | 813 (50) | 1/40 (2.5) | 20 | 1 *Ur. novobscura* |
| 4 | Tokyo | Jan. 6, 1992 | 1,291 | 1,032 (80) | 1/80 (1.3) | 13 | 1 *Ae. albopictus* |
| 5 | Yokohama | Jan. 16, 1992 | 1,172 | 703 (60) | 0/45 | — | — |

* This was also the date the container arrived at the tire company and was opened.

containers with imported tires are opened up. These are Cape Town, Johannesburg and Pretoria.

We thank the following staff of the Port Health Office, Durban, in the Department of National Health and Population Development for their splendid assistance in the project: Stella Simes, Mimie van der Merwe, Debbie Naidoo, Mark Morganthal, Susan Kukard and Alvin Louve.

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4.5

# Results of a preliminary ovitrap survey for Aedes albopictus (Diptera: Culicidae) at two tyre retreading companies in Durban

by

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## INTRODUCTION

The mosquito, Aedes (Stegomyia) albopictus (Skuse), is an important vector of dengue virus in Asia. It has also been shown an efficient laboratory vector of, among others, four African arboviruses: chikungunya, Rift Valley fever, West Nile and yellow fever. The species has adapted readily to urban and peri-urban environments in a range of climates ranging from the tropical to those with severe winters. Both artificial containers, including discarded tyres, and natural container sites are utilized for breeding and the adults feed avidly on a wide range of vertebrate hosts including man (Hawley 1988). The increased exportation of used tyres since 1985 by countries in the Far East, particularly Japan, has led to the establishment of breeding populations of Ae. albopictus in the United States of America (Francy et al. 1990), Brazil (Forattini 1986) and Nigeria (Savage & Miller 1991). Because of its ecological and vectorial attributes, there is a danger that Ae. albopictus could become involved in arboviral disease transmission in countries that were previously free of the species and that it might possibly outperform the local vectors or introduce a formerly enzootic virus to a susceptible human population.

Routine surveillance by the Department of National Health in South Africa has recently demonstrated the presence of Ae. albopictus and other exotic mosquitoes, including Asian Aedes (Stegomyia) aegypti (Linnaeus), in Cape Town and Durban in used tyres imported from Japan (Cornel & Hunt 1991; Jupp & Kemp, in press). In view of this, additional surveillance was considered necessary at the premises of tyre importers to

determine whether breeding populations of Ae. albopictus had become established. We decided to begin this surveillance in Durban by means of ovitraps.

## MATERIALS and METHODS

Two tyre retreading companies, Natyre and Smythe's Tyres, situated respectively in the suburbs of Jacobs and Congella, are the biggest importers in Durban of used tyres from the Far East. Because of this they were selected as the two sites for our surveillance. Bamboo pot ovitraps containing dechlorinated water and a few dry poplar leaves were suspended at heights ranging from 1,0-2,5 metres above ground level at Natyre and in two premises adjacent to Smythe's Tyres that belong to Durban City's Department of Parks, Recreation and Beaches. Ovitraps were hung from walls, in trees and amongst shubbery in groups of 25 per site for four periods ranging from 34 to 49 days, namely 03/12/91 to 14/01/92 (period A), 14/01/92 to 25/02/92 (B), 25/02/92 to 31/03/92 (C) and 31/03/92 to 19/05/92 (D).

For periods A and B, ovitraps not sheltered from the sun (40/50) were suspended two centimetres below small squares of hardened masonite to help retard evaporation. During periods C and D, 13 of the ovitraps at Natyre were suspended from two 1,0 x 1,5 $m^2$ frames covered with shade-cloth and the remainder hung in trees. At Smythe's Tyres, 18 ovitraps were placed inside carports and the remainder left attached to outside walls. The 40 ovitraps exposed to direct sunlight at the two sites during periods C and D were fitted with masonite hoods.

Staff of the Durban Port Health Office inspected the ovitraps every fortnight and topped them up with dechlorinated water to two thirds full. Ovitraps removed at the end of each of the four periods were emptied and mosquito larvae and pupae harvested for rearing in the laboratory. The ovitraps were then allowed to dry out partially before being flooded with tap water in the laboratory. They were left for two days to allow the eggs to hatch. Once the ovitraps had dried, they were reflooded a second time. All the resulting larvae were reared to adults before being identified.

## RESULTS

No evidence of Ae. albopictus was found in the survey. The most prevalent species in the ovitraps was Ae. aegypti with bamboo pot indices ranging from 28-52% and abundance indices from 0,14-0,60 mosquitoes/pot/day for the combined collection sites (Table 1). Bamboo pot indices increased during the survey period at Smythe's Tyres but not at Natyre; abundance indices were essentially unchanged except for a peak in March at Natyre. The next most prevalent mosquito was Culex (Culex) quinquefasciatus Say, with abundance indices of 0,08-0,36 mosquitoes/pot/day but bamboo pot indices all below 24%. The other species collected were, in order of prevalence: Ae. (Stegomyia) simpsoni (Theobald), Culex (Culiciomyia) nebulosus Theobald, Aedes (Diceromyia) furcifer (Edwards) group and Aedes (Stegomyia) metallicus (Edwards).

## DISCUSSION

The highly anthropophilic Ae. aegypti and Cx. quinquefasciatus, both potential vectors of pathogens, were present in significant numbers at two industrial sites where there were virtually no artificial or natural containers for larval habitats except for the bamboo pots. The only trees at Natyre were Casuarina equisetifolia J.R. & G. Forster (Casuarinaceae) and a solitary guava, Psidium guajava L. (Myrtaceae), neither being suitable phytotelmata. One of the plots adjacent to Smythe's Tyres had no vegetation at all and the other only had shubbery and small monocotyledons. The buildings at both sites could conceivably provide resting sites for adults and company employees could provide bloodmeals, especially for the diurnal Stegomyia species.

The period between the opening of the shipping containers in which the tyres are imported and the drying of the tyre casings during heat treatment prior to retreading is reportedly about four days. This is too short a time for local rainfall to hatch out eggs present on the walls of the tyres and for the resultant larvae to develop into adults. However, imported tyres are occasionally water-logged and could contain fourth stage larvae and pupae which could emerge within two or three days after arrival. Adult mosquitoes that may have emerged inside the shipping containers could also be released when the containers are opened in the factory yard.

In spite of the dearth of containers for oviposition at the two factories, alternative larval habitats in the form of artificial containers such as discarded beverage cans and cardboard cartons (of the kind used to contain milk or sorghum beer) are available in the close vicinity of the two tyre retreading companies. Natural larval habitats in the form of tree-holes or leaf-axils are also present within a few hundred metres of Smythe's Tyres.

An earlier bamboo pot survey conducted in the grounds of Natal University at Congella, Durban (Kemp & Jupp 1991) from 07/12/89-0?/02/90 produced comparable bamboo pot index (53%) but lower abundance index (0,14 mosquitoes/pot/day) for Ae. aegypti. The two species, Ae. simpsoni and ?. nebulosus were as rare, but Ae. metallicus and Cx. quinquefasciatus were absent in the previous study. No evidence was found of the predacious larvae of Toxorhynchites (T hynchites) brevipalpus Theobald which occurred in 40% of the ovitraps in 1989/1990. One female specimen of the Ae. furcifer group was obtained from a pot from Natyre. Unfortunately, discrimination between the sympatric species, Ae. furcifer and Aedes (Diceromyia) cordellieri Huang is only possible for the male imago (Jupp et al., in press). However, only Ae. furcifer sensu stricto has been recorded this far south before (Kemp & Jupp 1991) and in December 1991, an Ae. furcifer male was recorded from a discarded tyre at Sprin Durban (Jupp, unpublished).

The absence of Ae. albopictus from our survey would suggest that the species has not become established in the Durban area. Although other companies in Durban import used tyres from the Far East, they do so only on a much smaller scale. However, the establishment of permanent populations of Ae. albopictus in the future cannot be ruled out. Therefore, the Department of Public Health intends continuing the survey in Durban and expanding it to include other major ports and cities in South Africa.

ACKNOWLEDGEMENTS

We wish to thank the following: the staff of the Durban Port Health Office for their conscientious assistance in the field; the helpful cooperation of Messrs T. Flint of Natyre, T. Hore of Smythe's Tyres and L. Lauderdale of the Durban City Department of Parks, Recreation and Beaches; Messrs E. Chauke and J. Mahlangu of the Arbovirus Unit.

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TABLE 1. Bamboo pot indices[1] and abundance indices[2] (in parentheses) for mosquitoes at two tyre retreading companies in Durban from 03/12/91 to 19/05/92.

| | Smythe's Tyres | | | | Natyre | | | |
|---|---|---|---|---|---|---|---|---|
| Period[3] | A | B | C | D | A | B | C | D |
| No. of pots | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 21 |
| No. of days exposed | 42 | 42 | 34 | 49 | 42 | 42 | 34 | 49 |
| Ae. furcifer group | - | - | - | - | - | - | - | 4 (<0,01) |
| Ae. aegypti | 28 (0,28) | 28 (0,14) | 32 (0,24) | 40 (0,25) | 40 (0,25) | 52 (0,60) | 44 (0,40) | 40 (0,25) |
| Ae. metallicus | - | - | - | - | - | 4 (<0,01) | - | - |
| Ae. simpsoni | - | 8 (0,15) | - | 4 (<0,01) | - | - | - | - |
| Cx. nebulosus | - | - | - | - | 4 (0,10) | - | - | - |
| Cx. quinquefasciatus | 16 (0,08) | - | 12 (0,12) | 24 (0,36) | 8 (0,22) | - | 16 (0,33) | - |

1. Percentage of pots containing the species.
2. No. mosquitoes/pot/day.
3. See Materials and Methods for dates.

J. Med. Ent. Vol. 9, no. 2: 155–159 28 April 1972

# FURTHER ISOLATIONS OF ARBOVIRUSES FROM MOSQUITOES COLLECTED IN TONGALAND, SOUTH AFRICA, 1960-1968[1]

By B. M. McIntosh, P. G. Jupp and J. de Sousa[2]

*Abstract:* Over a period of 8.5 years 168,231 mosquitoes, including at least 52 species, were collected in a Game Reserve and tested for infection with arboviruses. Fifty-two isolations, including strains of Sindbis, Middelburg, Ndumu, Wesselsbron, Spondweni, Bunyamwera, Shokwe, Germiston, Pongola, Mossuril, Lebombo, Ingwavuma and Simbu viruses were made from 5 species of mosquito. The most frequently infected species, *Aedes* (*Neomelaniconion*) *circumluteolus* (Theobald), yielded 37 strains which included strains of 11 arboviruses. The appearance of particular viruses was intermittent and it was not possible to incriminate any single species fundamentally in the survival of these viruses. Seven species comprised 96% of the total number of mosquitoes tested and it seemed possible that unidentified vectors may be among species collected in small numbers.

[1]The studies and observations on which this paper is based were financed jointly by the South African Institute for Medical Research, the Poliomyelitis Research Foundation and the Council for Scientific and Industrial Research.

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Since 1956 a field study on arboviruses has been maintained by this Unit in the Ndumu Game Reserve situated in the tropical, eastern, coastal plain of South Africa. One aspect of this study has been a viral survey of the mosquito population. Worth et al. (1961) reported on the viruses isolated from 1956 through April 1960, briefly described the ecology of the area and discussed the epidemiological implications of their results. During this period 59 isolations, which included 9 identified viruses, were effected from 9 species of mosquito. Spondweni, Wesselsbron and Pongola viruses were most frequently recovered and *Aedes circumluteolus*,

*Culex univittatus* (= *Culex neavei*) and *Mansonia africana* were most productive of viruses. The incidence of viral isolates in time and from mosquito species was irregular and failed to reveal firm indications of host specificity. The present report carries the study for a further 8.5 years through to the end of December 1968 involving altogether a period of 13 consecutive years.

## METHODS

Worth et al. (1961) collected most mosquitoes by hand off vegetation in glass test-tubes, and mosquitoes intended for virus isolation were sorted without regard to stages of the gonotrophic cycle. During the first 4 years of the present study similar techniques were used. After that collecting sites and collecting methods varied depending upon what other objectives the studies were attempting to fulfill. For instance, following the disclosure that a high immune rate to chikungunya virus existed in the monkey population in the Reserve, the collecting sites were moved in March 1964 from savanna to riverine forest where mosquitoes were collected at ground level and in the canopy in an attempt to identify the vector of chikungunya virus. These collections were made off live bait and were therefore heavily biased towards the blood-seeking female. This study terminated in April 1967 and from August 1967 through December 1968 a further change ensued when collections were concentrated on a single species, *A. circumluteolus*, mostly in savanna, in a study on the population dynamics of this species through the seasons. These collections were made with portable suction traps sweeping low vegetation and the catch included a high proportion of blooded mosquitoes which in the pooling for virus isolation were kept separate from non-blooded specimens.

As shown in TABLE 2, during the 8.5 years of the present study there were 44 mosquito-collecting sessions with a total of 329 working days. The average duration of a session was 7.5 days and total working time was 10% of the elapsed time.

Mosquitoes were identified shortly after collection and stored on solid carbon dioxide until processed in the laboratory for inoculation intracerebrally into infant mice. Pool sizes were usually no larger than 50 mosquitoes although this was occasionally increased to 100 when catches were very large.

During the present study a significant ecological transformation occurred in the study areas as a result of the development of the Reserve as a wild life sanctuary. Over this period the domestic animal and human populations were reduced to

TABLE 1. Number of mosquitoes, by species, tested for virus from Ndumu, 1960–68.

| Species | Total |
|---|---|
| *Anopheles* (*Anopheles*) *coustani* | 89 |
| " " *tenebrosus* | 149 |
| " " *ziemanni* | 62 |
| " (*Cellia*) *funestus* group | 314 |
| " " *gambiae* complex | 102 |
| " " *marshallii* | 13 |
| " " *pharoensis* | 74 |
| " " *rivulorum* | 23 |
| " " *squamosus* | 73 |
| " spp. indet. | 254 |
| *Ficalbia* (*Mimomyia*) *mimomyiaformis* | 3 |
| " spp. indet. | 5 |
| *Coquillettidia* (*Coquill.*) *aurea/wahlbergi* | 15 |
| " " *chrysosoma* | 17 |
| " " *fuscopennata* | 16 |
| " " *metallica* | 264 |
| " " *microannulata* | 9 |
| *Mansonia* (*Mansonioides*) *africana* | 30,099 |
| " " *uniformis* | 2129 |
| *Eretmapodites quinquevittatus* | 76 |
| " *silvestris* | 146 |
| " *subsimplicipes* | 7 |
| *Aedes* (*Mucidus*) *luciamus* | 120 |
| " " *sudanensis* | 276 |
| " (*Stegomyia*) *aegypti* | 42 |
| " " *calceatus* | 1 |
| " " *metallicus* | 39 |
| " " *simpsoni* | 18 |
| *Aedes* (*Aedimorphus*) *albocephalus* | 6242 |
| " " *cumminsii* | 3539 |
| " " *durbanensis* | 25 |
| " " *filicis* | 1 |
| " " *kennethi* | 253 |
| " " *leesoni/dentatus** | 1111 |
| " " *marshallii* | 14 |
| " " *minutus* | 159 |
| " (*Neomelaniconion*) *circumluteolus* | 91,443 |
| " " *lineatopennis* | 282 |
| " (*Dicrormyia*) *furcifer/taylori* | 247 |
| " (*Pseudarmigeres*) *natalensis* | 8 |
| " spp. indet. | 98 |
| *Culex* (*Lutzia*) *tigripes* | 57 |
| " (*Neoculex*) *horridus* | 6 |
| " " *insignis* | 290 |
| " (*Culiciomyia*) *nebulosus* | 1 |
| " (*Culex*) *annulioris* | 56 |
| " " *antennatus* | 628 |
| " " *guiarti* | 68 |
| " " *neavei*** | 25,434 |
| " " *perfuscus* | 1 |
| " " *pipiens* | 27 |
| " " *poicilipes* | 446 |
| " " *thalassius* | 16 |
| " " *theileri* | 27 |
| " " *zombaensis* | 2420 |
| " spp. indet. | 894 |
| Total | 168,231 |

*Both *leesoni* and *dentatus* occur at Ndumu but *leesoni* is by far the more prevalent. No attempt was made to differentiate these species.

**_neavei_ was previously referred to by us as _univittatus_.

insignificant numbers while there was a gradual increase in wild animals, particularly antelope.

## RESULTS

TABLE 1 gives the total number of females of each mosquito species tested for virus. *A. circumluteolus* constituted 54% of the total catch and this species, together with *M. africana* and *C. neavei*, composed 87% of the total. A further 4 or 5 species, *A. albocephalus*, *A. cumminsii*, *C. zombaensis* and *A. leesoni/dentatus*, in descending order of abundance, together made up a further 8%. Hence the remaining 43 or more species comprised only some 5% of the total.

From TABLE 2 it can be seen that 52 isolations, which included strains of 13 arboviruses, were made. The isolation of individual viruses on a time basis was irregular with no tendency towards an annual isolation pattern. For instance, 6 viruses, Ndumu, Spondweni, Germiston, Lebombo, Ingwavuma and Simbu were only isolated during a single year and another 4 viruses, Sindbis, Bunyamwera, Shokwe, and Mossuril only during 2 years. Even those viruses more frequently isolated such as Middelburg, Wesselsbron and Pongola showed a high proportion of years during which they failed to appear.

TABLE 3 shows the virus isolation frequency from the 5 species of mosquito found infected over the the whole period of study. As in the previous study of Worth et al. (1961) *A. circumluteolus* was revealed as the most frequently infected species. . produced 37 of the 52 isolations and all but 2 of the viruses were isolated at least once from this species. Nevertheless, considering the large numbe of specimens of this species tested, with a yearly average of about 10,000, the infection rate with each virus was low Even with Pongola virus the overall rate was 1 in 5000 which, considered together with the irregular appearance of this virus over the years, hardly justifies firm conclusions with regard to host specificity. A further factor tending to diminish the importance of *A. circumluteolus* as vector is that in 1968 when the entire collecting effort was directed towards this species, a total of 33,617 specimens produced only a single isolation of virus. The isolation frequency of different viruses from the remaining 4 species found infected was also low, and consequently with these species also there are no substantial indications of host specificity.

## DISCUSSION

The ..ppeara e of different viruses in the mosquitoes ··er the years of observation was quite intermittent. Even Pongola, the most frequently isolated virus, failed to appear during 4 years. This is in accord with the results of Worth et al. (1961) and after 13 years it must be concluded that there were no firm indications of enzooticity by any particular virus. Nor was there definite evidence of host specificity. Generally, the most frequently infected species were the most abundant and frequency of infection seemed more related to numbers of each species tested than to host specificity. This is the pattern of infection to be expected if the species concerned were incidental vectors or were merely carrying virus without necessarily transmit-

TABLE 2. Virus isolations, collecting sessions and days, total mosquitoes and number of *Aedes circumluteolus* tested, on an annual basis, from Ndumu, 1960–68.

| | | | | | | Virus isolations | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Group A | | | Group B | | Bunyam-wera | | | Ilwamba | Simbu | | Unc. | | |
| Year | No. sessions yearly | No. collecting days | Total mosq. | *A. circumluteolus* Total | *A. circumluteolus* % of Total | Sindbis | Middelburg | Ndumu | Wesselsbron | Spondweni | Bunyamwera | Shokwe | Germiston | Pongola | Ingwavuma | Simbu | Mossuril | Lebombo | Total |
| 1960 | 1 | 8 | 3557 | 3334 | 94 | | | 1 | | | | | | | | | | | 1 |
| 1961 | 3 | 30 | 14,720 | 10,834 | 74 | | | | | | | | | 2 | | | | 1 | 3 |
| 1962 | 5 | 42 | 30,678 | 18,397 | 44 | 3 | 4 | | 1 | 4 | | 2 | | 1 | 1 | | 1 | | 17 |
| 1963 | 5 | 44 | 29,670 | 10,167 | 34 | 1 | 1 | | | | | | | | | | 1 | | 3 |
| 1964 | 6 | 46 | 13,898 | 6456 | 46 | | | | 2 | | 4 | 1 | | 11 | | 1 | | | 19 |
| 1965 | 4 | 41 | 20,446 | 2877 | 14 | | 1 | | | | 1 | | | | | | | | 2 |
| 1966 | 2 | 14 | 4785 | 1135 | 24 | | | | | | | | | 4 | | | | | 4 |
| 1967 | 7 | 44 | 16,860 | 9626 | 57 | | | | 1 | | | | | 1 | | | | | 2 |
| 1968 | 11 | 60 | 33,617 | 33,617 | 100 | | | | | | | | 1 | | | | | | 1 |
| Totals | 44 | 329 | 168,231 | 91,443 | 54 | 4 | 6 | 1 | 4 | 4 | 5 | 3 | 1 | 19 | 1 | 1 | 2 | 1 | 52 |

TABLE 3. Frequency of isolation of different viruses from mosquito species at Ndumu, 1960–68.

| | VIRAL ISOLATIONS | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | GROUP A | | | GROUP B | | BUNYAMWERA | | | BWAMBA | SIMBU | | UNGROUPED | | |
| SPECIES | Sindbis | Middelburg | Ndumu | Wesselsbron | Spondweni | Bunyamwera | Shokwe | Germiston | Pongola | Ingwavuma | Simbu | Mossuril | Lebombo | TOTAL |
| *Aedes circumluteolus* | 2 | 1 | 1 | 3 | 4 | 4 | 1 | 1 | 18 | | 1 | | 1 | 37 |
| " *leesoni/dentatus* | | 3 | | | | | | | | | | | | 3 |
| " *cumminsii* | 1 | ? | | | | | 1 | | | | | | | 4 |
| *Mansonia africana* | | | | | | 1 | 1 | | 1 | | | | | 3 |
| *Culex neavei* | 1 | | | 1 | | | | | | 1 | | 2 | | 5 |

ting. There is therefore little evidence to implicate any species as a maintenance or primary vector.

Several possible explanations come to mind as to why the study failed to reveal definite enzooticity or host specificity but comment will be confined to only one possible reason. This concerns the question of adequate sampling among the entire mosquito fauna at Ndumu. Most collecting techniques used in a study of this kind tend to favor the prevalent species and there are valid reasons for the use of methods which yield large numbers of mosquitoes. However, should the prevalent species be relatively unimportant as vectors it seems quite possible that excessive emphasis on these species could result in important vectors among the rarer species being overlooked. Worth & Paterson (1961) listed 75 culicine species from Ndumu so the total of about 44 species tested for virus is only slightly more than 1/2 the culicine fauna. Furthermore, of those tested, 37 species were only tested in very small numbers. Therefore, it does seem possible that collecting methods used at Ndumu failed to yield sufficient numbers of the right species. It is among those species which exist continuously as adults, such as *Culex*, that maintenance vectors are most likely to occur and on this basis it would seem that efforts should be directed towards collecting more of such species as *C. horridus*, *C. insignis*, *C. antennatus*, *C. guiarti* and *C. perfuscus* which were barely sampled in the present study. These species may not be quite as rare as collections have hitherto indicated.

The frequency of infection observed in *A. circumluteolus* over several years strongly implicates this species as a vector, even if, as seems likely, this is only in an incidental capacity. Apart from infection in the field, experimentally it has been shown capable of transmitting Wesselsbron (Muspratt et al. 1957), Pongola, Bunyamwera and Spondweni viruses (unpubl. work) so it is highly probable that it is responsible for a significant amount of natural transmission. However, there are factors in its biology tending to diminish its importance as a vector. Its high feeding preference for the large mammal serves to decrease its chances of feeding on such hosts as rodents, birds, bats or reptiles which are the more likely viral hosts; during the long, dry season in Tongaland the adult population falls to such low levels that it is doubtful whether significant transmission by this species could occur at this time; although it appears in vast numbers at Ndumu it is largely restricted to the flood plains of the Usutu, Pongola and Ingwavuma rivers which would limit its importance over the remaining area of Tongaland; it is essentially a bush-haunting species so infection of man by it in a domestic environment is probably slight; and finally considering the large numbers tested, the 37 isolations from it divided among as many as 11 viruses, fail to implicate it fundamentally in the ecology of any particular virus.

Furthermore, during the last 18 months of the study there was evidence that *A. circumluteolus* was not becoming infected as frequently as before. From August 1967 through December 1968, 41,600 *A. circumluteolus*, comprising 45% of the total number of this species tested for virus, were collected. Yet during this period, involving 16 sessions with a total of 58 working days, only 1 strain of virus was isolated. A possible reason for this diminished infectivity was a major change in blood-feeding pattern by this species as a result of the transformation of the Reserve into a wild-life sanctuary which was complete by mid-1967. By this time precipitin tests on 1018 blood meals of *A. circumluteolus* revealed that 96% were of antelope origin (unpubl. work) whereas an earlier survey of 360 meals (Paterson et al. 1964) had indicated a more general host range. The conclusion seems justified that in areas where it occurs in large numbers *A. circumluteolus* is an im-

portant but incidental vector of several viruses and is probably not deeply involved in the survival of these viruses.

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J. Med. Ent. Vol. 12, no. 6: 637–640 20 February 1976

# *CULEX (EUMELANOMYIA) RUBINOTUS* THEOBALD AS VECTOR OF BANZI, GERMISTON AND WITWATERSRAND VIRUSES

## I. Isolation of virus from wild populations of *C. rubinotus*[1]

By B. M. McIntosh, P. G. Jupp, I. S. L. Dos Santos and G. M. Meenehan[2]

*Abstract:* From 19,226 *Culex (Eumelanomyia) rubinotus* collected in South Africa and Rhodesia over 3 years, 80 strains of Banzi virus, 74 of Germiston virus and 67 of Witwatersrand virus were isolated. It is concluded that the regularity of infection in different geographical areas over this period and its frequency among individuals of several different natural populations indicate that *C. rubinotus* is a maintenance vector of all 3 viruses. Four isolations of Arumowot virus and 2 of a Bunyamwera-like virus were also made from *C. rubinotus*.

In this series of papers evidence is presented which we believe strongly implicates *Culex rubinotus* as a maintenance or primary enzootic[3] vector of Banzi (BAN), Germiston (GER) and Witwatersrand (WIT) viruses among wild rodents in southern Africa.

In 1958 isolations of BAN, GER and WIT viruses were made from pools of *Culex rubinotus* alone and a mixed pool of *C. rubinotus* and *Cule. theileri* collected at localities near Johannesburg on the inland, temperate plateau region of South Africa (Smithburn et al. 1959, Kokernot et al. 1960, McIntosh et al. 1960).

This was the first implication of *C. rubinotus* as a possible viral vector and the first isolations of GER and WIT viruses. If it is assumed, as now seems likely, that *C. theileri* in the single mixed pool was not implicated, infection rates in *C. rubinotus* were high, as only 89 *C. rubinotus*, grouped into 6 pools, yielded 1 strain of BAN virus, 2 of GER and 1 of WIT. Although attempts were made shortly afterwards to obtain further specimens of *C. rubinotus* in the environs of Johannesburg, these were unsuccessful, and the apparent rarity of the species there and elsewhere in South Africa seemed confirmed by the collection of only occasional individuals for several years thereafter.

Indeed, it was not until 1971 that *C. rubinotus* was again encountered in some numbers. This occurred at Nyabira, 40 km north of Salisbury, on the Rhodesian highlands. On this occasion 563 *C. rubinotus* tested in 15 pools yielded 3 isolations of BAN, 5 of GER and 3 of WIT viruses. This 2nd episode involving the same 3 viruses aroused suspicions that *C. rubinotus* was deeply concerned in the transmission of these viruses and further efforts were made to obtain more specimens. Success was achieved the following summer at Port Shepstone, on the coast of the province of Natal, South Africa, in estuarine habitats and once again high infection rates with the same viruses were demonstrated. Shortly afterwards the same viruses were isolated from *C. rubinotus* populations in lake habitats on the coastal plain of northern Natal.

The existing situation, therefore, is that whenever wild populations of *C. rubinotus* in southern Africa have been sampled for infection, at least 1 and frequently all of these viruses have been isolated. The nature of these isolations led us to investigate other aspects of the ecology of these viruses, including their transmission by *C. rubinotus*, the biology of this mosquito, the exposure of hamsters as virus sentinels among wild populations of *C. rubinotus*, and attempts at determining the natural vertebrate hosts, which are described in the companion papers (Jupp et al. 1976, McIntosh et al. 1976a, b).

### METHODS

Females of *C. rubinotus*, as well as other species, were caught by a variety of collecting methods which included the following: (1) portable light traps; (2) the same traps with carbon dioxide as additional attractant; (3) test tube collections off human bait; and (4) net traps baited either with carbon dioxide, goat or sheep.

Mosquitoes were killed, identified and stored under refrigeration on the day of collection. Storage was either on solid carbon dioxide or liquid nitrogen. Mosquitoes were processed in the laboratory, and the suspensions inoculated intracerebrally into infant mice, 2–3 days old. Attempts were made to keep mosquito pool sizes as uniform and small as possible but since numbers of mosquitoes in pools varied,

[1]The studies and observations on which this paper is based were financed jointly by the South African Institute for Medical Research, the Poliomyelitis Research Foundation and the State Health Department.

[2]Arbovirus Research Unit, South African Institute for Medical Research, Johannesburg, South Africa.

[3]These terms are used synonymously in the sense that Smith (1964) defined "maintenance host," i.e., a host that "is essential for the continued existence of a virus," without any claim that we can explain the precise survival mechanism of these viruses in nature.

infection rates are expressed merely as averages per 1000 mosquitoes.

Virus isolates were identified by their immunological reactions with immune sera to the prototype strains of BAN, GER and WIT viruses, viz., H336, AR1050 and AR 1062, respectively. With some BAN and GER isolates, reactions to closely related viruses were also studied. All isolates were tested in complement-fixation tests using an antigen of the isolate prepared from brains of sick infant mice and prototype strain immune ascitic fluids obtained by the hyperimmunization of adult mice. With some isolates, complement-fixation reactions were confirmed by neutralization tests conducted in mice and/or hemagglutination-inhibition tests using immune sera prepared in guinea pigs or monkeys by a single inoculation of virus.

Evidence was obtained indicating that some mosquito pools actually contained more than 1 virus. In these cases no attempt was made to identify the other virus, since too few pools were concerned to have justified the work involved.

## RESULTS

TABLE 1 shows the number of isolations of BAN, GER and WIT viruses effected from *C. rubinotus* collected at Nyabira, Rhodesia and at 6 localities in Natal Province, South Africa. These collections took place during 10 collecting sessions involving 72 collecting days spread over 3 years, from April 1971 through February 1974.

It can be seen that there was a remarkable regularity of isolation of each virus with regard to locality as well as collecting session. The localities varied ecologically from the Rhodesian highlands, in tropical savanna, to the subtropical coastal plains of Natal, some 1600 km southwards. At the single collecting session in Rhodesia and at each of the 3 at Port Shepstone all 3 viruses were consistently isolated. At these localities, where only moderate numbers of *C. rubinotus* were present, infection rates were always at levels we regard as high, considering that enzootic viral activity was probably involved. They varied from the lower value of 5.3 with BAN and WIT viruses in Rhodesia to 24.0 per 1000 mosquitoes with BAN at Port Shepstone.

The study sites of Kwambonambi, Lake Teza, Monzi, St. Lucia estuary and Lake Insese are located close to each other on the coastal plain of northern Natal and, with the exception of Kwambonambi and Monzi, are on the edges of large permanent lakes and marshes where conditions are apparently eminently favorable for *C. rubinotus*, since extremely high population densities were encountered there.

At these localities there was some irregularity in isolation of virus, and lower infection rates were present compared with those in Rhodesia and Port Shepstone. Nevertheless, it is evident that infection rates were still significantly high and that all 3 viruses were associated with *C. rubinotus* on much the same scale and manner as in the other 2 areas.

From a total of 19,226 *C. rubinotus* from all localities and tested in 1004 pools, 80 strains of BAN, 74 of GER and 67 of WIT viruses were isolated.

In addition to these isolations, 4 isolations of Arumowot virus were made from *C. rubinotus* collected in Natal. Two isolations occurred in 1972 at Port Shepstone and 2 in 1973 at Lake Teza. Two further strains of virus isolated from *C. rubinotus* collected at Nyabira have been tentatively classified as Bunyamwera virus.

At all these collecting sessions several thousand mosquitoes belonging to many species were collected and also tested for virus. With regard to BAN, GER and WIT viruses the outcome of all this was the isolation from *Culex poicilipes* collected at Nyabira

TABLE 1. Virus isolations, with infection rates, from *Culex rubinotus*, at various localities.

| Locality | | Date of collections | No. collecting days | Total mosq. | No. mosq. pools | Mean pool size | Virus isolations: BAN | | Infection rates GER | | WIT | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Nyabira | Rhodesia | Apr. 1971 | 4 | 563 | 15 | 50 | 3 | 5.3* | 5 | 8.9 | 3 | 5.3 |
| Port Shepstone | S. Africa | Feb. 1972 | 9 | 603 | 63 | 10 | 10 | 16.6 | 11 | 18.2 | 5 | 8.3 |
| | | Apr. 1972 | 9 | 812 | 167 | 5 | 8 | 9.8 | 12 | 14.8 | 13 | 16.0 |
| | | Feb. 1974 | 20 | 1374 | 169 | 8 | 33 | 24.0 | 9 | 6.6 | 20 | 14.6 |
| Kwambonambi | " " | Dec. 1972 | 7 | 145 | 30 | 5 | 1 | 6.9 | | | | |
| Lake Teza, Matubatuba | " " | Jan. 1973 | 6 | 6567 | 254 | 25 | 15 | 2.3 | 14 | 2.1 | 13 | 1.9 |
| Monzi, Matubatuba | " " | Jan. 1973 | 2 | 461 | 20 | 25 | 1 | 2.2 | 1 | 2.2 | 1 | 2.2 |
| St. Lucia Estuary | " " | Jan. 1973 | 3 | 2078 | 85 | 25 | 1 | 0.5 | 1 | 0.5 | | |
| Lake Insese, Empangeni | " " | Apr. 1973 | 2 | 1695 | 106 | 25 | | | 7 | 3.7 | 7 | 3.7 |
| | | May 1973 | 10 | 4728 | 101 | 15 | 8 | 1.7 | 14 | 3.0 | 5 | 1.1 |
| Total | | | 72 | 19,226 | 1004 | 20 | 80 | 4.5 | 74 | 3.8 | 67 | 3.5 |

*5.3 per 1000 mosquitoes.

of 1 strain each of BAN and GER virus and a strain of GER virus from *Culex pipiens* collected at Lake Inseze in May 1973.

## DISCUSSION

When wild populations of maintenance vectors are sampled for infection, the nature of infection revealed is likely to be regular in space and time and of a high frequency among individuals of these populations. The nature of infection by BAN, GER and WIT viruses shown to exist among *C. rubinotus* populations in our studies conforms to this concept and suggests, therefore, that *C. rubinotus* is maintaining these viruses. In support of this viewpoint, we present evidence in the companion papers (Jupp et al. 1976, McIntosh et al. 1976a, b) concerning the vector capability and biology of *C. rubinotus*, as well as evidence of infection in the probable vertebrate host. Underlining these positive aspects is the large volume of virtually negative evidence implicating other species of mosquitoes, many of which were tested for virus at a time and place when infection was shown to be present in *C. rubinotus*.

It is almost certain that the viral activity uncovered in the present study concerns the wild transmission cycles, involving *C. rubinotus* and wild rodents. There was no evidence at the time that human beings were ever involved with the 2 viruses known to be pathogenic for man. At Port Shepstone man rarely entered the rather restricted and inaccessible habitats to which *C. rubinotus* was apparently confined and in any case *C. rubinotus* seems not to feed on man in this area. At Port Shepstone also, domestic animals were virtually absent from the main collecting site. Furthermore, blood meals of *C. rubinotus* collected at Port Shepstone and in northern Natal at the time infection was demonstrated in *C. rubinotus* were predominantly of rodent origin and evidence showing that wild rodents are infected with these 3 viruses is produced in the following paper (McIntosh et al. 1975a).

Infection rates were consistently higher at Port Shepstone than in the northern Natal localities. Under natural conditions rates can obviously be expected to vary geographically and factors causing the difference in this instance seem likely to have been the relative availability of rodents and the age and size of vector populations, both of which would have tended towards the lowering of rates in northern Natal. In the latter area the natural habitat has been destroyed on a far greater scale by sugarcane cultivation than in the Port Shepstone area and, as a result, it is likely that rodent numbers were higher at Port Shepstone. However, probably of greater effect is the vast extent of suitable aquatic habitats in northern Natal which ensured the presence there of much larger and younger *C. rubinotus* populations.

The similarities in *C. rubinotus* infection rates by the 3 viruses were remarkable. It is perhaps the type of situation which could be expected to occur in transmission cycles in which the same vector, transmitting immunologically distinct viruses with somewhat equivalent efficiency, was the principal means of transmission between the same or a limited number of vertebrate species. Because there is no immunological overlap between the 3 viruses, which *C. rubinotus*, according to our observations, apparently transmits at a somewhat comparative level of efficiency, and because the number of rodent species in any one area is generally limited, this might well be the explanation for this similarity in infection rates.

The survival of virus through the dry winter occurring in southern Africa was not investigated. This is still largely an unsolved problem with even well-studied arboviruses, and its solution in the case of BAN, GER and WIT viruses is likewise unlikely to be achieved without considerable effort and expertise. There are indications, however, that these viruses are maintained by a continuous cycling of virus between vector and rodents during the summer, perhaps with lowered or temporarily suspended transmission during the winter. There are factors present that promote prolonged transmission. That the vector is a *Culex* breeding in permanent waters suggests that for most if not the whole year, adults would be active. Moreover, the protected and humid nature of the *C. rubinotus* habitat suggests that even an extended dry season might not entirely eliminate adult *C. rubinotus*. The fact that all 3 viruses were isolated from sentinel hamsters exposed at Mopeia in Mocambique during the dry season (McIntosh et al. 1975a) shows that in that area, at least, transmission occurred at this time, and there is no reason to exclude *C. rubinotus* as vector of these infections.

Until more information is available on the distribution of these viruses and *C. rubinotus*, the extent of the geographical area in which *C. rubinotus* is maintaining these viruses must remain unknown. At least in southern Africa, a large area encompassing markedly different ecological zones is involved. The isolation of GER and WIT viruses from *C. rubinotus* in Uganda (Henderson et al. 1972) and of BAN virus from *C. rubinotus* in Kenya (Metselaar et al. 1974) suggests that *C. rubinotus* is a vector over a large part of Africa.

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J. Med. Ent. Vol. 12, no. 6: 647–651 20 February 1976

# *CULEX (EUMELANOMYIA) RUBINOTUS* THEOBALD AS VECTOR OF BANZI, GERMISTON AND WITWATERSRAND VIRUSES

## IV. Observations on the biology of *C. rubinotus*

By P. G. Jupp, B. M. McIntosh and D. Anderson[1]

*Abstract:* Because of its importance as a vector of viruses, a study was made on aspects of the biology of *Culex rubinotus* in southern Africa. Catches showed that this species is prevalent in the subtropical coastal plain of Natal where the adult is commonly found within dense marshland and adjoining sugarcane fields. Larvae were also found in reed beds. The active female is ground-frequenting, occurring rarely in the tree canopy. Comparative animal-baited catches and precipitin tests on natural blood meals showed it to feed primarily on wild rodents and to a moderate extent on domestic ungulates. Different geographical populations differed in their feeding preferences for man, varying from very low to moderate. It is concluded that *C. rubinotus* has biological characteristics consistent with the viewpoint that it is the maintenance vector of Banzi, Germiston and Witwatersrand viruses among wild rodents. The other principal mosquito species collected are briefly mentioned.

When it seemed from the high infection rates of Banzi, Germiston and Witwatersrand viruses detected in wild populations of *Culex rubinotus* that this mosquito is an important viral vector (McIntosh et al. 1976), the need arose for information on its biology. Neither in South Africa nor apparently elsewhere in Africa has this mosquito been collected frequently and as a result little has been recorded of its habits (Muspratt 1955, Worth & de Meillon 1960). For a long time, despite much mosquito collecting, *C. rubinotus* was regarded as a rare species in southern Africa and it was only in 1971 in Rhodesia that fair numbers were first encountered by us. High virus infection rates in these mosquitoes led to searches for *C. rubinotus* in South Africa and this resulted in the finding of large populations in the subtropical coastal plain of Natal Province where most of the present studies on its biology were made.

The observations reported here on the biology of *C. rubinotus* were made either as a result of collections intended primarily for virus studies or from collections designed specifically to obtain information on biology. They include observations on geographical distribution, habitat association, prevalence, host preferences and the vertical distribution of active females.

### METHODS

*Study sites*

Observations were made at 6 localities between March 1971 and March 1974. The first of these was Nyabira, 40 km north of Salisbury, Rhodesia, where collections were made in open grassland in a small swampy area containing rushes and rank grass. The others were in South Africa. At the Port Shepstone area in southern Natal, the sites utilized were in estuarine marshes along the Boyboyi, Izotsha and Mtentweni rivers, while in the coastal plain of northern Natal collecting sites were mostly on the edge of large riverine lakes. These were Lake Teza, near Mtubatuba, with an area of about 200 ha, Lake Insese, near Empangeni, of about 300 ha and the area of marshland at St. Lucia estuary which is even larger. Monzi, the remaining locality, is situated 16 km east of Mtubatuba and is a much smaller marsh. The area around Lake Teza is under intense sugarcane cultivation and most of the observations at this locality were made on the eastern side of this lake.

*Mosquito collecting*

(1) *Light-$CO_2$ trap:* This consisted of a portable battery-operated light trap with carbon dioxide as additional attractant. The trap was hung so that the entrance was about 1 m above the ground. The $CO_2$ was released through 3-mm bore polythene tubing close to this opening. Source of the $CO_2$ was a tin insulated with corrugated cardboard containing about 2 kg of solid $CO_2$. The mosquitoes, sucked downwards into the collecting cage, were mainly blood-seeking females, but also collected were some replete or gravid females and some males.

(2) *Net trap:* This trap was set overnight baited either with a goat or a sheep or 3 kg of solid $CO_2$. The animal was restrained within a wire cage.

(3) *Lard-can type trap:* This is based on the lard-can trap designed by Bellamy et al. (1952) but is smaller, being 48 cm long and 37 cm square in cross section. These traps with various animal species as bait were set overnight on or just above the ground. Bait animals were enclosed in wire cages of 12-1/2 mm mesh except for snakes which were enclosed by 10-1/2 mm mesh. The following animals were employed: 3 rodents (*Saccostomus campestris*), 3 fruit bats (*Rousettus aegyptiacus*), 1

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TABLE 1. Relative activity of *C. rubinotus* in different habitats according to light-$CO_2$ traps.

| | Site | | | | | |
|---|---|---|---|---|---|---|
| | | | 200 m from lake | | 500 m from lake | |
| | Inside reeds | Reeds' edge | Open track | Sugarcane | Open track | Sugarcane |
| Total mosquitoes collected | 605 | 1186 | 300 | 1231 | 232 | 367 |
| No. trap-nights | 10 | 11 | 8 | 9 | 7 | 5 |
| Mw* | 43 | 85 | 33 | 119 | 25 | 70 |

*Mw = Williams' mean.

domestic pigeon (*Columba livea*), 2 brown house snakes (*Boaedon fuliginosus*) and 5 toads (*Bufo carens*).

(4) *Human-baited catches:* These were undertaken with 2 or more catchers. Each man operated alone, using a torch darkened with a red hood, and collected mosquitoes as they alighted in test tubes. Collections started at sunset and lasted for up to 2 hr.

*Analysis of results*

The male mosquitoes collected were not included in the results. For the series of catches made with light-$CO_2$ traps in different habitats (TABLE 1), mean catches are expressed as Williams' modification of the geometric mean (Mw) as recommended by Haddow (1960). In the catches made in the lard-can type traps (TABLE 2), the proportion of engorged mosquitoes is expressed as the arithmetic mean of the percentages engorged from all catches of each series. Relative attraction for human bait is expressed as the number of mosquitoes biting per man-hour.

*Precipitin test*

The method used for the preparation of antisera for the test and the technique employed for the test were as described by Anderson (1967).

## RESULTS

*General observations*

Early in the studies it became apparent that unless catches were made within or on the immediate periphery of its characteristic habitat, appreciable numbers of *C. rubinotus* would not be collected. This reluctance of *C. rubinotus* to leave its habitat was strikingly demonstrated at Port Shepstone when a net trap set only about 10 m from the edge of a reed bed caught 1.5 *C. rubinotus* per trap-night, whereas when it was moved to within the adjacent reed bed 54 per trap-night were taken. However, it was subsequently observed at the study sites in northern Natal that when *C. rubinotus* occurred in very large numbers there was a tendency for it to also occur at some distance from its usual habitat, particularly where cultivated cane was present.

The preferred habitat of *C. rubinotus* was found to be marshland associated with permanent waters such as lakes, rivers or streams. A dominant, perhaps essential feature, is the dense tall stand of reeds (*Phragmites* spp.), papyrus (*Cyperus papyrus*) or rushes or in less moist situations, of rank grasses. Forest, wooded savanna and dry grassland seem unsuitable, and the species is thus very localized despite its occurrence in zones of markedly different ecologic type, such as coastal lowland and inland plateau.

Numbers of *C. rubinotus* collected on windy nights were extremely low. For example, a mean catch of 500 mosquitoes in light-$CO_2$ traps 1 night fell to only 12 mosquitoes the following night which was windy, while other mosquito species were collected in almost undiminished numbers. Relative to other species, it was also observed that *C. rubinotus* died quickly after collection. These observations, together with its reluctance to leave its habitat, seem to suggest that this species is unusually sensitive to low humidities and is perhaps also a weak flyer.

Although *C. rubinotus* was reared in an insectary to the adult stage from gravid, wild-caught females, difficulty was experienced in passing it through the larval stage; emerged adults failed to feed on a hamster and soon died. From this it seems that

TABLE 2. Total numbers and mean percentage fed of *C. rubinotus* collected in lard-can type traps with various animal bait.

| Type of bait | No. trap nights | Total mosq. collected | Mean % fed |
|---|---|---|---|
| Rodent | | | |
| *Saccostomus campestris* | 17 | 232 | 30 |
| Pigeon | | | |
| *Columba livea* | 17 | 17 | |
| Bat | | | |
| *Rousettus aegyptiacus* | 11 | 14 | |
| Snake | | | |
| *Boaedon fuliginosus* | 5 | 0 | |
| Toad | | | |
| *Bufo carens* | 5 | 0 | |
| Control | | | |
| (empty) | 2 | 0 | |

TABLE 3. Relative attraction of *C. rubinotus* to . .an according to human-bait catches at 3 localities.

| | Rhodesia, Nyabira | Natal, Port Shepstone | | Natal, Lake Teza | |
|---|---|---|---|---|---|
| | Apr. 1971 | Feb. 1972 | Apr. 1972 | Jan. 1973 | Mar. 1974 |
| Total mosquitoes collected | 510 | 1 | 11 | 10 | 5 |
| No. of man-hours | 45 | 7.5 | 15 | 4 | 2 |
| No. mosquitoes per man-hour | 11 | <1 | <1 | 2.5 | 2.5 |

colonization of this species would be difficult.

In regard to the efficacy of the various collecting methods, it was evident that goat-baited net and light-$CO_2$ traps were very effective and a high proportion entering the net traps fed on the goat. Light traps without $CO_2$ yielded poor catches, as did animal-baited lard-can traps at Port Shepstone where populations were low.

Larvae of *C. rubinotus* were found in shallow water in reed beds at Lake Teza and in reed beds on the edge of the Boyboyi River near Port Shepstone.

*Relative prevalence at various localities*

Catches in light-$CO_2$ traps and/or baited net traps set at various localities in Natal to collect female mosquitoes for virus studies yielded information on the prevalence of *C. rubinotus*. These showed that it occurred at high densities at Lake Teza, Lake Insese and St. Lucia estuary, all of which are large marshes, but at only moderate densities at Port Shepstone and Monzi where study sites were located in much smaller marshes. For example, collections in light-$CO_2$ traps at Lake Teza yielded a mean catch of 336 *C. rubinotus* per trap-night, while the same trap at Port Shepstone yielded a mean of only 8. High densities at Lake Insese were also demonstrated by the collection there in goat-baited net traps of about 1000 *C. rubinotus* per trap-night. The conclusion drawn from these collections is that *C. rubinotus* is widely distributed along the entire coastal zone of Natal, although confined to specific habitats within this zone, and that it occurs in vast numbers in the larger marshes.

At Lake Teza more than 20 species of mosquitoes other than *C. rubinotus* entered the light-$CO_2$ traps. The 4 most prevalent of these species were as follows: *Culex* (*Culex*) *thalassius* and *Mansonia* (*Mansonioides*) *africana*, which were collected in about equal numbers with *C. rubinotus*; *Anopheles* (*Anopheles*) *tenebrosus* at twice this level; and *Mansonia* (*Mansonioides*) *uniformis* which was about 18 times more abundant. The occurrence and relative proportions of these species were very similar at the other Natal localities.

*Activity of females in different habitats*

A study was undertaken on a sugar farm bordering Lake Teza in which light-$CO_2$ traps were set in different habitats at varying distances from the lake to investigate to what extent *C. rubinotus* activity is influenced by habitat. The habitats chosen were as follows: (1) deep within the reed bed where the water was about 30 cm deep; (2) ashore at the extreme edge of the reed bed; (3) & (4) within 1.8-m high sugarcane at distances of 200 m and 500 m, respectively, from the lake; and (5) & (6) in open breaks consisting of tracks 3–4 m wide at distances of 200 m and 500 m, respectively, from the lake. The numbers and geometric means of *C. rubinotus* collected in these habitats are given in TABLE 1. It can be seen that *C. rubinotus* was more active on the edge of the reed bed than deeper in and even greater activity was present in cane 200 m from the lake's edge and was still high at 500 m. Activity was lowest in the open breaks.

*Vertical distribution*

A series of catches was made with light-$CO_2$ traps over 6 nights at Lake Teza to compare *C. rubinotus* activity at ground level and at 7.5 m in the canopy of a tree. The total number of *C. rubinotus* collected at ground level was 698, while in the canopy it was 30.

*Host preferences*

To determine the host preferences of *C. rubinotus* among various classes of wild animals, a series of catches was made at Lake Teza using lard-can traps baited with 3 rodents, a pigeon, 3 bats, 2 snakes or 5 toads. The results of these catches are shown in TABLE 2. It can be seen that the rodents attracted most *C. rubinotus*, with a total of 232 mosquitoes, of which 30% had engorged. Blood meals from 10 of the mosquitoes were identified as being of rodent origin with the precipitin test. Small numbers were attracted to the pigeon and bats and none to the other animals.

With respect to other mosquito species, *M. uniformis* was attracted to and fed on rodents, bats and pigeons, *M. africana* on bats and, to some extent, on rodents and *C. thalassius* on bats. Of interest also were 2 freshly engorged mosquitoes collected in the toad-baited traps—1 *Ficalbia* (*Mimomyia*) *lacustris* and 1 *Uranotaenia pallidocephalus*.

In regard to the preference of *C. rubinotus* for rodents, it was also observed that it fed readily on hamsters placed on the ground. In a series of catches with hamster-baited lard-can traps set over 8 nights, an average of 68 *C. rubinotus* was taken nightly and 80% of the total catch was engorged.

At 3 localities, viz., Nyabira, Rhodesia, and Port Shepstone and Lake Teza, South Africa, catches were made using man as bait. As shown in TABLE 3, on no occasion were large numbers collected, although at Nyabira a fair degree of attraction to man was evident. At the other 2 localities, numbers collected were extremely low considering that the catches took place in reed beds and, at Lake Teza, among very high population densities. Thus, although different geographical populations of *C. rubinotus* apparently vary in their preference for man, at its highest level this preference is only moderate.

The blood meals of 174 engorged *C. rubinotus* collected in $CO_2$-baited traps at 7 localities in Natal were tested with the precipitin test to determine host species. With 140 meals, antisera to rodent, goat/cow, man, bird, rabbit, cat, dog, pig and horse were used in the test, while for the other 34 meals only rodent, goat/cow and human antisera were used. The percentages of meals reacting positively with the different antisera were as follows: rodent 66%, goat/cow 9%, man 3%, bird 0.6%, pig 0.6%, and unidentified 16%.

At 6 localities the percentage of meals reacting with rodent antiserum far exceeded all other reactions, ranging from 65% to 88%. The single exception occurred among 36 meals collected at Lake Insese, where large numbers of goats and cattle were present and in which 36% reacted with goat/cow antiserum and only 30% with rodent. Among the 16% of unidentified meals were probably bloods from wild vertebrates for which antisera were not available.

## DISCUSSION

Contrary to previous experience, the studies showed that in Natal, at least, *C. rubinotus* is a very prevalent mosquito indeed, and within its habitat is widely distributed on the coastal plain. The discovery of such large populations was unexpected, as to us it had seemed unlikely that a species existing in such large numbers could have escaped notice for so long. It seems that the failure to locate these populations earlier can be attributed to the rather spatially restricted occurrence of *C. rubinotus* combined with an omission by collectors to operate within or on the immediate peripheral of the *C. rubinotus* habitat.

This habitat is quite characteristic and presumably provides the sheltered environment which *C. rubinotus* apparently requires. It is available on an abundant scale in the numerous estuaries and riverine lakes present along the coast of Natal. The shallow and calm water present in this habitat is almost certainly the principal larval habitat. Cultivated sugarcane also provides a suitable environment for adults at some distance from the natural habitat.

Collections indicated that *C. rubinotus* showed a tendency to avoid far-ranging flights which would entail leaving the dense vegetation and high humidity of its habitat. It seems to be exceptionally sensitive to low humidities and presumably the presence of its preferred host within or close to its habitat eliminates the necessity for such flights.

The evidence on host preferences clearly showed that *C. rubinotus* is primarily a rodent feeder and that feeding is at ground level. It will also feed to a lesser extent on goats and sheep and presumably on other large ungulates and to a small extent also on birds and bats, but the paucity of active females at the higher vertical levels suggests that natural feeding on bats and tree-roosting birds is probably insignificant.

There were indications that while the degree of anthropophilism was generally low it varied among different geographical populations. On the inland plateau of southern Africa, viz., Salisbury, Rhodesia and apparently at Johannesburg, South Africa (Kokernot et al. 1960), it was attracted fairly readily to man. On the coastal plain of Natal Province, however, it showed a very low preference for man. Nevertheless, because of its prevalence in cultivated sugarcane in Natal it seems that it might feed significantly on man in cane fields because of the practice of cane-cutters to start work well before sunrise.

Because of its prevalence, host preferences and feeding habits, it is evident that *C. rubinotus* possesses biological characteristics consistent with its probable role as a vector of rodent-associated arboviruses.

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*J. ent. Soc. sth. Afr.* 1987
*Vol.* 50, *No.* 2, *pp.* 393–397

# Isolations of Middelburg virus from *Aedes (Ochlerotatus) juppi* McIntosh (Diptera: Culicidae) suggestive of a reservoir vector

by

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Seventeen alphavirus isolates (16 Middelburg and 1 Sindbis) were obtained from 3957 *Aedes juppi* mosquitoes collected at a single site on a farm at Tweespruit in the south-western Orange Free State Highveld. As collections were made only one month after the first significant rain of an unusually dry summer, these mosquitoes were thought to be the first brood of the season and the appearance of Middelburg virus due to the presence of transovarially infected mosquitoes. This could indicate that *Ae. juppi* serves as a reservoir vector to carry this virus through dry inter-epizootic periods.

## INTRODUCTION

As part of a long term study on the ecology of Rift Valley fever virus in South Africa, the Arbovirus Unit has been investigating floodwater aedine mosquitoes to ascertain whether they act as reservoir vectors of this virus during dry inter-epizootic periods. Floodwater *Aedes* deposit their eggs in the flood plains of streams and rivers as well as at the water's margin in pans. Pans are natural shallow depressions in grassland with no outlets which are not associated with a river drainage system. They are seasonally flooded by rainfall which causes the eggs to hatch and develop into adult mosquitoes. Subsequently the pans dry up mainly through water evaporation. In a project recently completed on farms in the Tweespruit area of the Orange Free State province, 20 000 eggs of *Aedes (Ochlerotatus) juppi* McIntosh were successfully recovered from soil samples, mainly from those samples taken in pans as opposed to vleis (swamps) (Gargan, Novak and Jupp, unpublished). These eggs were collected during the winter in August, 1984, when pans and vleis were dry. However, as no virus of any kind were isolated from mosquitoes reared from them, evidence implicating *Ae. juppi* as a transovarial vector was not obtained.

A follow-up visit was made to the same Tweespruit farms at the end of March, 1985, a month after the fist significant summer rains had fallen. One of the objectives of this visit was to trap adult floodwater *Aedes*. It was hoped to make isolations of Rift Valley fever virus from *Ae. juppi* mosquitoes which represented what was thought to be the first brood of the summer, and to take live mosquitoes back to the laboratory for vector competence experiments. On arrival it was found that the rains had been inadequate to affect any of the pans which remained devoid of any standing

water although the inundated area of the vleis and a dam had increased slightly. Surprisingly, moderately large numbers of *Ae. juppi* were taken in bait collections, the majority of which were subsequently assayed for virus with the isolation of the alphaviruses Middelburg (MID) and Sindbis (SIN). These isolations and their significance form the topic of this paper.

## METHODS

Tweespruit is located at the south-western edge of the Highveld region 29° 11' 27° 0: the Orange Free State Province. The term Highveld refers to the high inland plateau region with an altitude of from 900–1 600 metres. It is grassland with a temperate climate and summer rains; mean annual temperature is 13–17 °C. Pans are plentiful in the Tweespruit area but they have been empty since about the summer of 1980 owing to below average rainfall.

Mosquito collecting was carried out on the farm Prospect from 26–28th March, 1985, using three different methods. These were net-traps (Jupp and McIntosh 1967) and NIV light traps (Jupp 1986), both baited with solid carbon dioxide and set overnight. Thirdly, human-baited catches were carried out from late afternoon till dark by two operators using battery-operated aspirators. One person acted as bait and the other collected the mosquitoes which alighted on him to bite. All the mosquitoes were taken at one site near a dam and adjacent vleis, the margins of which had experienced slight flooding from rainfall at the end of February.

Mosquitoes were segregated according to species and feeding status and placed into pools which were stored in liquid nitrogen. Pool size was 100 unless the total number of a species collected was less. Subsequently, suspensions of the pooled insects were inoculated intracerebrally into 2-day-old suckling mice in attempts to isolate virus. Brain from sick mice was used for passage of virus in mice and as a source of virus for identification. Antigens were prepared by the sucrose-acetone method described by Clark and Casals (1958). Virus isolates were identified by complement-fixation tests in which hyperimmune mouse ascitic fluids were used as a source of known antibody. These tests were done according to the method of Bradstreet and Taylor (1962) adapted to a micro technique using 0,025 ml volumes.

The statistical mosquito infection rate was calculated by the method of Chiang and Reeves (1962).

## RESULTS

Table 1 shows the spring and summer rainfall which occurred up to the end of March when mosquitoes were collected. Table 2 shows the numbers of the 4 floodwater aedine mosquito species collected which were tested for virus and gives details of the isolations made. Smaller numbers of *Culex* and *Anopheles* were also collected but these were discarded. Sixteen isolations of MID virus and one of SIN virus were obtained from 3957 *Aedes juppi* which accounted for 98,5% of the total *Aedes* catch. Two *Aedes (Ochlerotatus)* and two *Aedes (Neomelaniconion)* species were present in the collections. The minimum infection rates with MID virus for the total *Ae. juppi* catch was 4,04 per 1 000 mosquitoes while it was 3,85 for the unengorged *Ae. juppi*. The statistical infection rate for unengorged *Ae. juppi* was 4,84.

TABLE 1. Spring–summer rainfall on a Tweespruit farm until mosquito collecting at end of March, 1985

| | Dates | No. mm. |
|---|---|---|
| October | 5, 11 | 38 |
| | 24, 29, 31 | 28 |
| November | 15 | 13 |
| | 29 | 42 |
| December | 19, 21 | 28 |
| January | 16, 17, 22 | 47 |
| February | 7, 8 | 21 |
| | 20, 23, 27, 28 | 82 |
| March | 11–15 | 45 |
| | 25, 26 | 19 |

TABLE 2. Floodwater *Aedes* collected at Tweespruit in March 1985: number of mosquitos tested for virus and number of viruses isolated.

| Species | No. Tested | No. Pools | Pool Size | Virus Isolates | | | |
|---|---|---|---|---|---|---|---|
| | | | | SIN | MID | | |
| | | | | No. | No. | MIR[a] | IR[b] |
| Ae. (Och.) juppi | 3900 | 39 | 100 | 1 | 15 | 3,85 | 4,84 |
| | 20 | 1 | | | | | |
| | 37[c] | 1 | | | 1 | | |
| | 3957 | 41 | | 1 | 16 | 4,04 | |
| Ae. (Och.) caballus | 20 | 1 | | | | | |
| Ae. (Neo.) unidentatus | 12 | 1 | | | | | |
| Ae. (Neo.) albothorax | 28 | 1 | | | | | |

a=minimum infection rate = no infected mosquitoes/1000.
b=statistical infection rate/1000 calculated by method of Chiang and Reeves (.962).
c=engorged mosquitoes.

## DISCUSSION

In 1973 McIntosh described *Ae. juppi*, a new species very similar to *Ae. caballus*. Hence collections of mosquitoes made before that date and called *Ae. caballus* may have consisted only of one of the species or they may have been mixed so they should be referred to as *Ae. caballus sensu lato*.

The infection rate of *Ae. juppi* with MID virus is less than that of the *Ae. caballus sensu lato* collected in the Karoo region at Middelburg, Cape, in 1957 when MID vi-

rus was first identified as a new virus (Kokernot *et al.* 1957). The present isolates are the first made from an *Aedes* species collected on the South African Highveld. It is noteworthy that both species were present at Tweespruit in the 1985 collections as no *Ae. caballus* were reared from the eggs collected during the previous winter or were taken there as adults in 1974/75 (McIntosh *et al.* 1980). MID virus was isolated 17 times in South Africa and twice in Zimbabwe during the period 1957–76 and except for four isolations from *Culex theileri*, all the isolations were from floodwater *Aedes* mosquitoes belonging to the *Neomelaniconion*, *Aedimorphus* and *Ochlerotatus* subgenera (Worth *et al.*, 1961 and Arbovirus Unit, unpublished data).

It seems that the first significant raints which were sufficient to inundate the margins of the dam and vleis, albeit to a limited extent, causing aedine eggs to hatch, occurred from 20–28th February when 82 mm was measured. Previous to this, hatching is unlikely to have taken place because falls were too small and too spread out temporally (Table 1). Hence it is thought that the *Ae. juppi* mosquitoes collected one month later, at the end of March, represented the first brood of aedine mosquitoes and that they included individuals that might have been trans-ovarially infected with MID virus. This interval seems rather longer than that required for development of adults after submersion of the eggs, since 9–15 days are needed in the laboratory at a constant water temperature of 23 °C. There is some laboratory evidence that rodents might be involved in MID transmission cycles (McIntosh 1961). However, it seems unlikely that the mosquitoes would have been alive long enough as adults to have initiated a rodent-mosquito transmission cycle. These mosquitoes would have represented populations distinct from those sampled as eggs during the previous winter since the great majority of eggs were recovered from pans which were dry when the adults were taken in the 1985 baited catches. The virus has almost invariably been isolated from floodwater *Aedes* rather than from *Culex* mosquitoes which do not overwinter in the egg stage. This suggests transovarial transmission. *Ae. caballus sensu lato* has transmitted the virus horizontally in the laboratory (Kokernot *et al.* 1957) but tests to demonstrate transovarial transmission remain to be done.

An explanation for the pool of *Ae. juppi* infected with SIN virus is probably that this single infection was acquired when *Ae. juppi* fed on viraemic birds. *Culex (Culex) univattatus* Theobald also occurred in our collections and a *Cx. univittatus*–bird transmission cycle would be expected with this mosquito breeding throughout the summer in the available permanent water.

## ACKNOWLEDGEMENTS

We wish to thank Mr Henri Jacobs for allowing us to work on his farm. The senior author acknowledges a grant from the South African Medical Research Council which contributed towards the study.

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Accepted 5 November 1986

# TICKS AND ARTHROPOD-BORNE VIRUSES IN SOUTH AFRICA

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## ABSTRACT

To date 6 arthropod-borne viruses have been isolated from argasid, but none from ixodid ticks in South Africa. Apart from African Swine Fever and Pretoria viruses isolated by other laboratories, 4 viruses have been isolated by our Unit from bird-feeding *Argas* or *Ornithodoros* ticks. These are an Orbivirus, Chenuda virus, and 3 unclassified viruses — Soldado, Quaranfil and Nyamanini. Details concerning our isolation of these 4 viruses are described as well as the extent of our knowledge of their ecology.

In searching for natural arthropod maintenance vectors of the medically important mosquito-borne viruses Chikungunya, Rift Valley fever and Wesselsbron viruses, we have considered argasid ticks and have undertaken laboratory vector experiments on *Ornithodoros savignyi*. With Chikungunya and Rift Valley fever viruses first nymphal instars failed to become infected but with Wesselsbron virus the infection rate of the subsequent second instars, was 22% showing that trans-stadial transmission had occurred. Four ticks infected with Wesselsbron virus did not transmit the virus to 3 hamsters 76 days after the infective feed but further tests at shorter periods of extrinsic incubation are in progress.

## INTRODUCTION

In South Africa all the arthopod-borne viruses of major medical importance are mosquito-borne and ticks have yet to be implicated in their transmission. This is in contrast to several other countries where there are arboviruses causing important human diseases which are tick-borne, such as Russian Spring Summer Encephalitis virus in eastern Europe and Russia, Congo virus in Central and West Africa and South-eastern Europe and Colorado tick fever virus in North America, all of which are transmitted by ixodid ticks. It is unusual for a virus to have as its vectors arthropods belonging to widely divergent taxons and this also seems to apply even between different families of Diptera. This is clear if reference is made to a catalogue of virus isolations made from arthropods the world over (Berge, 1975) which shows that only a very few viruses have been recovered consistently from both ticks and mosquitoes, from both mosquitoes and *Culicoides* or from both mosquitoes and phlebotomines. Furthermore, very few tick-borne viruses have been isolated from both argasid and ixodid ticks. The specificity of the virus for its arthropod host is indeed usually restricted to one or 2 closely related species.

Six of the 37 different arboviruses isolated to date in Southern Africa have come from argasid ticks, the remainder originating from mosquitoes (24) and *Culicoides* (7). These 6 viruses will be discussed in this paper, 4 of which were isolated by our Unit and have not been reported on before. Probably more viruses would have been recovered from ticks by our Unit if as much effort had been devoted to their collection as has been devoted to the collection of blood-sucking flies. This limited regard to ticks has been due to the absence of any epidemiological evidence in South Africa to suggest that they are involved in transmission cycles of any of our medically important arboviruses. However, because of our failure to gather evidence to implicate mosquitoes as maintenance vectors of Rift Valley fever (RVF), Wesselsbron (WESS) and Chikungunya (CHIK) viruses, we have given consideration to argasid ticks as being possible maintenance vectors. Accordingly we have conducted experiments to test the vector competence of *Ornithodoros savignyi*, the sand tampan, with these viruses in the laboratory to assess whether ticks could be involved in transmission. The results of our experiments are reported in this paper. It should perhaps be emphasized that we do have good evidence in the case of RVF and CHIK viruses that mosquitoes act as epizootic-epidemic vectors and it is likely that the same would apply to WESS virus.

**Table 1** Arboviruses isolated from ticks in South Africa.

| Virus | Classification | Vector yielding isolations | Vertebrate host* | Locality | Reference |
|---|---|---|---|---|---|
| African Swine Fever | Iridovirus (Iridoviridae) | *Ornithodoros moubata* | Warthog | Transvaal | Pini & Hurter 1975 |
| Chenuda | Orbivirus (Reoviridae) | *Argas arboreus* | Cliff swallow | Hartebeespoortdam, Halfway House, Transvaal | Arbovirus Unit N.I.V. |
| Quaranfil | Unclassified | *A. walkerae* | Cattle egret | Naboomspruit, Transvaal | Arbovirus Unit N.I.V. |
| Nyamanini | Unclassified | *A. walkerae* | Cattle egret | Naboomspruit. Ndumu, Natal. | Arbovirus Unit N.I.V. |
| Soldado | Unclassified | *Ornithodoros* sp ⊢ | Cormorant | Lamberts Bay, Cape | Arbovirus Unit N.I.V. |
| Pretoria | Unclassified | *A. africolumbae* | Cape rock pigeon | Pretoria, Transvaal | Converse et al., 1975 |

* Virus was isolated from those animals underlined

⊢ Probably *O. capensis*

Table 1 summarizes details concerning the 6 viruses isolated in South Africa from argasid ticks. The first, African Swine fever, is a porcine virus while the 5 others are all avian viruses. Each will be discussed in turn below.

### African Swine Fever virus (ASF)

ASF virus is classified as an Iridovirus in the family Iridoviridae and is related to the insect viruses. This virus, which periodically causes outbreaks of disease in domestic pigs in the Transvaal, is believed to be maintained in a cycle between *Ornithodoros moubata* and warthogs *(Phaecchoerus aethiopicus)* (Plowright, 1977; Pini and Hurter, 1975). *O. moubata* is also responsible for the transmission of the virus from warthogs to domestic pigs. Transovarial and venereal transmission of the virus by *O. moubata* has been demonstrated in the laboratory (Plowright et al., 1970) and these may be important for the natural maintenance of the virus. Doubt exists whether *O. moubata* can acquire virus from warthogs as viraemia levels in warthogs are apparently insufficient to infect these ticks (Plowright, 1977).

### Chenuda virus (CNU)

CNU virus is an Orbivirus in the family Reoviridae. Twenty one isolates of the virus were obtained form *Argas arboreus* collected by our Unit from nests of the cliff swallow, *(Petrochelidon spilodera)* under road culverts between Johannesburg and Hartebeespoort Dam, Transvaal, in the summer of 1960. Nests of several colonies of swallows were sampled. Seventeen more isolates were made from ticks taken from empty nests the following winter in August 1961 just before the swallows returned to South Africa after their annual migration to Central Africa. These second recoveries of virus showed that CNU virus had overwintered in the ticks. The cliff swallow is the only known host of *A. arboreus*.

### Quaranfil virus (QRF)

QRF is an unclassified tick-borne virus and has been isolated by our Unit once from a cattle egret *(Bubulcus ibis)* and 7 times from *Argas walkerae* ticks. A heronery in the tree tops on a farm at Naboomspruit, Transvaal, was the source of both the cattle egret as well as the ticks which were recovered from under the bark adjacent to the cattle egret nests. Adults and nymphal ticks were collected.

In Egypt this virus was isolated from the same avian and tick species and was isolated twice from human beings (Taylor et al., 1966). Isolations were made from 2 children who were bled during a mild febrile illness and serological surveys of the human population living in close proximity to heroneries gave evidence of occasional human infection. Furthermore, experiments by the same workers showed that although the virus could be transmitted trans-stadially on moulting it was not transmitted transovarially.

### Nyamanini virus (NYM)

This unclassified virus was first isolated by our Unit from a cattle egret collected at Nyamanini pan in the Ndumu Game Reserve in 1957. This reserve is situated in Northern Natal on the Mocambique border. Subsequently 4 further isolations came from cattle egrets collected at Naboomspruit as well as 14 isolations from *Argas walkerae* collected at the same site. The virus has also been isolated from an egret and *Argas arboreus* in Egypt (Taylor et al., 1966) and

from *A. arboreus* in Nigeria (Kemp et al., 1975).

### Soldado virus (SOL)

To date SOL virus has remained in the unclassified group but recently Casals and Tignor (personal communication) have shown on the basis of serological relationships that this virus should be placed together with other viruses in the new genus *Nairovirus* of the family Bunyaviridae. This genus has recently been described by Bishop et al., (in press).

Eight isolations of SOL virus were made by our Unit from *Ornithodoros* ticks, probably *O. capensis*, collected from cormorant *(Phalocrocorax africanus* and *P. capensis)* nests on an island in Lamberts Bay, Cape Province. This virus has also been isolated from *O. capensis* infesting marine birds living on islands in Trinidad (Jonkers et al., 1973) and the Seychelles (Converse et al., 1975[1]) as well as from *O. maritimus* infesting herring gulls on Puffin Island in North Wales (Converse et al., 1976). The extensive migrations of these marine birds accounts for the remarkably extensive distribution of the virus. Converse et al., (1975[1]) transmitted the virus to domestic chicks by infected *O. capensis* removed from terns, the virus causing death of these chicks.

### Pretoria virus (PRE)

This virus, heretofore placed in the unclassified category, has like SOL virus recently been shown to belong to the genus *Nairovirus* of the family Bunyaviridae (Casals and Tignor, personal communication).

It was isolated by Converse and co-workers from a pool of 2 *Argas africolumbae* ticks sent to them from South Africa (Converse et al., 1975[2]). These ticks were taken near a nest of a Cape Rock pigeon *(Columba guinea phaeonata),* in a house at Derdepoort in Pretoria.

It can be seen that each of these bird-associated viruses is probably maintained by a species of argasid tick which is restricted to the particular avian host population. The tick vector also provides for dissemination of virus to further birds. Man is unlikely to readily acquire infection of any of these viruses, because of the host specificity of the vector and the specialised habitat of the avian host.

**Table 2** Vector competence tests with 1st instar nymphs of *Ornithodoros savignyi* and 3 viruses

| | | | Infection | | Transmission |
|---|---|---|---|---|---|
| Expt. No. | Virus | Titre of viraemic host in logs* | Days after infective feed ticks tested | Infection rate (2nd instars) | Result |
| 1 | RVF (Bunyavirus-like) | 5,8 (hamster) | 64-67 | 0/26 ⊢ | 12 groups of 1-5 ticks failed to transmit to hamsters on the 64-66th day |
| 2 | WESS (Flavivirus) | 5,6 (hamster) | 63-78 | 11/50 (22%) | 3 groups of 1-2 infected ticks failed to transmit on the 76th day |
| 3 | CHIK (Alphavirus) | 6,6 *(Mystromys albicauditus)* | 50-61 | 0/11 ⊢ | N.D. |

* expressed as $\log_{50}$ per 0,02 ml of mouse inoculum.

⊢ numerator = No. ticks infected, denominator = No. ticks tested.

⊢ these ticks failed to moult and remained 1st instars.

N.D. = not done.

Table 2 shows the results of experiments to test the vector competence of *Ornithodoros savignyi* with 3 viruses which are medically important in South Africa. First instar nymphs were allowed to feed on viraemic syrian hamsters (RVF and WESS viruses) or viraemic *Mystromys albicaudatus* (CHIK virus). A blood sample was taken from each animal immediately prior to this infective feed and titrated in infant mice to determine the concentration of virus in the blood. Titres were expressed as $\log_{50}$ per 0,02 mls of mouse inoculum. After feeding, the engorged ticks were kept in sand and allowed to moult to second instars. This occurred in the case of those ticks infected with RVF and WESS viruses (experiments 1 and 2) but not with those ticks infected with CHIK virus (experiment 3), in which this virus might have inhibited moulting and caused considerable mortality among the ticks.

Transmission was attempted to hamsters in the case of RVF and WESS viruses. With RVF virus, between day 64 and 66 after the infective feed 12 groups of 1-5 ticks failed to transmit to hamsters which confirmed the subsequent finding that none of the ticks was infected with virus. With WESS virus 3 groups of 1-2 ticks, subsequently shown to be infected, failed to transmit to hamsters on the 76th day.

One or 2 days after the transmission attempts the 2nd instar nymphs (experiments 1 and 2) and the 1st instars (experiment 3) were killed and tested individually for the presence of virus by intracerebral inoculation into infant mice. No ticks were shown to be infected with RVF and CHIK viruses but 11 out of 50 (22%) were infected with

WESS virus indicating that trans-stadial transmission had occurred with this virus.

The results of these experiments indicate that argasid ticks were unlikely to be involved as maintenance or other vectors of RVF, WESS or CHIK viruses. In South Africa there is no evidence that ticks play a role in secondary transmission cycles for spread of mosquito-borne viruses as has been suggested by Camicas (1978). Because of a few isolations of West Nile virus made from ticks in the U.S.S.R. and Egypt and a few of Snowshoe Hare virus made from ticks in the northern U.S.A. and Canada, Camicas has suggested that ticks could intervene in the ecology of a mosquito-borne virus. He postulated that there are tick-vertebrate cycles which act as secondary transmission cycles capable of "relaying" the major mosquito-vertebrate cycles from one area to another.

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this parasite is limited to the western Cape where its effect on the bark beetle population is negligible (G. Tribe, personal communication).

During June–July 1984 I collected natural enemies of *O. erosus* from infested *Pinus halepensis* Miller and *P. brutia* Tenore at several sites in the Coastal Plain, Judean Hills and the Upper Galilee of Israel and sent them to South Africa. After quarantine the following natural enemies were released in a pine plantation at Bergvliet near Sabie: *Dendrosoter caenopachoides* Rusch. (Hymenoptera: Braconidae); *Metacolus unifasciatus* Foerster (Hymenoptera: Pteromalidae); *Aulonium ruficorne* Ol. (Coleoptera: Colydiidae); *Corticeus pini* Panz. (Coleoptera: Tenebrionidae); *Platysoma oblongum* F. (Coleoptera. Histeridae); *Lyctoris* sp. (Hemiptera: Anthocoridae). In addition 5 releases of *C. caenopachoides* were made between January and April 1985 at Lebanon and Nuweberg State Forests in the western Cape Province. It is not known yet if these natural enemies have become established. – Rami Kfir, Plant Protection Research Institute, Private Bag X134, Pretoria, 0001.

**Identity of Argasid ticks yielding isolations of Chenuda, Quaranfil and Nyamanini viruses in South Africa.** – In 1981 we published a review on 'Ticks and Arthropod-borne viruses in South Africa' (Jupp, P. G. and McIntosh, B. M. 1981. *Proceedings of an International Conference, 27–29 January, 1981, Tick Research Unit, Rhodes University, Grahamstown, Ed. Whitehead & Gibson* pp. 177–180.) In this paper we reported the first recorded isolations of four particular viruses obtained from ticks in this country. In the case of three of these viruses, it has since become clear that the tick vectors were incorrectly identified.

The isolations of Chenuda virus originated from *Ornithodoros (Alveonasus) peringueyi* Bedford and Hewitt 1925 and not from *Argas arboreus*. This is evident after an examination of records of other collections made at the same locality. The latter consists of nests of the cliff swallow *(Petrochelidon spilodera)* situated between Johannesburg and Hartebeespoort Dam. Further, the isolations of Quaranfil and Nyamanini viruses were from *Argas (Persicargas) arboreus* Kaiser, Hoogstraal and Kohls 1964, and not from *Argas walkerae* as previously reported. This is clear from records of other ticks collected in or near the same cattle egret *(Bulbulcus ibis)* heronery in Naboomspruit.

Chenuda virus is an Orbivirus in the family Reoviridae while Quaranfil and Nyamanini viruses are still unclassfied arboviruses. – P. G. Jupp and B. M. McIntosh, National Institute for Virology, Private Bag X4, Sandringham, Johannesburg, 2131.

***Pachycentrinus convexicollis* Hustache (Coleoptera: Curculionidae), a possible biocontrol agent against jointed cactus.** – While searching for suitable cactophagous insects for the biological control of the jointed cactus weed, *Opuntia aurantiaca* Lindley, in Argentina between 1970 and 1973, we observed characteristic feeding-damage on some low growing opuntiae caused by a weevil recently identified as *Pachycentrinus convexicollis* Hustache of the subfamily Baridinae (Zimmermann, H. G., H. E. Erb & R. E. McFadyen. 1979. *Acta Zoologica Lilloana* **33**: 101–112). This was the only cactophagous beetle found in Argentina on jointed cactus-like opuntiae (series Aurantiacae) and hence our interest in its possible use for biocontrol of jointed cactus in South Africa.

*P. convexicollis* was described from specimens collected at Rio Tapenagá in the eastern Chaco of Argentina (59° 40′ W, 27° 30′ S) from an unknown host plant (Hustache, A. 1949. *Boletin do Museu Nacional, Zoologia, Brasil* **97**: 111–112). It has subsequently been collected further north (23°) and west (63°) in the Chaco from the follow-

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*Am. J. Trop. Med. Hyg.*, 32(6), 1983, pp. 1407–1415
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# CRIMEAN-CONGO HEMORRHAGIC FEVER IN SOUTH AFRICA

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*Abstract.* Crimean-Congo hemorrhagic fever virus was isolated for the first time in South Africa in February 1981, from the blood of a 13-year-old boy who died in Johannesburg after attending a camp in a nature reserve in the western Transvaal. Virus was isolated from 21/120 pools of questing ticks from the nature reserve, the infected species being *Hyalomma marginatum rufipes* and *H. truncatum*. Virus was also isolated from 4/38 pools of partially engorged ticks and other ectoparasites collected off hosts, the infected species being *H.m. rufipes, H. truncatum* and *Rhipicephalus evertsi*. Antibodies were found in the sera of 5/74 humans, 8/26 wild vertebrates, 74/270 sheep, and 109/170 cattle from the reserve and surrounding farms. Antibodies were also found in 28/200 hares from various locations in the country. It was concluded that the virus is widely prevalent in South Africa, but the full medical and veterinary significance of its presence has yet to be determined.

Crimean-Congo hemorrhagic fever (CCHF) was diagnosed for the first time in South Africa by isolation of the virus from the blood of a 13-year-old boy who died in a hemorrhagic state in Johannesburg on 19 February 1981, after attending a nature study camp (veldschool) at the S. A. Lombard Nature Reserve near the town of Bloemhof in the western portion of the Transvaal province from 5–13 February. Field investigations which followed recognition of the disease included an antibody survey on sera collected from man and livestock in the vicinity of the veldschool and a survey of the prevalence of virus in ticks. Further investigations were undertaken in September 1981, after reports were received that deaths had occurred in antelope and gazelle at the nature reserve.

A clinical description of the fatal case of CCHF has been published elsewhere.[1] This paper presents the results of laboratory and field investigations.

## MATERIALS AND METHODS

### *History and specimens*

The patient became ill on the morning after he returned home and what is believed to have been a *Hyalomma* tick was found attached to his scalp. He had found a similar tick crawling on himself while attending the camp and had crushed it. A medical practitioner consulted at the onset of illness made a tentative diagnosis of tick-borne typhus (*Rickettsia conori* infection) and instituted appropriate antibiotic therapy. The condition of the patient deteriorated over the next few days and by 18 February, when he was in a frankly hemorrhagic state, suspicion had turned to the African viral hemorrhagic fevers, CCHF in particular, and accordingly, blood, urine and pleural fluid were submitted for virological examination. He died on the following day, and since permission for autopsy was refused the only other specimen submitted for virological examination consisted of tracheal aspirate.

The patient had been one of 320 boys of similar age from schools in the Johannesburg area who had attended the camp together. An epidemic of meningoencephalitis, associated with ECHO virus 9 infection, was known to be occurring in school children in the Johannesburg area at the time, and seven of the children from the veldschool group were affected within 2 weeks of their return home. Appropriate specimens for the isolation of enteroviruses were submitted from affected children, as well as sera to be tested for the presence of CCHF virus and antibody. Sera were also collected and stored from all medical personnel who had direct or indirect contact with the CCHF patient.

The S. A. Lombard Nature Reserve (27°35′S; 25°30′E), which covers an area of 4,000 hectares and has an annual precipitation of 300 to 400 mm, is located 18 km north-west of Bloemhof on the western edge of the high-veld plateau at an altitude of 1,283 meters. It consists of flat or gently

Accepted 26 April 1983.

undulating grassland with a few sparsely wooded areas adjacent to pans: shallow accumulations of surface water in depressions which lack drainage. The veldschool is sited on a subdivision of the nature reserve, and during their stay school children undertook adventure trails in the reserve and spent nights outdoors sleeping on the ground.

A team of investigators spent a week at the nature reserve immediately following the death of the patient, collecting questing ticks plus ectoparasites, blood and organ samples from a few birds, reptiles and small mammals. Partially engorged ticks were also collected from the few sheep and cattle kept at the veldschool. During March blood samples were collected from all staff and their families at the veldschool and nature reserve, as well as from sheep and cattle at the veldschool and on adjoining farms.

At the end of August, it was reported that the following deaths had occurred in antelope and gazelle at the nature reserve over a period of two months: 0/222 black wildebeest (*Connochaetes gnou*), 3/26 gemsbuck (*Oryx gazella*), 2/132 hartebeest (*Alcelaphus buselaphus*), 3/88 impala (*Aepyceros melampus*), 10/1,000 springbuck (*Antidorcas marsupialus*), and 23/80 eland (*Taurotragus oryx*). For some species, notably springbuck, the losses did not exceed what could normally be expected during the dry winter months, but the losses in eland, in particular, caused concern and were the first deaths recorded in this species at the nature reserve. The team which investigated the deaths at the beginning of September included a veterinary pathologist, a veterinary parasitologist, and a pasture specialist. Two weak eland were killed for autopsy and blood and organ samples were taken. A serum sample which had been collected earlier from a third eland was also obtained and partially engorged ticks were collected from the fresh and some putrified eland carcases.

Finally, 200 sera which had been collected from hares in various locations in South Africa between 1977 and 1979 in a study of the taxonomy of hares were obtained from Dr. T. J. Robinson of the Mammal Research Institute of the University of Pretoria.

### *Reagents*

Reference arthropod-borne virus grouping fluids in the form of pools of mouse immune ascitic fluids (MIAFs) were obtained from the National Institutes for Health, Bethesda, MD, U.S.A. Reference human antisera to CCHF, Rift Valley fever (RVF), Ebola (EBO), Lassa fever (LAS) and Marburg (MBG) viruses were obtained from the Centers for Disease Control, Atlanta, GA, U.S.A., as were multivalent and monovalent cell culture antigen slides of the same viruses for use in indirect immunofluorescence (IF) tests. CCHF antigen slides were also obtained from the Centre for Applied Microbiology, Porton Down, U.K. Reference strains of CCHF virus (Ib Ar 10200) and Hazara virus (JC280) were obtained from the Yale Arbovirus Research Unit, New Haven, CT, U.S.A. as was MIAF to Hazara virus. Fluorescein-labeled anti-species immunoglobulins were obtained from Wellcome Reagents Ltd, Beckenham, Kent, U.K. Anti-rabbit conjugate was used in IF tests with hare sera.

Mouse immune ascitic fluids to viruses isolated in this study and to the reference Ib Ar 10200 strains of CCHF virus, were prepared as described by Sartorelli et al.,[2] except that the first two doses of mouse brain virus used for hyperimmunizing mice were inactivated with beta-propriolactone.[3] Antigens for complement-fixation (CF) and immunodiffusion (ID) tests consisted of sucrose-acetone extracted[4] infected mouse brain or liver, or crude 10–20% saline extracts of these organs.

### *Virus isolations*

Human body fluids were inoculated intracerebrally into day-old mice and into cultures of Vero cells[5] in micro-slide culture chambers (Bellco Glass Inc., Vineland, NJ). Cultures were fixed in cold acetone and stained by IF with the antisera to CCHF, RVF, LAS, EBO, and MBG viruses on days 5, 8 and 14 after inoculation, and supernatant fluid was subcultured twice, serially. Viruses isolated in mice were subcultured in mice and in Vero, primary calf kidney (CK) and CER[6] cells and stained as above. Ten percent suspensions of animal organs (brain, spleen, liver, kidney and lung) were prepared in cell culture medium, centrifuged at 10,000 × *g* for 1 hour at 4°C, and the supernatant was inoculated into cell cultures and mice. Suspensions of tick pools, most consisting of 30 individuals of a particular species, were prepared in 5 ml cell culture medium, centrifuged as above and the supernantant was inoculated into mice only. Where necessary, subsamples of immature ticks were cleared and mounted for identification by microscopy.

*Serological tests*

Complement-fixation tests were performed as described by Blackburn and Swanepoel,[7] IF tests as described by Johnson et al.,[8] reversed passive hemagglutination inhibition (RPHI) tests as described by Swanepoel et al.,[9] and ID tests as described by Clarke.[10] MIAFs used in neutralization tests in mice[11] or in fluorescent focus reduction (FFR) tests[12] in CER cells were subjected to acetone-ether extraction for removal of non-specific inhibitors of the virus.[11]

## RESULTS

*Virology*

Deaths in infant mice inoculated with the blood of the patient started on the 6th day after inoculation and continued over the next few days, a total of 15/32 inoculated mice dying. In CF tests on the 7th day, antigen prepared from mouse brain reacted to a titer of 1:64 with both Congo grouping fluid and the reference human CCHF anti-serum. CF tests were negative with other grouping fluids and with antisera to a range of local arthropod-borne viruses. CCHF virus had not previously been cultured in the laboratory and reference virus had not even been acquired at the time. The virus was not successfully isolated in cell cultures, but could be subcultured from mouse brain in Vero, CK and CER cells. The virus was designated strain 4/81. A second strain of the virus, designated 6/81, was obtained from the pleural fluid of the patient and this killed only 3/14 infant mice in the original passage. Urine and tracheal aspirate failed to yield virus.

No viruses were isolated from the sera of meningoencephalitis patients, but ECHO virus 9 was isolated from one cerebrospinal fluid and two stool specimens in the general diagnostic section of the Institute.

No viruses were isolated from the organs of 10 cattle egrets (*Bubulcus ibis*), a multimammate mouse (*Mastomys coucha*) or the two eland antelope which had been killed for examination. The two eland were emaciated and there was marked hemosiderosis of the spleen in both animals. The presence of antibodies, reported below, indicated that they had undergone CCHF infection. It was felt that eland had coped less well than smaller species with the stress brought on by dry winter grazing and cold spells.

The results of attempts to isolate virus from ticks and other ectoparasites are summarized in Table 1. Adult ticks were highly active at the veldschool and nature reserve during February, and a total of 3,037 questing ticks were collected in the course of a week. The ticks were overwhelmingly (2,964/3,037) *Hyalomma* spp. and only five were immature (*Hyalomma truncatum* nymphs) despite express efforts to capture immature stadia by blanket-dragging. The ticks were abundant on the bare ground between trees where the children had slept and they readily approached when investigators stamped their feet on the ground.

Preliminary sorting of ticks into species was carried out in the field without the aid of a microscope, and thus it transpired that 28 pools of *Hyalomma* ticks were tested for virus in the laboratory before it was realized that two species were involved: *H. marginatum rufipes* and *H. truncatum*. The first 28 pools are, therefore, designated *Hyalomma* spp. in Table 1. The remaining ticks were identified in the laboratory and tested as separate species. The 21 isolations of CCHF virus which were made from questing ticks, all came from the 109 pools of *Hyalomma* tested and the incidence of virus was not significantly different in the two species (Table 1).

Crimean-Congo hemorrhagic fever virus was also isolated from a pool of 30 partially engorged *Rhipicephalus evertsi* ticks taken off sheep in February and from three pools of *Hyalomma* ticks taken off eland antelope in September. All other parasites taken off hosts failed to yield virus (Table 1).

The majority of the strains of virus isolated from ticks took 8–14 days or longer to kill mice in the original passage, and most could not be subcultured or reisolated. Isolation of CCHF virus could, nevertheless, be confirmed in three ways: brain and liver suspensions of dead mice reacted in CF tests with MIAF to the reference Ib Ar 10200 strain of virus, producing antigen titers of up to 1:128; the same suspensions produced precipitation lines of identity in ID tests with Ib Ar 10200 MIAF and antigen; and, finally, characteristic lesions occurred in mice. In this last respect, a peculiarity of all strains isolated in the present study, from both human and tick specimens, was the marked pathology seen in mouse livers. Macroscopically, some livers appeared normal, but most were mottled with 2 mm diameter or larger white areas of necrosis and a few livers were entirely white or

TABLE 1

*Results of attempts to isolate Crimean-Congo hemorrhagic fever virus from ticks and other ectoparasites from the S. A. Lombard Nature Reserve and Veldschool*

| Parasite | Description | Number | Isolations/ Pools (%) |
|---|---|---|---|
| Questing ticks: | | | |
| *Hyalomma* spp. | Adults | 715 | 6/28 (21) |
| *Hyalomma marginatum rufipes* | Adults | 541 | 5/22 (23) |
| *Hyalomma truncatum* | Adults | 1,703 | 10/58 (17) |
| *Hyalomma truncatum* | Nymphs | 5 | 0/1 |
| *Rhipicephalus evertsi* | Adults | 52 | 0/5 |
| *Amblyomma marmoreum* | Adults | 20 | 0/5 |
| *Aponomma latum* | Adult | 1 | 0/1 |
| Host: cattle | | | |
| *Hyalomma marginatum rufipes* | Adults | 12 | 0/1 |
| *Hyalomma truncatum* | Adults | 10 | 0/1 |
| *Rhipicephalus evertsi* | Adults | 30 | 0/1 |
| Host: sheep | | | |
| *Rhipicephalus evertsi* | Adults | 90 | 1/3 |
| Host: hares (*Lepus* spp.) | | | |
| *Hyalomma marginatum rufipes* | Nymphs | 100 | 0/3 |
| *Hyalomma marginatum rufipes* | Larvae | 22 | 0/3 |
| *Rhipicephalus evertsi* | Nymphs | 69 | 0/2 |
| *Rhipicephalus evertsi* | Larvae | 82 | 0/2 |
| *Ctenocephalides felis damarensis* | Fleas | 20 | 0/1 |
| Host: ground squirrel (*Xerus inauris*) | | | |
| *Haemaphysalis leachii* | Nymphs | 7 | 0/1 |
| *Neohaematopinus faurei* | Lice | 250 | 0/1 |
| Host: springhare (*Pedetes capensis*) | | | |
| *Rhipicephalus evertsi* | Nymph | 1 | 0/1 |
| *Radfordilaelaps meridionalis* | Mites | 120 | 0/1 |
| Host: yellow mongoose (*Cynictus penicillata*) | | | |
| *Rhipicephalus evertsi* | Nymph | 1 | 0/1 |
| Host: leopard tortoise (*Testudo pardalis babcocki*) | | | |
| *Amblyomma marmoreum* | Adults | 26 | 0/1 |
| *Amblyomma marmoreum* | Larvae + nymphs | 51 | 0/1 |
| Host: monitor lizard (*Varanus exanthematicus albigularis*) | | | |
| *Amblyomma marmoreum* | Nymphs | 5 | 0/1 |
| Host: multimammate mouse (*Mastomys coucha*) | | | |
| *Echinolaelaps muricola* | Mites | 11 | 0/1 |
| *Laelaps liberiensis* | Mite | 1 | |
| Host: eland antelope (*Taurotragus oryx*) | | | |
| *Hyalomma marginatum rufipes* | Adults | 60 | 2/4 |
| *Hyalomma truncatum* | Adults | 71 | 1/4 |
| *Margaropus winthemi* | Adults | 217 | 0/3 |
| *Rhipicephalus evertsi* | Adults | 46 | 0/3 |

pale yellow in appearance. Microscopically, there was massive necrosis of hepatocytes with karyorrhexis. Despite the presence of extensive lesions and demonstrable antigen in livers, the infective virus content was low and virus could be subcultured from liver with difficulty and in few instances only.

One strain of virus, 128/81/7, which was iso-

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lated from *H. m. rufipes* ticks off eland antelope, grew better than the others and killed mice in 5 days but, despite passaging the virus at limiting dilutions, the highest titers achieved were $10^5$ mouse ic $LD_{50}$/0.02 ml and 7.. cell culture infective doses ($TCID_{50}$/ml). This strain was compared more closely with strain Ib Ar 10200 in order to make a definitive identification of the virus as CCHF virus. Identical homologous and heterologous antigen titers of 1:128 and antibody titers of 1:512 were obtained in reciprocal CF chessboard titrations with antigens and MIAFs of the two viruses, whereas antigen titers of 1:8 and antibody titers of 1:64 were obtained in tests with Hazara MIAF. However, the MIAFs lacked potency in neutralization tests and nonsignificant homologous and heterologous $\log_{10}$ neutralizing indices of 0.8 to 1.0 were obtained in tests in mice, and almost identical results were obtained in FFR tests in cell cultures.

*Serology*

No antibody to CCHF virus was detected by IF test on the serum sample of the patient which yielded virus, nor were CCHF antibodies detected in acute and convalescent sera from children with meningoencephalitis or medical personnel who suffered minor illnesses following contact with the patient.

The incidence of antibody to CCHF virus detected by RPHI technique in human and a variety of wild vertebrate sera from the veldschool and nature reserve is shown in Table 2. The human sera were tested by both RPHI and IF techniques and five of 74 sera were positive by both techniques while the remaining sera were uniformly negative. IF titers were similar to RPHI titers: 8/8, 16/16, 32/64, 64/128, 64/64 (IF/RPHI titers of individual sera). None of the people with antibody could recall having suffered illness suggestive of CCHF, but it is noteworthy that three of the people with antibody were related to each other. A few of the residents could recall having found ticks attached to themselves. The four RPHI-positive hare sera from the nature reserve had IF/RPHI titers of: 8/8, 128/256, 128/128 and 256/256 while six sera were negative by both techniques. Officials at the nature reserve felt that hares had been particularly numerous in 1981, but this was a purely subjective impression. The single positive ground squirrel serum had a minimal RPHI titer of 1:8 and the three eland sera had RPHI titers of 1:128, but lack of suitable anti-species conjugates prevented confirmatory IF tests being performed on the sera.

TABLE 2

*Prevalence of reversed passive hemagglutination inhibition antibody to Crimean-Congo hemorrhagic fever virus in human and wild vertebrate sera from the S. A. Lombard Nature Reserve and Veldschool*

| Species | No. positive/tested |
|---|---|
| Humans | 5/74 |
| Hares (*Lepus* spp.) | 4/10 |
| Ground squirrels (*Xerus inauris*) | 1/4 |
| Springhare (*Pedetes capensis*) | 0/5 |
| Monitor lizard (*Varanus exanthematicus albigularis*) | 0/1 |
| Yellow mongoose (*Cynictis penicillata*) | 0/1 |
| Pygmy mouse (*Mus minutoides*) | 0/1 |
| Mole rat (*Cryptomys hottentotus*) | 0/1 |
| Eland antelope (*Taurotragus oryx*) | 3/3 |

The results of RPHI tests on sheep and cattle sera from the veldschool and neighboring farms are summarized in Table 3. The incidence of antibody in sheep averaged 27% and was consistently lower on all properties than the incidence of antibody in cattle, which averaged 64%. In confirmatory IF tests, sheep sera with RPHI titers of 1:8 and 16 were frequently negative, while sheep sera with RPHI titers greater than 1:16 had IF titers which were the same or within one or two dilutions of RPHI titers. Agreement between RPHI and IF titers was less good in cattle sera. Sera with RPHI titers of up to 1:32 were negative in IF tests and in sera with RPHI titers greater than this, IF titers were frequently 4- to 8-fold lower than RPHI titers.

The results of RPHI tests on hare sera collected between 1977 and 1979 are summarized in Table 4. Positive sera were recorded in all four provinces of South Africa, and occurred only in the two species of *Lepus* recognized as valid.[13] Fewer individuals of the three recognized species of *Pronolagus* hares were tested, but all lacked antibody. It is of interest that among the hare sera, antibodies were found in 2/7 *Lepus saxatillus* sera collected in the S. A. Lombard Nature Reserve at least 2 years prior to the occurrence of the fatal case of CCHF. The hare sera were also screened in undiluted form for ID antibody and at a dilution of 1:8 for IF antibody. The results are shown in relation to RPHI titers in Table 5.

The known distribution of CCHF virus in South

TABLE 3

*Prevalence of reversed passive hemagglutination inhibition antibody to Crimean-Congo hemorrhagic fever virus in cattle and sheep sera from the veldschool and adjoining farms, and titers of sera*

| Property | No. positive/no. tested (% positive) | |
|---|---|---|
| | Cattle | Sheep |
| Veldschool | 9/12 (75) | 17/46 (37) |
| Bospan | 18/29 (62) | 16/77 (20) |
| Panfontein | 20/35 (57) | 20/50 (40) |
| Kareefontein | 54/78 (69) | 21/97 (22) |
| Swartlaagte | 8/16 (50) | — |
| Totals | 109/170 (64) | 74/270 (27) |

| Species | Titers of sera | | | | | | | | | | Total pos./tested (% positive) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | <8 | 8 | 16 | 32 | 64 | 128 | 256 | 512 | 1,024 | 2,048 | |
| Cattle | 61 | 1 | 5 | 11 | 20 | 31 | 31 | 8 | 1 | 1 | 109/170 (64) |
| Sheep | 196 | 2 | 3 | 10 | 14 | 29 | 13 | 1 | 2 | — | 74/270 (27) |

Africa, based on the present virological and serological findings, is shown in Figure 1.

## DISCUSSION

The most urgent problem raised by the diagnosis of CCHF in the present study, concerned the advisability of continuing to use the veldschool for visits by school children and, indeed, the next group of children were already at the school by the time that the investigations were started. The problem centered on whether or not the occurrence of CCHF could be regarded as an isolated incident, possibly related to recent introduction of infected ticks on migratory birds.[14] In the event, the children were sent home immediately and the veldschool was subsequently closed when it emerged that CCHF virus was highly prevalent.

An incidental problem, faced at the outset of the study, concerned the need for a convenient serological test for investigating the disease. The difficulties encountered in testing for antibody to CCHF virus in surveys are discussed in another paper,[9] which reports reinvestigation of the RPHI technique of Gaidamovich et al.[15] and describes a standardized RPHI test used in the present study. The results obtained here tend to confirm the impression that the RPHI test is more sensitive than the IF and ID tests. Correlation between the results obtained by IF and RPH. techniques was found to be acceptably good in human, sheep and hare sera, but IF titers were much lower than RPHI titers in cattle sera and in all species there is need to regard RPHI titers of 1:32 or less with caution unless the finding can be confirmed by a second technique.

The lack of potency of antisera observed here in neutralization tests is a well known phenomenon with CCHF virus,[12, 16-20] and may be related to the low infective titers achieved with the virus. It is notable that in some recent reports of the isolation of CCHF virus, neutralization tests were

TABLE 4

*Prevalence of reversed passive hemagglutination inhibition antibody to Crimean-Congo hemorrhagic fever virus in hare sera collected in 1977–1979 in various locations in South Africa*

| Species | Province | | | | Total pos./tested (% positive) |
|---|---|---|---|---|---|
| | Cape | Orange Free State | Natal | Transvaal | |
| *Lepus saxatilus* | 4/16 | 6/22 | 3/45 | 4/34 | 17/117 (15) |
| *Lepus capensis* | 4/24 | 6/10 | - | 1/10 | 11/44 (25) |
| *Pronolagus rupestris* | 0/12 | 0/2 | 0/9 | — | 0/23 |
| *Pronolagus crassicaudatus* | — | — | 0/13 | — | 0/13 |
| *Pronolagus randensis* | — | — | — | 0/3 | 0/3 |

TABLE 5

*Numbers of hare sera which reacted in immunofluorescent (IF) and immunodiffusion (ID) screening tests shown in relation to reversed passive hemagglutination inhibition (RPHI) titers of the sera*

| Test* | RPHI titer | | | | | | Total positive (RPHI 28) |
|---|---|---|---|---|---|---|---|
| | <8 (n = 172) | 8 (n = 3) | 16 (n = 13) | 32 (n = 3) | 64 (n = 8) | 256 (n = 1) | |
| IF | 0 | 2 | 10 | 3 | 7 | 1 | 23 |
| ID | 0 | 1 | 9 | 3 | 8 | 1 | 22 |

* Sera were screened undiluted in ID tests and at a dilution of 1:8 in IF tests.

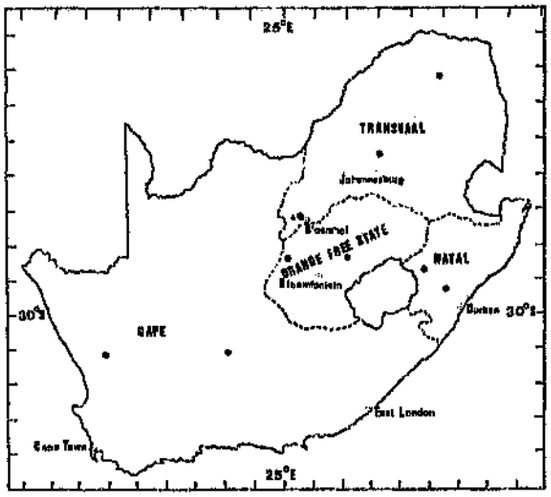



FIGURE 1. Sites at which antibodies (●) to Crimean-Congo hemorrhagic fever virus were detected or virus was isolated (▲) in South Africa.

omitted and reliance was placed instead on IF and CF tests for the identification of virus.[21, 22] Viruses isolated in the present study cross-reacted strongly in CF, ID and IF tests with a reference strain of CCHF virus and less completely in CF tests with Hazara virus. Identification of strains 4/81 and 128/81/7 as CCHF virus has been confirmed by CF tests against a range of antisera at the Centre for Applied Microbiology, Porton Down, U.K. (Dr. D. I. H. Simpson, personal communication).

Crimean-Congo hemorrhagic fever virus is widely distributed in eastern Europe and in Asia, and it has been isolated in a belt of countries in tropical Africa extending from Senegal in the west to Kenya in the east.[14] Prior to the present investigation, the furthest south that evidence of the virus had been found in Africa had been antibodies in Tanzania.[14] Subsequent to the isolation of virus in South Africa, IF antibodies were found in rural children in Zimbabwe.[23] The implication is that the virus may be even more widely distributed in Africa than at present realized.

The results obtained with hare sera indicate that the virus is widely distributed within South Africa, yet the disease has not been recognized previously. It has been suggested that African strains of the virus may be less pathogenic than Eurasian strains, but Hoogstraal rejects this suggestion on the grounds that investigations in Africa have been too limited to allow such a conclusion.[14] It is surprising that recognized cases of the disease did not occur earlier among the thousands of school children who passed through the veldschool annually, but possibly the present patient was particularly susceptible. Elsewhere in South Africa it is quite possible that even severe or fatal cases of CCHF could have passed undiagnosed in relatively unsophisticated rural populations who are at greatest risk. Moreover, there has been a lack of awareness of the disease even where sophisticated medicine applies, and specific laboratory tests were not previously available.

The high incidence of antibodies found in livestock raises the question of the possible veterinary significance of the virus. The role which farm animals play as amplifiers of both virus and ticks appears to be well established, and there are numerous records of human infection arising from contact with apparently healthy animals.[14] The possibility that the virus is pathogenic for animals is implied in a few publications. Thus, in at least two incidents in the Soviet Union, human infection followed the butchering of sick cattle,[14] while in Kenya, the virus was isolated from the blood of a febrile cow in a herd where abortions were occurring.[16] Possibly the present observations on eland should be placed in the same inconclusive category. In the limited experimental infections which have been produced in livestock,[14] emphasis has been placed on viremia studies and on infection of ticks, but it was noted in one study that the virus produced mild febrile illness in two calves.[24] Clearly, there is need for further investigation of infection in livestock, including pregnant animals.

The available evidence suggests that hares and hedgehogs serve as reservoir hosts in Eurasia,[14] and the virus has been isolated from a hedgehog in Nigeria.[24] The present results suggest that hares may also serve as reservoir hosts in Africa, but it

is necessary to extend the observations and to determine whether there is a real difference in the roles played by *Lepus* and *Pronolagus* species.

Crimean-Congo hemorrhagic fever virus has been isolated previously[14] from both of the *Hyalomma* tick species found to be infected here, and one species, *H. m. rufipes*, has been proven to be a vector and to be capable of transmitting the virus transovarially.[24] It appears that the virus has not previously been isolated from *R. evertsi*, but in this instance true infection cannot be distinguished from possible recent engorgement of infected blood in ticks which were removed from sheep. The virus has been associated with a wide variety of ticks elsewhere,[14] and there is need for systematic study of the vector potential of South African ticks.

Investigations of the medical and veterinary significance of the virus and of the roles played by various tick species and potential reservoir hosts in South Africa are in progress.

ACKNOWLEDGMENTS

We thank officials of the Transvaal Departments of Nature Conservation and of Education for their cooperation. Staff of the Department of Health collected human sera, and staff of the Division of Veterinary Services collected livestock sera. Drs. J. A. W. Coetzer and I. Carmichael of the Veterinary Research Institute, Onderstepoort, and G. P. Viljoen of the Department of Agricultural Technical Services, cooperated in investigating antelope deaths.

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*Reprinted from the "SA Medical Journal", Vol. 53, 15 April 1978, pages 598 - 600*

# Infection of the Common Bedbug *(Cimex lectularius* L*)* with Hepatitis B Virus in South Africa

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## SUMMARY

**A total of 1 368 bedbugs of the species *Cimex lectularius* L were collected mainly from huts in villages or on farms at 6 localities in the northern Transvaal. They were tested in pools of 10 for the presence of hepatitis B surface antigen (HBsAg). None of 20 pools from Pietersburg was HBsAg-positive, but 32 out of 120 pools from the 5 other localities were HBsAg-positive. Estimated infection rates per 1 000 bugs were 17,1 (Messina), 24,9 (Waterpoort), 28,4 (Letaba), 54,5 (Potgietersrus) and 67,0 (Louis Trichardt), with an overall rate of 30,6. Seventeen out of 57 pools of the engorged bugs (infection rate 34,8) and 14 out of 62 pools of the unengorged bugs (infection rate 25,3) were HBsAg-positive. These very high infection rates, even in unengorged bugs, suggest that *C. lectularius* could be a vector of hepatitis B virus in the Transvaal, and that the varying degrees of infestation could explain the markedly different HBsAg-positive frequencies previously shown in sera collected from different population groups in that province.**


*S. Afr. med. J.*, 53, 598 (1978).

Evidence is accumulating that bedbugs could be vectors of human hepatitis B virus (HBV).[1] Both the common bedbug, *Cimex lectularius* L — the only domestic species found in temperate regions but which also occurs in some tropical areas — and the tropical species, *Cimex hemipterus* F, have been incriminated. In laboratory experiments, Newkirk *et al.*[2] demonstrated that hepatitis B surface antigen (HBsAg) persisted in *C. lectularius* for 5 weeks after the insects had fed on HBsAg-positive blood. They also obtained evidence for possible replication of the virus. More recently Wills *et al.*[3] collected *C. hemipterus* from the huts of village dwellers in Senegal and showed high field infection rates of HBV. They detected HBsAg in 12 out of 55 unengorged bugs, 8 out of 17 engorged bugs and 3 out of 89 insects which were denied blood for 30 days after they had been collected.

In South Africa *C. lectularius* is the principal bedbug species, and Newkirk *et al.*'s findings prompted us to evaluate this insect as a possible vector in our country. This seemed particularly worth while, since no wholly satisfactory explanation was available to account for the marked difference in prevalence of HBsAg among different population groups in the country.[4] HBV transmission between humans by the bedbug could explain the much higher HBsAg-positivity among the Black population, since bedbug infestations are commoner in housing used by Blacks.

In this article we report high field infection rates of HBV as detected by the presence of HBsAg in pools of *C. lectularius* collected from huts in villages or on farms at 5 out of 6 localities in the northern Transvaal.

## MATERIAL AND METHODS

Bedbugs were collected at 6 localities in the northern Transvaal (Fig. 1). At all of these, except Pietersburg, bedbugs were removed from huts in two or more villages or on farms during June and July 1977 when visits were made for routine malaria control spraying. In April 1977, during bedbug control operations, collections were made at Pietersburg in rooms in hostels situated within the municipal area. The bedbugs were captured alive just before spraying, killed shortly afterwards by freezing, and kept at −20°C. They were transported at 4°C from Pietersburg to Johannesburg where they were stored at −70°C.

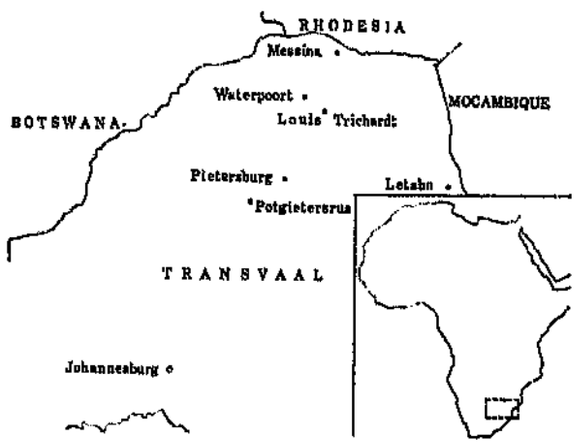



**Fig. 1. Map of the Transvaal, showing the localities where bedbugs were collected.**

Frozen bugs were examined under a stereo microscope for identification and separation into pools, which usually contained 10 insects, according to stage of development (nymph or adult) and state of engorgement. Each pool of insects was then ground in 1,0 ml physiological saline in a mortar and the homogenate was centrifuged at 2 500

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rpm for 30 minutes. The supernatants were immediately frozen at −70°C. Later these were thawed and tested in duplicate by radio-immunoassay (Ausria 11 - 125, Procedure B, Abbott Laboratories, North Chicago, Illinois). However, the criterion of positivity differed from that recommended by Abbott Laboratories in that only those values greater than 3,0 times the negative control mean were considered unequivocally positive. This higher cut-off value was chosen because of the results obtained when repeat tests were carried out on borderline positives, as described in the 'Results' section. The reactivity of selected positive specimens was confirmed with the Ausria 11 - 125 confirmatory test kit.

The infection rates for the bedbugs sampled were estimated statistically by means of the method recommended by Chiang and Reeves[6] for mosquito vectors.

## RESULTS

Several thousand bedbugs were collected, but only a sample of 1 368 (all the common bedbug *C. lectularius*) was identified and tested. According to Usinger's[5] map *C. lectularius* is the only domestic species which occurs in South Africa, except in the extreme northern or north-eastern part of the Transvaal where the tropical bedbug, *C. hemipterus*, occurs as well. Although some of our collections came from these areas, i.e. Messina and Letaba, they did not contain *C. hemipterus*. This probably indicates that the tropical bedbug occurs only rarely within South Africa and that we would need to examine large samples from these localities to encounter it.

The use of either bovine phosphate albumin or physiological saline for processing the bugs gave essentially the same results in the radio-immunoassay test. Comparison of freshly prepared supernatants with supernatants which had been frozen at −70°C for 2 days indicated that freezing did not lower the reactivity of the supernatants. However, when 20 of the 31 specimens which showed slight positive reactivity (values of 2,15 - 3,0) were re-tested on different days, variations from 0,02 to 0,91 were obtained. Owing to this, only values greater than 3,0 were regarded as positive. Seven such specimens were confirmed as HBsAg-positive by means of the confirmatory neutralization test.

TABLE I. RESULTS OF HBsAG TESTS ON *CIMEX LECTULARIUS* COLLECTED IN THE NORTHERN TRANSVAAL

| Locality | Proportion of villages/ farms with HBsAg (+) bugs | Unengorged (U) or engorged (E) | Adults (A) or nymphs (N) | No bugs collected | No pools | | No pools HBsAg (+) | | Infection rate per 1 000* |
|---|---|---|---|---|---|---|---|---|---|
| Pietersburg municipality | | U | A | 80 | 7 | 20 | 0 | | 0 |
| | | E | A | 71 | 4 | | 0 | | |
| | | U | A/N | 36 | 4 | | 0 | | |
| | | E | N | 52 | 5 | | 0 | | |
| Potgietersrus | 2/2 | U | A | 58 | 5 | 21 | 1 | 9 | 54,5 |
| | | E | A | 71 | 7 | | 1 | | |
| | | U | N | 49 | 5 | | 4 | | |
| | | E | N | 36 | 3 | | 2 | | |
| | | U/E | N | 7 | 1 | | 1 | | |
| Letaba | 2/2 | U | A | 120 | 8 | 20 | 2 | 5 | 28,4 |
| | | E | A | 80 | 8 | | 3 | | |
| | | U | N | 10 | 1 | | 0 | | |
| | | E | N | 30 | 3 | | 0 | | |
| Waterpoort | 2/2 | U | A | 140 | 10 | 18 | 1 | 4 | 24,9 |
| | | E | A | 30 | 3 | | 1 | | |
| | | U | N | 10 | 1 | | 0 | | |
| | | E | N | 40 | 4 | | 2 | | |
| Messina | 2/3 | U | A | 70 | 7 | 19 | 0 | 3 | 17,1 |
| | | E | N | 50 | 5 | | 1 | | |
| | | U | A | 31 | 3 | | 0 | | |
| | | E | N | 40 | 4 | | 2 | | |
| Louis Trichardt | 3/5 | U | A | 89 | 7 | 22 | 3 | 11 | 67,0 |
| | | E | A | 53 | 5 | | 3 | | |
| | | U | N | 20 | 3 | | 2 | | |
| | | E | N | 98 | 5 | | 2 | | |
| | | U | A/N | 10 | 1 | | 1 | | |
| | | E | A/N | 5 | 1 | | 0 | | |
| Total | | | | 1 368 | 120 | | 32 | | 30,6† |

* Estimated statistically (see 'Material and Methods' section).
† Overall infection rate. (Infection rate for unengorged bugs only = 25,3 and that for engorged bugs only = 34,8.)

The use of the HBsAg test on bedbugs collected at the 6 localities is shown in Table I. No bugs from Pietersburg were HBsAg-positive, but HBsAg-positive bugs occurred in two collections from villages and farms at Potgietersrus, Letaba and Waterpoort. Insects from 2 of the 3 farms sampled at Messina were also HBsAg-positive, as were those from 3 of the 5 farms at Louis Trichardt. An overall total of as many as 32 out of 120 pools contained HBsAg-positive bugs and the infection rates per 1 000 bugs were very high, varying from 17,1 at Messina to 67,0 at Louis Trichardt, with an overall rate of 30,6.

Table I also shows the proportion of the pools of unengorged and engorged insects which were HBsAg-positive at each locality. Overall, these proportions were 14 out of 62 pools for unengorged bugs, 17 out of 57 pools for engorged bugs and 1 remaining HBsAg-positive pool which was a mixture of engorged and unengorged insects. The infection rate per 1 000 for unengorged insects was 25,3 and that for engorged insects 34,8, indicating a very high rate, even in unengorged insects.

## DISCUSSION

Bersohn *et al.*[4] reported on the prevalence of HBsAg in sera from rural southern African Blacks. Our results for localities in the Transvaal tend to concur with their findings as shown in their map, except in the case of Potgietersrus for which they found a 2,1 - 3,0% HBsAg-positive frequency but from which locality we collected bedbugs that possessed the second highest infection rate (54,5 per 1 000).

Apart from 89 bugs which were starved for a prolonged period after collection, Wills *et al.*'s[3] tests were carried out on only 72 specimens. Our results on a larger sample of another species give similar results, with high infection rates for HBsAg and an appreciably higher rate for engorged over unengorged bugs.

While the positive results for the engorged insects probably merely reflect HBsAg taken up with the insects' blood meal, the infections which occurred in both unengorged nymphal and adult bugs suggest that the bedbug may either be a biological vector of HBV in which the virus multiplies, or an unusually efficient mechanical vector by virtue of the extraordinary stability of HBV. Further laboratory experiments are necessary to confirm that multiplication of the virus occurs in bedbugs and to investigate whether transmission can take place to animals. In addition, concurrent testing for HBsAg both in bedbugs and in sera from humans living in the same hut, if done on a large enough scale, might well yield epidemiological evidence indicating transmission of HBV by these insects. Positive evidence for virus replication in the bedbug and transmission by this insect would place HBV in the arbovirus category.

We wish to thank Dr G. Joubert, Director of Regional Health Services for the northern Transvaal, for arranging the bedbug collecting and the health inspectors in the various regions for undertaking it. Collecting in Pietersburg municipality was arranged by Mr D. J. Fraser, to whom we are most grateful. We are also indebted to the staff of the Arbovirus Unit of the Institute for Virology for technical assistance and to Dr J. de Beer, Secretary for Health, for permission to publish.

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*Reprinted from the "SA Medical Journal", Vol. 56, 14 July 1979, pages 54 - 57*

# Transmission Experiments with Hepatitis B Surface Antigen and the Common Bedbug *(Cimex lectularius L)*

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## SUMMARY

**Hepatitis B surface antigen (HBsAg)-positive blood meals were fed to a colony of the common bedbug (*Cimex lectularius* L) in a series of 5 experiments. Antigen persisted in the bugs for at least 7½ weeks, but was undetectable after 18 weeks. Trans-stadial transmission was demonstrated through one moult only, and transovarial transmission did not occur. Antigen was successfully transmitted by adult bugs into 3 out of 35 canisters of HBsAg-negative blood. Antibody of HBsAg was detected in the serum from a rabbit on which HBsAg-positive adult bugs had fed as well as in the serum of 2 out of 10 guinea-pigs on which HBsAg-positive 4th and 5th nymphal instars had fed The results as a whole indicate that biological multiplication and biological transmission do not occur in C. *lectularius*, but mechanical transmission has been demonstrated. This is probably an important means of hepatitis B virus transmission among humans in South Africa.**


*S. Afr. med. J.*, 56, 54 (1979).

Recent work in our laboratory[1] showed high positivity rates for hepatitis B surface antigen (HBsAg) in the common bedbug, *Cimex lectularius* L, collected from huts located in the northern Transvaal. Other workers[2] demonstrated the antigen in the tropical bedbug, *Cimex hemipterus* F, collected from huts in Senegal. The presence of HBsAg is usually considered to indicate infection with hepatitis B virus. However, infection alone in these insects is insufficient evidence for accepting the bedbug as a vector of hepatitis B virus, although it suggests that this is a possibility. Firmer evidence would be a demonstration of the insect's ability to transmit HBsAg by bite. In this article we report the results of laboratory experiments with *C. lectularius* designed primarily to investigate this problem, but also to study the persistence of the antigen in this bedbug and its possible trans-stadial and transovarial transmission.

## MATERIALS AND METHODS

### Bedbug Colony

A subcolony of *C. lectularius* was established with adults taken from a colony maintained at the South African Bureau of Standards in Pretoria. The bugs were reared and maintained in the dark in an insectary where the temperature was 25 - 26,5°C and the relative humidity 75 - 80%. Rabbits were provided as a source of bloodmeals.

### Bedbug Feeding

HBsAg-positive blood was obtained from either renal dialysis patients or, more often, patients who no longer required dialysis because they had undergone kidney transplants. In experiment 1, 0,6 ml of HBsAg-positive serum from a patient was added to 30 ml of negative defibrinated blood, while in experiments 2 - 5 blood was defibrinated or heparinized on bleeding and offered to the bedbugs the same day. In each experiment a sample of the blood used was stored at −70°C for subsequent antigen assay and in experiments 1 and 2 some groups of bugs were fed on antigen-negative blood as controls. A membrane feeding method was used for feeding the bugs; this was essentially the same as that used in our laboratory for feeding mosquitoes.[3] With this method bugs were placed in Perspex canisters closed with organdie netting at one end and with Badruche membrane at the other. The blood was placed within a Perspex ring inside a plastic dish. The canister was then placed on top of the ring so that full contact was achieved between blood and membrane. The dishes were held in a water bath so that the blood temperature was 32 - 33°C. Feeding took place in the insectary in the dark; afterwards groups of bugs were kept for varying periods of extrinsic incubation and allowed to moult or oviposit before being refed in the transmission attempts. For the latter, either the bugs were allowed to refeed on HBsAg-negative defibrinated blood through a membrane or they refed on a rabbit or on guinea-pigs. The membrane feeding technique for transmission feeds was the same as that used for the first feed, except in the cases where a smaller, 0,2 - 0,5-ml volume of blood was used. In this case the blood was placed in smaller Perspex rings, with an internal diameter of 10 mm and an internal height of 4,5 mm, cemented on a Perspex sheet, and the insects in canisters of the same diameter. As bugs engorged they were replaced by further unfed insects. For transmission feeds on animals, the bugs were placed in glass centrifuge tubes with a 5 cm internal diameter and the tops were closed with organdie netting. These tubes were inverted and applied to the ears of a rabbit or the shaved abdomen of anaesthetized guinea-pigs.

### Virological Techniques

Bugs were killed for antigen assay at varying intervals after the HBsAg-positive blood-meal, those which fed

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a second time in the transmission tests usually being killed on the day of the second feed. The insects were killed by placing them in a −70°C freezer where they were stored until extracts could be prepared from them for antigen assay. For this, each bug was ground in a tissue-grinding tube in bovine phosphate albumin (BPA). The volume of BPA was 0,5 ml for pools of 1 - 5 first nymphal instars or single specimens of 2nd or 3rd instars, and 1,0 ml for pools of 29 - 62 first instars or single 4th instars, 5th instars and adult bugs. The homogenates were centrifuged at 3 000 rpm for 30 minutes and the supernatants frozen immediately at −70°C. Later they were thawed and tested for HBsAg by radio-immunoassay (Ausria II - 125 (Procedure B); Abbott Laboratories, North Chicago, Illinois) and the results were interpreted according to the manufacturers' instructions. Apart from the bug supernatants, other specimens tested were HBsAg-positive defibrinated blood samples used for the first blood-meal and control blood samples, as well as the blood samples used in transmission attempts with their controls.

The guinea-pigs used in transmission attempts were bled 2 days before and 21 and 61 days after the transmission feed, while the rabbit was bled 1 hour before the feed and 39 and 110 days afterwards. These samples were tested in duplicate for hepatitis B surface antibody (HBsAb) by radio-immunoassay (Ausab, Abbott Laboratories).

## RESULTS

### Antigen in Adult Bugs and Transmission by Adults and Nymphal Instars

Bedbug feeding rates were rather higher on defibrinated blood than on heparinized blood, so the former was used in most experiments. Furthermore, rates were much lower on blood obtained from patients undergoing dialysis (experiment 2). Batches of insects were given antigen-positive blood-meals in 5 experiments on different dates.

Table I shows the antigen positivity rates in adult bedbugs in three experiments at intervals after the HBsAg-positive blood-meal, control bugs fed HBsAg-negative blood in experiment 2 all being antigen-negative. These positivity rates remained high for 7 weeks. After varying periods of extrinsic incubation, attempts were made to transmit the antigen through a membrane to HBsAg-negative blood and in two of these attempts three successful transmissions were accomplished. The first occurred in experiment 3, when 0,5 ml of blood became HBsAg-positive after two infected bugs had engorged. The second and third were in experiment 4, when one and two infected bugs fed on 0,2 and 0,4 ml samples of blood respectively. A fourth transmission occurred when 25 bugs were refed on a rabbit 24 days after their first HBsAg-positive blood-meal. The rabbit serum was HBsAb-negative on the 39th day, but HBsAb-positive on the 110th day after the transmission feed.

TABLE I. PERSISTENCE OF HBsAg IN ADULT BUGS AND ATTEMPTS TO TRANSMIT ANTIGEN BY BITE TO HBsAG-NEGATIVE BLOOD AND A RABBIT

| | | | Transmission attempts to HBsAg (−) blood | | | |
|---|---|---|---|---|---|---|
| Experiment No.¶ | Days after HBsAg (+) feed on which bugs tested | HBsAg positivity rate in bugs | Days after HBsAg (+) feed | Number of HBsAg (+) bugs which fed on each blood-meal | Number of canisters of blood (volume in brackets) | Number of samples becoming HBsAg (+) |
| 2 | 3 | 3/3* (0/1)† | | | | |
| | 10 | 6/6 (0/1) | | | | |
| | 17 | 3/4 (0/1) | | | | |
| | 24 | 2/3 (0/1) | | | | |
| | 33 | 1/1 | 33 | 2,3 | 2 (8 - 10 ml) | 0 |
| | 35 | 5/5 (0/5) | 34 | 1,2 | 2 (8 - 10 ml) | 0 |
| | 36 | 3/3 | | | | |
| | 42 | 4/7 (0/3) | 42 | 1 | 3 (0,5 ml) | 0 |
| 3 | 10 | 12/13 | 10 | 1 | 1 (0,5 ml) | 0 |
| | | | | 2 | 1 (0,5 ml) | 1 |
| | | | | 46‡ | 1 (c. 7,0 ml) | 0 |
| 4 | 11 | 24/24 | 10 | 1 - 5 | 10 (0,2 - 0,4) ml) | 2 |
| | 15 | 54/55 | 15 | 1 - 7 | 15 (0,2 - 0,4 ml) | 0 |
| | 27 | 6/6§ | | | | |
| | 34 | 0/6 | | | | |

* Number positive/number tested.
† Control bugs fed HBsAg (−) blood.
‡ Six of these bugs tested and found positive.
¶ In each experiment a batch of bugs was fed on positive blood from 1 or 2 patients on a different date.
§ On the 24th day 25 bugs, of which these 6 bugs were a sample, were fed on a rabbit. The rabbit's serum was HBsAb (−) on the 39th day, but HBsAb (+) on the 110th day after this transmission feed.

TABLE II. ATTEMPTS TO TRANSMIT HBsAg BY BITE FROM BUGS WITH POSITIVE BLOOD TO GUINEA-PIGS (EXPERIMENT 5)

| Bug instar | Days after HBsAg (+) feed on which bugs tested | HBsAg positivity rate in bug sample tested | Transmission attempts (52)* | | |
|---|---|---|---|---|---|
| | | | Guinea-pig No. | Number of bugs engorged | HBsAb guinea-pig |
| 3rd | 52 | 2/2 | 321 | 40 - 80 | Negative |
| 3rd | | 2/2 | 330 | 40 - 80 | Negative |
| 3rd | | 1/2 | 334 | 40 - 80 | Negative |
| 3rd | | 2/2 | 343 | 40 - 80 | Negative |
| 3rd | | 1/1 | 347 | 40 - 80 | Negative |
| 4th and 5th | | 0/1 | 328 | 40 - 80 | Negative |
| 4th and 5th | | 1/1 | 344 | 40 - 80 | Positive† |
| 4th and 5th | | 1/1 | 345 | 40 - 80 | Positive‡ |
| 4th and 5th | | 1/1 | 346 | 40 - 80 | Negative |
| Adults | | 1/1 | 332 | c. 20 | Negative |

* Number of days after HBsAg (+) feed.
† Negative for antibody at 21 days after transmission feed and positive at 61 days.
‡ Positive for antibody at 21 days and negative at 61 days.

Table II shows the results of attempted transmission to 10 guinea-pigs by means of nine tubes each containing 40 - 80 nymphal instars and one tube containing about 20 adult bugs. A sample of one or two of the bugs which fed on each guinea-pig was tested for positivity (Table II) and the results of these tests are included in the positivity rates for each instar shown in Table IV. It is evident that most insects tested were still HBsAg-positive on day 52 when the transmission feeds occurred. Of the 10 guinea-pigs 2 became HBsAb-positive, one 21 days and the other 61 days after 4th and 5th nymphal instars had engorged in the transmission feed. Antibody had disappeared in the first guinea-pig by the 61st day.

## Antigen in Nymphal Instars and Trans-stadial Transmission

The results of experiment 1 are shown in Table III. A large number of 1st nymphal instars were given a HBsAg-positive blood-meal. Some of the 2nd instars subsequently obtained were tested for antigen, as were samples of the succeeding instars and adults obtained after blood-meals on an HBsAg-negative rabbit. This rabbit was HBsAb-negative when tested 3 months after the last feed. Antigen was present in 60% of the 2nd nymphal instars tested and then it disappeared from the bugs after the next moult.

TABLE III. TRANS-STADIAL TRANSMISSION OF HBsAg FROM FIRST INSTAR NYMPHS (EXPERIMENT 1)

| Instar | Days after HBsAg (+) meal on which bugs tested | HBsAg positivity rate in bugs |
|---|---|---|
| 2 | 11 | 6/10 (0/10)* |
| 3 | 19 | 0/10 (0/5) |
| 4 | 27 | 0/10 (0/5) |
| 5 | 60 | 0/5 (0/5) |
| Adults | 74 | 0/5 (0/5) |

* Control bugs fed HBsAg (−) blood.

Table IV gives the results of experiment 5. Groups of bugs belonging to each of the nymphal instars were given an antigen-positive blood-meal on the same day, and the subsequent instars produced as each group moulted were tested for antigen. All the bugs tested among the succeeding instars were HBsAg-positive on the 10th day after the blood-meal, and it was shown that the 3rd, 4th and 5th instars and adults were still mostly positive on the 52nd day. On the 127th day, however, all 5th instars and adult bugs tested were negative. One batch of 3rd instars among the succeeding instars was refed on an HBsAg-negative guinea-pig and the subsequent 4th instars were negative on the 127th day.

TABLE IV. TRANS-STADIAL TRANSMISSION OF HBsAg AND ITS PERSISTENCE IN THE NEXT NYMPHAL INSTAR (EXPERIMENT 5)

| Instar given HBsAg (+) feed | Succeeding instar tested for HBsAg | Days after HBsAg (+) feed on which bugs tested | HBsAg positivity rate in bugs |
|---|---|---|---|
| 1st | 2nd | 10 | 8/8 |
| 2nd | 3rd | 10 | 8/8 |
| | 3rd | 52 | 9/10 |
| | 4th | 127 | 0/10 |
| 3rd | 4th | 10 | 8/8 |
| | 4th | 52 | 8/11 |
| 4th | 5th | 10 | 8/8 |
| | 5th | 52 | 7/7 |
| | 5th | 127 | 0/10 |
| 5th | Adults | 10 | 12/12 |
| | Adults | 52 | 7/7 |
| | Adults | 127 | 0/10 |

## Transovarial Transmission

In experiments 2, 3 and 4, adult bugs were given antigen-positive feeds and when the eggs subsequently deposited by them hatched into 1st nymphal instars these were

TABLE V. ATTEMPTED TRANSOVARIAL TRANSMISSION: ANTIGEN TESTS IN FIRST NYMPHAL INSTAR PROGENY OF ADULT BUGS PREVIOUSLY FED AN HBsAg-POSITIVE MEAL

| | Experiment | | |
|---|---|---|---|
| | 2 | 3 | 4 |
| Age (days) of 1st instars when tested ... ... ... ... | 11 - 17 | 1 - 2 | 1 - 8 |
| Interval (days) after parent bugs fed HBsAg (+) meal | 33 | 17 | 14 - 15 |
| Number of pools of 1st instars ... ... ... ... ... | 7 | 3 | 13 |
| Number of insects per pool ... ... ... ... ... ... | 1 - 5 | 29 - 30 | 50 - 62 |
| Result of antigen test ... ... ... ... ... ... ... | Negative | Negative | Negative |

assayed for antigen. The results of tests on 23 pools of bugs were all negative as shown in Table V.

## DISCUSSION

There are two previous reports of laboratory studies on *C. lectularius* and HBsAg. Downe[4] reported the detection of antigen in bedbug faeces, while Newkirk *et al.*[5] showed that antigen persisted for 5 weeks in adult bugs and that 4th or 5th nymphal instars retained antigen after moulting. Our work confirms the results obtained by Newkirk *et al.*,[5] as well as adding new findings. Our findings are as follows: (*i*) HBsAg persisted for at least 6 weeks in adult *C. lectularius* and for at least 7½ weeks in adults and nymphal instars fed antigen while in the preceding instars; (*ii*) antigen persisted after one moult (trans-stadial transmission) but was not retained by bugs after two successive moults; (*iii*) antigen was not transmitted transovarially; (*iv*) antigen was transmitted by adult bugs to HBsAg-negative blood and to a rabbit, and by 4th or 5th instars to guinea-pigs, although the results indicate that the transmission rate was low.

Our results indicate that there was no biological multiplication of hepatitis B virus in *C. lectularius* and no biological transmission by the bug. Rather it seems that HBsAg persists in these insects and can be transmitted mechanically. The evidence for this is as follows: firstly, positive/negative values for the radio-immunoassays done on the bugs of differing instars (experiment 5) were approximately proportional to the size of the bugs. If multiplication of antigen had occurred in the different instars this relationship wouldn't have been expected. Secondly, after the bugs had been kept for an extended period (127 days) after the HBsAg-positive blood-meal, the antigen disappeared. Thirdly, transmission of antigen into HBsAg-negative blood should have been more frequent if antigen had multiplied in the salivary glands of the bug prior to the transmission feed. The limited number of transmissions which occurred are thought to have been mechanical. Villarejos *et al.*[6] came to the same conclusion as regards the multiplication of hepatitis B virus in the reduviid bug *Rhodnius prolixus* after they were unable to demonstrate HBsAg in either the haemolymph or the salivary glands of this insect.

The successful transmissions obtained in our experiments suggest that mechanical transmission by bedbugs could well play an important role in the epidemiology of human serum hepatitis due to hepatitis B virus. In our work the Dane particle had been identified by electron microscopy in large numbers in the positive serum which was mixed with defibrinated blood for the HBsAg-positive feed in experiment 1. However, it was not known whether the blood-meals in experiments 2 - 5 contained this particle. Furthermore, measurement of the B antigen[7] was not attempted, although this will be done in future experiments. Downe[4] found that the Dane particle could pass unchanged from a blood-meal into the faeces of *C. lectularius*; this suggested to us that it might also be transmissible mechanically by bite. If complete Dane particles were inoculated by bugs into susceptible humans they would be expected to multiply, causing a higher transmission rate than could be inferred from our work on non-susceptible rabbits and guinea-pigs. It is not possible to demonstrate a transmission rate which can be equated with the natural one except with chimpanzees, the only host laboratory animal.[8] Transmission of hepatitis B virus under natural conditions could be due to mechanical transmission by bite of *C. lectularius* as well as by contamination from infective faeces. Both these routes could be important in the spread of hepatitis B virus in South Africa.

We wish to thank Linda Stannard of the University of Cape Town for a supply of serum containing Dane particles, Professor A. M. Meyers and Sister S. Blom of the Johannesburg General Hospital for a supply of HBsAg-positive blood specimens and the donors for their co-operation. We are also grateful to Mr G. M. T. Moerdyk of the South African Bureau of Standards for supplying bedbugs and to Professor O. W. Prozesky and Dr J. J. Alexander of the National Institute for Virology for their helpful suggestions and encouragement. Lastly we thank Dr J. de Beer, Secretary for Health, for permission to publish.

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# Briewerubriek : Correspondence

*Die menings gelug in die Briewerubriek van die SAMT is nie noodwendig dié van die Mediese Vereniging van Suid-Afrika nie.*—Redakteur

*The views expressed in the Correspondence published in the SAMJ are not necessarily those of the Medical Association of South Africa.*—Editor

## ABSENCE OF BIOLOGICAL MULTIPLICATION OF HEPATITIS B VIRUS IN THE COMMON BEDBUG

To the Editor: As was stated in a recent review,[1] there is now a fair amount of evidence to incriminate both the common bedbug *Cimex lectularius* L.[2-4] and the tropical bedbug *C. hemipterus* F.[5,6] as vectors of human hepatitis B virus (HBV). In recent experiments in our laboratory[4] we achieved a few transmissions of the hepatitis B surface antigen (HBsAg) by *C. lectularius* from antigen-positive blood into canisters of antigen-negative blood as well as to guinea-pigs and a rabbit. We concluded that these transmissions were mechanical and that biological multiplication and biological transmission of HBV probably do not occur in the bug. We have since performed a further experiment with *C. lectularius* which we consider provides stronger evidence for the absence of biological replication of HBV in this insect.

Our experiment was carried out in an insectary at 25 - 26°C with a relative humidity of 75 - 80%. *C. lectularius* 4th and 5th nymphal instars from a laboratory colony were allowed to feed through a membrane on defibrinated infected blood, using a technique previously described.[4] The blood had been obtained from the carrier donor on the day of the experiment, but a sample tested 2 months earlier possessed the following characteristics which indicated it was highly infected: HBe-positive, anti-HBe-negative, HBsAg (PHA) log$_{10}$: 5, hep. B-DNA polymerase pM/h/ml: 0,197.

On radio-immunoassay the P/N value for HBsAg in the blood on the day of the feed was 24,9. Ten days after this infective feed, when the bugs had moulted to 5th nymphal instars and adults, they were killed by being placed in a −70°C freezer, where they were stored. Ten of these adult bugs were tested individually for HBsAg and the resulting P/N values were all positive and high, with a mean of 34,8 (Table I). The remaining 179 bugs were ground in bovine phosphate albumin, 45 - 56 bugs per 2 ml, and centrifuged at 3 000 rpm for 30 minutes. The supernatant fluid obtained was then added to antigen-negative defibrinated blood in the proportion 1 : 2 to provide the second serial blood meal.

A second new batch of 4th and 5th nymphal instars was fed this second serial blood meal, which had a P/N value of 51,7. Thirteen days later the 5th instars and adult bugs were killed and stored at −70°C, except for a sample of 10 bugs which were tested for antigen. Only 7 out of these 10 were positive and P/N values had dropped so much that the mean value was only 3,0. The 262 bugs remaining were then used to prepare the third serial blood meal as described above, and a third batch of new bugs were fed this blood-virus mixture which had a P/N value of 9,2; this was significantly lower than that for the first and second blood meals. After 10 days the third batch of new 5th instars and adult bugs was killed and stored. All 22 bugs in a sample tested for HBsAg were negative, with a mean P/N value of 1,2 (Table I).

We consider that the disappearance of HBsAg after three passages in these insects indicates the absence of replication of HBV in *C. lectularius*. However, it is possible that the extrinsic incubation periods of 10 - 13 days allowed in our experiment may have been too short to allow the completion of viral multiplications within the tissues of the bugs. For this reason, we plan to repeat our experiment using a 60-day extrinsic incubation period, which is within the range for the duration of incubation of HBV in the human host.

Even though it may not be able to support biological multiplication of the virus, *C. lectularius* is probably of considerable importance as a mechanical vector of HBV among humans.

TABLE I. P/N VALUES BY RADIO-IMMUNOASSAY IN BLOOD MEALS AND BEDBUG SAMPLES

| | | Passage level | | | |
|---|---|---|---|---|---|
| | | 1 | 2 | 3 | |
| Blood meals | | 24,9 | 51,7 | 9,2 | |
| Individual bugs in samples | 1 | 26,2 | 3,5 | 1,1 | |
| | 2 | 38,5 | 4,3 | (22 | [illegible] |
| | 3 | 40,9 | 3,9 | | |
| | 4 | 36,6 | 1,8* | | |
| | 5 | 43,3 | 1,9* | | |
| | 6 | 35,4 | 3,0 | | |
| | 7 | 38,6 | 4,1 | | |
| | 8 | 36,6 | 1,5* | | |
| | 9 | 45,1 | 4,0 | | |
| | 10 | 5,2 | 2,3 | | |
| Mean for bug samples | | 34,8 | 3,0 | 1,2 | |

* Negative P/N values.

We acknowledge with thanks the suggestion of Professor K. R. Dumbell of St Mary's Hospital, London, that we perform an experiment of the type described, and permission to publish granted us by the Secretary for Health. We are also indebted to Professor J. Desmyter of the Katholieke Universiteit te Leuven for arranging for sera to be tested for the e antigen and DNA polymerase level.

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Reprinted from the "S.A. Medical Journal", Vol. 63, 15 January 1983, pp. 77-81.

# The mechanical transmission of hepatitis B virus by the common bedbug (*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.


*S Afr Med J* 1983; 63: 77-81.

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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[1] 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.[1] 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 (2,8) | 2,9 | | |
| | | 14,5 | 2,3 | | |
| | | 12,9 (1,5‡) | 2,3 | | |
| | | 12,4 (3,7) | 2,0‡(1,2‡) | | |
| | | 9,9 (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 = $0,18 \times 10^{-1}$ 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%) | 0/3† | 0/33 |

*Donor blood had the following characteristics: HBsAg P/N = 76,0; HBeAg P/N = 18,5; 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*[1,2,5] 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 = 66.6; HBeAg P/N = 20.1; anti-HBeAg-negative, and hepatitis B DNA polymerase = 1.53 × 10⁻³ pmol/h/ml.
†On day 32 bugs were fed on uninfected blood
ND = not done; + = positive; - = negative

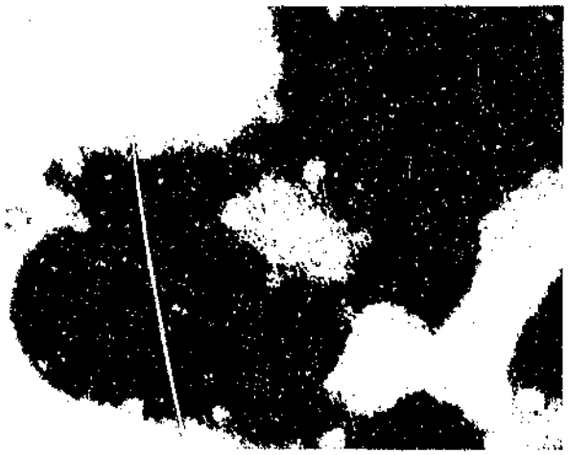


**Fig. 1. Spherical 22 nm particles from bedbug faeces negatively stained with phosphotungstic acid at pH 6,0 (× 180 000).**

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.[3,5,13] 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[14] 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.

We wish to thank Mr L. A. S. van Wyk, formerly of the Pietersburg office of the State Health Department, for arranging the bedbug collections, Mr. G. M. T. Moerdyk of the South African Bureau of Standards for supplying bedbugs, and Professor O. W. Prozesky of the National Institute for Virology for his helpful suggestions and encouragement. Lastly we thank Dr J. de Beer, Director-General of the Department of Health and Welfare, for permission to publish.

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*J. ent. Soc. sth. Afr.* 1990
*Vol. 53, No. 2, pp. 137–140*

# Detection of multiple blood-meals in experimentally fed bedbugs (Hemiptera: Cimicidae)

by

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The blood-meals of bedbugs (*Cimex lectularius*) were examined for serum protein haptoglobins (Hp) in order to detect potential multiple feeding by these insects. Hp types 2.1 and 2.2 and multiple feeds on both these types of blood were detectable up to at least 24 h after experimental feeding. If natural multiple feeds in bugs which have fed on human hosts with different Hp types can be detected this would indicate the likelihood of hepatitis B virus being transmitted mechanically between humans by interrupted feeding.

## INTRODUCTION

Studies have indicated that bedbugs (*Cimex lectularius* L. and *Cimex hemipterus* [Fabr]) may be vectors of hepatitis B virus (HBV) among the human population in certain parts of South and West Africa. Among such studies, *in vitro* vector competence experiments indicated that these insects could be capable of transmitting the virus (Jupp & McElligott 1979; Jupp *et al.* 1983; Ogston *et al.* 1979; Ogston 1981). More recently, Jupp *et al.* (in press) carried out *in vivo* experiments in which they attempted to transmit HBV to chimpanzees by the bite of *C. lectularius*. In the 2 experiments with *C. lectularius*, which were unsuccessful, a 10 day period was allowed to elapse to permit digestion of blood, egg development and oviposition before the bugs were refed on susceptible chimpanzees. However, it is possible that mechanical transmission between humans could occur by interrupted feeding. In such instances there would be an interval lasting only a few minutes between the start of feeding on an HBV carrier and the completion of the blood-meal on a non-immune person.

Although there is some evidence that interrupted feeding may occur in the laboratory (Dickerson & Lavoipierre, 1959), it is not known whether it occurs under natural conditions. One, and perhaps the only, way of proving whether interrupted or multiple feeds occur in bedbugs would be to collect engorged bugs in the field and demonstrate the presence of blood that originated from 2 different host individuals in such insects. By identifying the serum protein haptoglobin (Hp) types present in blood-meals, Boreham *et al.* (1978) have shown that a significant proportion of meals taken in the field by anthropophilic populations of mosquitoes may be multiple ones. There are 3 main haptoglobin (Hp) types in humans, 2.2, 2.1 and 1.1, although some people have no haptoglobins and are designated 0.0. Boreham and Lenahan (1976) identified multiple feeds by the detection of 2 different Hp types representing 2 human

hosts present in one mosquito blood-meal. The present paper describes the use of the Hp system to detect experimental multiple feeds in the common bedbug, *Cimex lectularius* L.

## MATERIAL and METHODS

### Haptoglobin identification

The haptoglobins were analysed by gradient gel electrophoresis in non-denaturing vertical gels essentially as described by Boreham and Lenahan (1976). The day prior to testing the bedbug specimens were transferred from liquid nitrogen to a freezer at −70 °C. Blood-meal samples were obtained by cutting off the bug's abdomen and submerging it in 50 $\mu\ell$ of electrophoresis buffer (0,089*M* tris/0,089*M* boric acid/0,002*M* EDTA) containing 15% glycerol. The sample was then lightly homogenized, the solid remnants removed and the sample of blood loaded on to the gel using a Hamilton syringe. The control serum samples were prepared by soaking Whatman Number 1 filter paper rectangles (8 × 6 mm) in the sera and inserting them into the gel. The Bio-Rad Protea II vertical slab gel cell which was used is 160 mm in length and is fitted with a central cooling core.

Gradient gels with various concentrations of polyacrylamide were compared in the electrophoresis in order to obtain the best resolution of the haptoglobin bands. Gradients of 5–28%, 5–12% and 5–10% were compared with and without the use of a 3,5% stacking gel. The best results were obtained using a 5–12% gradient gel with a 3,5% stacking gel. A series of gels were run for various times at various currents and it was found best to run gels overnight at 30 mA. Higher currents caused heating of the gel and reduced resolution of the bands in spite of cell-cooling. Gels were electrophoresed until the red haemoglobin band reached the bottom of the gel. It was found necessary to electrophorese at 4 °C with cold water circulating through the central core of the electrophoresis cell. After electrophoresis the gel was removed from the cell and stained with *o*-tolidine for the peroxidase activity of haemoglobin as described by Boreham and Lenahan (1976).

### Bedbugs and artificial feeding

The bedbugs used in the laboratory experiment came from a laboratory colony of *C. lectularius* maintained by the South African Bureau of Standards and were housed in an insectary throughout the experiment at 26 °C and 75–80% relative humidity. Adult male and female bugs were fed through a membrane as previously described (Jupp & McElligott 1979) except that the blood-meal consisted of defibrinated blood of known Hp type and the membrane used was of stretched parafilm. Three tubes each containing 25 bugs were allowed to feed in each of 4 groups as follows: (1) fed on blood of Hp type 2.1, (2) fed on blood of Hp type 2.2, (3) fed on an equal mixture of Hp type 2.1 and 2.2, (4) the bugs were allowed to feed for 3 min on Hp 2.1 before being transferred to blood of type 2.2. Group (4) represented an interrupted feed. A sample of bugs from each group was stored in liquid nitrogen immediately after feeding (0 h), at 2 h and at 24 h after feeding.

## RESULTS

Fig. 1 shows the first gel developed in the laboratory experiment. Four bugs were tested at three different intervals after feeding. Meals of blood which was Hp type

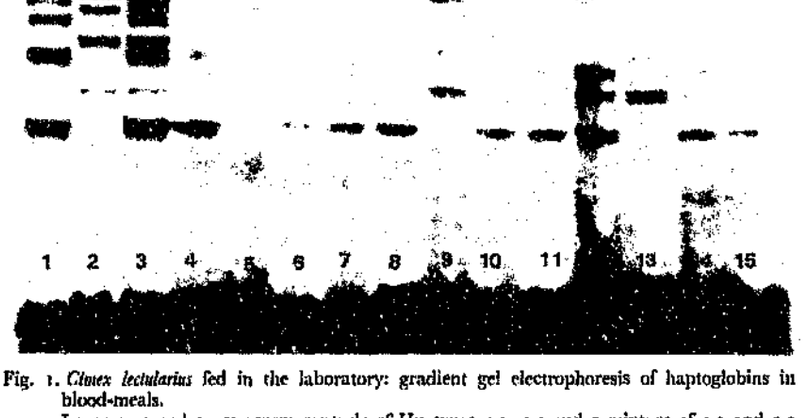



Fig. 1. *Cimex lectularius* fed in the laboratory: gradient gel electrophoresis of haptoglobins in blood-meals.
Lanes 1, 2 and 3 are serum controls of Hp types 2.1, 2.2 and a mixture of 2.1 and 2.2 respectively. Lanes 4–7 are meals from bugs fed on Hp types 2.1, 2.2, a mixture of 2.1 and 2.2 and a multiple feed on 2.1 and 2.2 with the bugs having been killed and stored in liquid nitrogen immediately after feeding. Lanes 8–11 and 12–15 are the same categories at 12 h and 24 h after feeding respectively.

2.1, type 2.2, an equal mixture of types 2.1 and 2.2 as well as a multiple meal on types 2.1 and 2.2 could all be identified up to 24 h after feeding. The whole experiment was repeated and the second gel obtained was very similar to the first.

## DISCUSSION

Polyacrylamide gradient gel electrophoresis proved a suitable method for determining Hp types in blood-meals of a laboratory colony of *C. lectularius*. Multiple blood-meals were clearly distinguishable in laboratory-reared bedbugs that had fed on a mixture of Hp types 2.1 and 2.2 and the haptoglobins were stable in the gut of *C. lectularius* for at least 24 h after feeding.

It is thought that analysis of the blood-meals of bedbugs collected from a hut occupied by hosts with differing Hp types would permit any multiple feeds to be identified provided a host was not Hp 0.0. It would not be possible to detect multiple feeds involving type 0.0, as this type with no haptoglobin would not show up in a mixed meal. If this study is to be continued it would be necessary to identify a number of such huts which would also have to be heavily infested with bedbugs.

## ACKNOWLEDGEMENTS

We are grateful to Mr D. van der Linde for supplying us with bedbugs from the colony at the South African Bureau of Standards, Pretoria, and to Prof T Jenkins and his staff of the South African Institute for Medical Research for helpful advice and technical

assistance. Finally we thank the Director-General of the Department of National Health for permission to publish.

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Accepted 2 February 1990.

manipulate the heart to regenerate damaged cells after injury.

The work had its beginnings in 1981 when we found that a protein (chalone) isolated from adult bovine hearts had the ability to inhibit growth (defined here as replication or DNA synthesis) of hearts in new-born hamsters.[1] The characterization of this protein showed it to be very large, with a molecular weight of approximately 720 000 daltons, and consisting of carbohydrate moeities attached to four protein subunits.[2]

Radiolabelled chalone was injected into new-born hamsters and found subsequently to be associated only with the hearts of these animals, indicating target specificity. This tissue specificity, but lack of species specificity, is a prerequisite for all chalones. Further work showed that the chalone was bound to the nuclear matrix of the heart cells — a protein skeleton found in the nucleus. Isolation of this matrix followed by binding experiments with chalone indicated the presence of receptor. for the chalone on the matrix.

We have been able to isolate a nuclear matrix preparation from hearts that has an endogenous DNA synthesis activity and we found that addition of chalone to this system strongly inhibits DNA synthesis.

Recent evidence has indicated that replication is initiated on the nuclear matrix and . .erefore we have concluded that since the myocardial chalone binds to this matrix and inhibits DNA synthesis there, the chalone must be a specific myocardial mitotic inhibitor. Further evidence to support this hypothesis was obtained when we succeeded in separating the major cell types of the heart, namely the cardiomyocytes (contractile) cells and the non-contractile cells (mainly fibroblasts). The chalone was found to inhibit DNA synthesis in nuclei and nuclear matrix preparations of only the cardiomyocytes. These results were extended to cultured cells, with which it was found that chalone inhibited replication in both primary cultures of cardiomyocytes and an established cardiomyocyte line, but not in primary cultures of fibroblasts or fibroblast cell lines. These findings are strongly suggestive of the presence of receptors for the chalone on myocyte nuclear matrices. It appears that when these receptors are activated by chalone, replication in these cells is inhibited.

Our group has also developed polyclonal and monoclonal antibodies to the chalone, with which we plan to characterize the molecule still further. In addition we intend to attempt to establish the exact source of the chalone. This knowledge may allow us to manipulate chalone concentration and could possibly lead to the therapeutic regeneration of contractile heart muscle. Clearly much is still to be learnt about this protein, but we hope that this work may become of benefit to victims of myocardial infarction.

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# Infection of Wild Populations of the Tampan, *Ornithodoros moubata* Murray, with Hepatitis B Virus in the Kavango, Northern Namibia

J.J. Joubert*, P.G. Jupp, O.W. Prozesky and J.G.H. Lourens

A high positivity rate to hepatitis B virus (HBV), as measured by various markers, particularly the viral surface antigen (HBsAg) and antibodies to this antigen (anti-HBs), has been reported for the human population of the territories in northern Namibia bordering on Angola.[1,2] In the case of Kavango, this finding has recently been confirmed and extended by us[3] and particular attention drawn to the exceptionally high positivity rate (95.5 – 99.4%) in the local population. The means whereby the virus is transmitted so effectively is unknown. The bedbug, *Cimex lectularis* L., may be a mechanical vector of HBV in tropical and subtropical areas of South Africa[4,5] and possibly also in the Kavango. However, the much higher prevalence of tampans, *Ornithodoros moubata* Murray, in this area, which infest about 80% of the huts of the local population, led us to investigate these argasid ticks as possible vectors. Tampans were tested for the presence of HBsAg and hepatitis e antigen (HBeAg) as indicators of HBV infection. These ticks inhabit the sand floors of the huts, feed exclusively on human blood, and are well-known as the vectors of *Borrelia duttonii*, the cause of endemic relapsing fever. This disease was reported in the Kavango by Ordman[6] in 1957 and is still prevalent (G. Newlands, personal communication).

## Materials and methods

From January to April, 1983, tampans were collected from 33 different wards in all five tribal areas in the Kavango, namely Kuangali, Mbunza, Shambyu, Gciriku and Mbukushu. These localities ranged from Namumtuntu near the Ovamboland border in the west, to Bagani near the border of the western Caprivi in the east. A ward is defined as a cluster of households under the jurisdiction of a local headman. Most of the samples were taken in a strip, about 5 km wide, along the banks of the Okavango river where more than 70% of the population is concentrated.[7] The tampans collected were separated according to ward and transported by air to Pretoria where they were killed and stored in liquid nitrogen until they could be tested. On the day of testing they were identified and separated into pools, which contained up to five ticks, according to stage of development (nymph or adult) and state of engorgement. Each pool was homogenised in 1 ml of bovine phosphate albumen (pH 7.0) and centrifuged. The resulting supernatant was subsequently tested for HBsAg and HBeAg by radio-immunoassay (RIA) (Ausria, Abbott Laboratories, North Chicago). Infection rates for the ticks were estimated statistically using the method of Chiang and Reeves.[8]

## Results and discussion

The results of the survey for the presence of HBsAg and HBeAg in the different pools of tampans collected at different localities are shown in Table 1. Seventy-eight pools were HBsAg-positive and 11 pools HBeAg-positive from the 33 different wards. The overall infection rate per 1 000 ticks for unengorged tampans was 142.1 and that for engorged tampans 191.2. All the HBsAg infection rates determined in this study were considerably higher than those first determined for bedbugs from the northern Transvaal.[4] However, a similar HBsAg infection rate was later reported for bedbugs collected in Modena village in the Louis Trichardt area (156.7) and a higher infection rate for HBeAg (137.7).[5]

This prevalence of HBsAg in natural tampan populations, as well as the presence of HBeAg in ticks from two of the tribal

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Table 1. Results of HBsAg and HBeAg tests on *Ornithodoros mouhata* collected in the Kavango.

| Tribal area | Proportion of wards with HBsAG(+) tampans | Unengorged (U) or engorged (E) | Adults (A) or nymphs (N) | No. tampans collected | No. pools | No pools HBsAG (+) | Infection rate per 1 000 | No pools of HBeAg (+) | Infection rate per 1 000 |
|---|---|---|---|---|---|---|---|---|---|
| Kuangali | 6/6 | E | A | 1 | 1 | 1 | | | |
| | | U | A | 9 | 3 | 3 | 275.3 | 0 | 0 |
| | | E | N | 0 | 0 | 0 | | | |
| | | U | N | 26 | 6 | 4 | | | |
| | | | | | 10 | 8 | | | |
| Mbunza | 5/6 | E | A | 0 | 0 | 0 | | | |
| | | U | A | 10 | 2 | 2 | 107.9 | 0 | 0 |
| | | E | N | 5 | 1 | 1 | | | |
| | | U | N | 100 | 20 | 7 | | | |
| | | | | | 23 | 10 | | | |
| Shambyu | 3/5 | E | A | 1 | 1 | 0 | | | |
| | | U | A | 23 | 5 | 0 | 129.5 | 0 | 0 |
| | | E | N | 0 | 0 | 0 | | | |
| | | U | N | 67 | 14 | 10 | | | |
| | | | | | 20 | 10 | | | |
| Gciriku | 10/12 | E | A | 18 | 5 | 4 | | 1 | |
| | | U | A | 19 | 5 | 4 | 215.5 | 0 | 6.8 |
| | | E | N | 5 | 1 | 1 | | 0 | |
| | | U | N | 128 | 26 | 17 | | 2 | |
| | | | | | 37 | 26 | | | |
| Mbukushu | 4/4 | E | A | 30 | 6 | 2 | | 2 | |
| | | U | A | 20 | 4 | 2 | 122.6 | 0 | 34.3 |
| | | E | N | 54 | 11 | 8 | | 4 | |
| | | U | N | 134 | 29 | 12 | | 2 | |
| | | | | | 50 | 24 | | | |
| Total: | | | | 650 | 140 | 78 | 150.4 | 11 | 26.7 |

areas, suggests that the tampan may play a role as a mechanical vector in the dissemination of the virus in the Kavango similar to that of the bedbug in the northern Transvaal. Preliminary experiments with tampans in the laboratory suggest that the virus does not multiply in these arachnids. Infection could probably be acquired by crushing an infected tick on one's person. Alternatively, infection might occur as the result of a bite, owing to the survival of virus on the tick's mouthparts. Such survival would be favoured by HBV's relatively high resistance to desiccation and elevated temperatures.[9,10] Whether the virus is present in the coxal fluid of infected ticks needs to be determined, since this could be a means of mechanical transmission.

Exposure of infected tampans in the Kavango may be promoted by the local habit of frequently changing sleeping quarters out of fear of witchcraft. The custom of moving the whole kraal to a new locality after a death, a practice formerly prevalent in the Kavango, has now become obsolete. This habit may have served as a preventive measure against the spread of the virus in that tampans infected with *Borrelia duttonii* and, perhaps, HBV were left behind in the move.

We thank the Director of Health and Welfare, S.W.A. Administration, for permission to carry out this study, General C.F. Scheepers and Brig. E.O. Prinsloo of the S.A. Medical Services for arranging air transport, and Messrs J. la Grange and E. Steyn for cooperation with the field work. This work is supported by a grant from the Medical Research Council.

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*Medical and Veterinary Entomology* (1987) 1, 361–368

# An experimental assessment of the tampan tick *Ornithodoros moubata* as vector of hepatitis B virus

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ABSTRACT. Wild-caught and colonized tampan ticks, *Ornithodoros moubata* (Murray), were fed on hepatitis B virus (HBV)-positive bloodmeans in a series of four experiments.

Hepatitis B surface antigen (HBsAg) persisted in nymphal and adult ticks for up to 779 days, while the epsmark antigen (HBeAg) persisted in mature nymphs up to 13 days, in adult males up to 11 days and in adult females up to 16 days. HBsAg was transmitted trans-stadially through two moults during the life cycle but transovarial transmission did not occur. The surface antigen was transmitted by two out of fifteen single ticks into 0.4 ml aliquots of HBV-negative blood, although six groups of ticks failed to transmit into 5.5 ml aliquots of blood: this antigen was not transmitted to hamsters. HBsAg was detected in samples of the ticks' coxal and rectal fluid secretions always at the infecting feed and usually at the second feed. HBeAg was only detected in one of two samples of coxal fluid collected at the infecting feed.

The results as a whole indicate that no biological multiplication of virus occurs in *O.moubata* but that mechanical transmission from ticks to man could occur by: (i) contamination of a person when crushing infected ticks; (ii) infection by bite; (iii) contamination with coxal fluid, especially by scratching bites. This is thought to take place among the Kavango tribe in their village huts in north-eastern Namibia where infestations of infected *O.moubata* occur.

**Key words.** Argasidae, ticks, *Ornithodoros moubata*, hepatitis B virus, mechanical transmission, Namibia.

## Introduction

Two earlier studies showed that the human population of the territories of Northern Namibia bordering on Angola had a high positivity rate for hepatitis B virus (HBV) (Bersohn *et al.*, 1974; Botha *et al.*, 1984). In 1985 further observations were reported in regard to one of these territories, the Kavango region, situated in the north-eastern corner of the country. These showed that only 1.9% of the subjects

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tested were negative for all the HBV markers used indicating that infection had been present at some stage in 98.1% of the sample (Joubert *et al.*, 1985a). Since none of the usual transmission methods for HBV seemed to explain this high positivity rate in Kavango, the hut tampan tick, *Ornithodoros moubata* (Murray) sensu Walton 1962 (Acari: Argasidae) came under consideration as a possible vector. This argasid tick was found to be very prevalent, occurring in about 80% of the huts of the local people where it also acts as a vector of *Borrelia duttoni* (Novy & Knapp) the cause of relapsing fever (Joubert *et al.*, 1985b). Samples of tampans were collected in huts and infection rates of 150.4 and 26.4 per 1000 ticks were demonstrated for the HBsAg and HBeAg respectively in these ticks (Joubert *et al.*, 1985b). These high infection rates suggested the tampan probably acted as a mechanical vector of HBV in a similar manner to that in which the bedbug, *Cimex lectularius* L., probably acts in the northern Transvaal (Jupp *et al.*, 1983). It was therefore decided to assess the vector competence of *O. moubata* in the laboratory to see whether such experiments would provide further evidence incriminating it as a vector of HBV.

### Life cycle

The following brief description of the life cycle of the hut tampan is included so that the remainder of the paper can be more readily comprehended. It is based on a review by Hoogstraal (1956) as well as observations by Walton (1959) and ourselves (unpublished) which all deal with ticks in the laboratory; there are almost no records for the life cycle under natural conditions. Copulation occurs before, during or after feeding. The hut tampan feeds exclusively on human blood, the male which is much smaller than the female taking a correspondingly smaller meal. As a result of taking a rapid blood-meal, the adult male moults while the female deposits a batch of eggs in or on the soil. A semi-active larva hatches from the egg and then becomes inactive until it moults to the first nymph. The first nymph, after a short rest, feeds on an available host for about half an hour, then returns to the soil to digest its meal. Subsequently the nymphs moult usually three times (those destined to become males) or four times (those destined to become females), with resting and feeding phases between each ecdysis. The adults emerge from the last moult and have hiding and feeding habits similar to those of the nymphs. Up to about 93 days are necessary for completion of the life cycle from copulation to emergence of the last females of the next generation and the ticks have a longevity of 14 months to 5 years. Coxal fluid is discharged from a coxal organ through a duct which passes between the first and second legs on each side of the body. This discharge starts about 15 min after feeding has begun and continues till completion of the meal and intermittently for about 1 h afterwards. The tick osmoregulates by secreting the coxal fluid in order to maintain the ionic balance of its haemolymph which is particularly necessary after a blood-meal. The coxal fluid is hypotonic with the haemolymph (Hagan *et al.*, 1982; Kaufman & Sauer, 1982). Defecation does not occur in the tampan as the small intestine has a blind ending. However, excretion is carried out by the malpighian tubes which connect to a rectal ampulla located posteriorly with a ventroposterior opening. Excretion of water as a white viscous rectal fluid ('urination') takes place occasionally during but always after each blood-meal and also sometimes when a tick is disturbed.

## Materials and Methods

### *Ticks*

Field-collected ticks from the Kavango were used in experiments 1 and 2, some of which might have been naturally infected with HBV. To eliminate this possibility the $F_1$ generation of an HBV-free tick colony (OM 1) was used for experiments 3 and 4. This colony was established by isolating thirty-one Rundu female ticks in separate tubes, also subsequently keeping their egg batches separate. After each egg batch had hatched a sample of five first nymphs was removed and tested for HBsAg negativity. The colony and ticks used for experiments 3 and 4 were kept in an insectary where conditions were 25–26°C and relative humidity 75–80°C. In experiments 1 and 2, after their infective feed, the ticks were held at a high humidity in plastic boxes kept at 28–30°C for 60 days and thereafter at 21°C. The tampans were maintained in fine sand inside plastic tubes, 6 cm high and 4.5 cm diameter, closed with Organdy mesh.

*Tick feeding*

For all infective meals ticks were fed through a membrane on defibrinated blood drawn from one or two HBV-carrier-donors on the day of the feed. This membrane feeding was carried out in the insectary, largely as described previously for bedbugs (Jupp & McElligott, 1979). Differences were that blood was warmed to 37°C and stretched Parafilm was used for membranes in experiments 3 and 4 rather than Budrache because a higher proportion of the ticks were found to feed through the Parafilm. *In vitro* attempts to transmit HBV were done by refeeding infected ticks on HBV-negative defibrinated blood, also through a membrane. *In vivo* attempts were carried out by refeeding the ticks on hamsters. The latter were anaesthetized and canisters containing the ticks, 4.0 cm diameter and 2.5 cm high with Organdy lids, were glued to the hamsters shaved abdomens.

*Virological techniques*

*Donor blood.* On the day of each infective feed a serum specimen was also obtained from the donor, upon which five tests were done to confirm that he was still a highly infectious HBV carrier. Each specimen was tested for HBsAg, HBeAg, Anti-HBs, Anti-HBe and Anti-HBc by solid phase radio-immunoassay ('AUSRIA II', 'HBeTM', 'AUSAB', HBeTM' and 'CORAB', Abbott, Chicago).

*Ticks.* Tampans were killed for antigen assay at varying intervals after the infecting meal, enough time usually being allowed for oviposition by females or moulting by males and nymphs. Those ticks which fed a second time in the transmission tests were killed the same day or up to 3 days after this feed. First stage nymphs, the progeny of infected females, were killed a few days after hatching. Tampans were killed by placing them in a −70°C freezer where they were stored until extracts could be prepared from them for antigen assay. For this, each adult or mature nymph or pool of fifty first-stage nymphs was homogenized in 1 ml bovine phosphate albumin, centrifuged at 3000 rpm for 30 min, and the supernatant frozen immediately at −70°C. Later they were thawed and tested from HBsAg and HBeAg. Six adults or mature nymphal ticks which had not received an infecting meal were also tested individually as controls.

*Transmission experiments.* In the attempts to transmit antigen to HBV-negative blood, such blood was obtained from appropriate donors. Samples of blood which had been exposed to infected ticks were tested for HBsAg and HBeAg along with control samples of the same blood which had remained unexposed. The hamsters used in transmission attempts were bled to obtain sera 2 days before and 21 and 61 days after the transmission feed. Sera were tested for HBsAg and Anti-HBs.

*Coxal and rectal fluids.* Coxal fluid was collected immediately after a mature nymph or adult tampan had finished feeding. Ticks were removed from membrane-feeding canisters or hamsters and transferred to empty plastic tubes from which coxal and also rectal fluid could be drawn up by means of fine glass capillary tubes.

Each sample of these fluids obtained came from a group of ticks and in some cases were diluted slightly with normal saline to bring volumes up to 0.4 ml before freezing the specimen at −70°C. These fluids were subsequently tested for HBsAg and HBeAg.

## Results

*Infectivity of adults and nymphs*

Table 1 shows the antigen positivity rates for three experiments in which adult and nymphal ticks were given an infective meal and tested at various intervals afterwards. Every tick killed from day 16 till day 779 (2 years 49 days) was HBsAg-positive except one solitary individual, but all were HBeAg-negative. As some of the adults were nymphs and the mature nymphs a younger nymphal stage when given the infective meal, trans-stadial transmission of the surface antigen through one moult had occurred. The six control ticks which had not fed on infective blood were HBsAg-negative. In experiment 3 infective blood came from two carriers, I+II, who had received transplanted kidneys. Ticks fed on blood from donor I failed to moult or failed to complete their moulting, the females failed to deposit eggs and many of the ticks were dead within 30 days. On the other hand, ticks fed on donor II's blood remained perfectly healthy and moulted or deposited their eggs. An inquiry as to the medicine being taken by each donor revealed that donor I's prescription included Cyclosporin A while donor II's did not.

TABLE 1. Tests for HBsAg and HBeAg in ticks at various intervals after infective meals.*

| Experiment no. (incubation temperature) | Tick stage† | Days after infective meal | No. of ticks HBsAg-pos. | No. of ticks HBeAg-pos. |
|---|---|---|---|---|
| 1 (28–30°C, 21°C)‡ | Field nymphs/ adults | 16–55<br>55–779 | 24/24<br>40/40 | 0/24<br>Not done |
| 2 (28–30°C, 21°C) | Field nymphs/ adults | 16–27<br>57–742 | 45/45<br>24/24 | 0/45<br>Not done |
| 3 (25–26°C) | Colony OM-1 | | | |
| | Mature nymphs | 22–27 | 8/8 | 0/8 |
| | Mature nymphs | 34–37 | 35/36 | 0/36 |
| | Males | 34–37 | 31/31 | 0/31 |
| | Females | 34–37 | 54/54 | 0/54 |

* Donor blood had the following characteristics: HBsAg-pos., HBeAg-pos., Anti-HBs-neg., Anti-HBe-neg., Anti-HBc-pos.

† Stage of tick tested for antigens.

‡ In these experiments ticks were held at 28–30°C for 60 days and subsequently at 21°C.

Cyclosporin A inhibits protein synthesis and thus appears to have interfered with the normal metabolism of the tampans in group I.

Since the ticks in experiments 1, 2 and 3, which all turned out HBeAg-negative, were only killed for assay from day 16 onwards, it was decided to undertake another experiment (no. 4). This was to determine how long the HBeAg remained detectable after the infective meal up until day 16. The results are shown in Tables 2 and 3 for individual ticks which had fed on blood from donors A and B respectively, and which were killed for assay on successive days. In Table 2 it can be seen that, although the surface antigen was always detected, the e antigen disappeared in females after day 12 and in males and nymphs after day 7. In contrast to this, in Table 3 ticks which fed on donor B, 90% (44/49) of which were HBsAg-positive, remained HBeAg-positive for longer. The antigen disappeared in nymphs after day 13, in males after day 11, but in females one of two ticks was still positive on day 16. Thus these results considered together with the findings of experiments 1 and 2 indicate that the 16th day is probably the limit of persistence of the HBeAg in ticks held at 25–26°C.

In view of the trans-stadial transmission of HbsAg through one moult which occurred in experiments 1–3, it was decided to ascertain whether this antigen would be transmitted through two moults. To do this some of the nymphal ticks which had been given an infective meal in experiment 4 were allowed to moult and on the 158th day post infection they were refed on uninfected blood. These tampans subsequently moulted a second time and on the 173rd day were killed and tested. All of the sample of eleven ticks, two nymphs and nine adults, were HBsAg-positive except one of the nymphs.

### *Transmission attempts*

In two experiments attempts were made to transmit HBsAg from HBV-positive to HBV-negative blood (Table 4). Ticks were either fed singly on small volumes of uninfected blood or in

TABLE 2. Experiment 4: tests for HBsAg (s) and HbeAg (e) in ticks killed daily after infective feed on blood from donor A. +, positive; −, negative; ND, no test done.

| | Days after infective meal | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1–5 | | 6 | | 7 | | 8 | | 9 | | 10 | | 11 | | 12* | | 13 | | 14 | | 15 | | 16 | |
| Antigens: | s | e | s | e | s | e | s | e | s | e | s | e | s | e | s | e | s | e | s | e | s | e | s | e |
| Nymph | + | + | + | + | + | + | + | − | + | − | + | − | ND | | + | − | + | − | + | − | + | − | ND | |
| Male | + | + | + | − | + | + | + | − | + | − | + | − | + | − | ND | | ND | | ND | | ND | | ND | |
| Females | + | + | + | + | + | + | + | + | + | − | + | − | + | − | + | + | + | − | + | − | | | | |
| | + | + | + | + | + | + | + | − | + | − | + | + | + | − | + | − | + | − | + | − | + | − | + | − |
| | | | | | | | | | | | | | + | + | + | − | | | | | | | | |

* From day 12 onwards some of the nymphs had moulted to adults.

TABLE 3. Experiment 4: tests for HBsAg (s) and HBeAg (e) in ticks killed daily after infective feed on blood from donor B. +, positive; −, negative; ND, no test done.

| | Days after infective meal | | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1–6 | | 7 | | 8 | | 9 | | 10 | | 11 | | 12 | | 13 | | 14 | | 15 | | 16 | | 126 | |
| Antigens: | s | e | s | e | s | e | s | e | s | e | s | e | s | e | s | e | s | e | s | e | s | e | s | e |
| Nymph | + | + | + | + | + | + | − | − | + | + | ND | | + | + | + | + | + | − | + | − | + | − | ND | |
| Male | + | + | − | − | + | + | + | − | + | + | + | + | ND | | − | − | + | − | + | − | − | − | + | − |
| | | | | | | | | | | | | | | | | | | | | | | | + | − |
| Females | + | + | + | + | + | + | + | − | + | + | + | + | + | − | + | + | + | + | + | + | + | + | + | − |
| | + | + | + | + | − | − | + | + | + | + | + | − | + | + | + | + | + | + | + | + | + | − | + | − |
| | | | | | | | | | | | | | + | ND | | | | | | | | | + | − |
| | | | | | | | | | | | | | | | | | | | | | | | + | − |

TABLE 4. Attempts to transmit HBsAg antigens from HBV-positive to HBV-negative blood by bite of *O. moubata*.

| Expt no. | Days after infective meal | Vol. negative blood used per sample (ml) | No. HBsAg-pos. ticks fed on each blood meal | No. samples becoming HbsAg-pos. |
|---|---|---|---|---|
| 2 | 27 | 0.55–0.65 | 1 | 0/12 |
| | | c. 4.5 | 5–7 | 0/3 |
| 3 | 31 | 0.4 | 1 | 2/15 |
| | | 5.5 | 6–20 | 0/6 |

groups on larger volumes 27 or 31 days after the infective feed. In experiment 3, two out of fifteen (13%) individual ticks – a female and a mature nymph – transmitted the surface antigen while feeding on 0.4 ml aliquots of blood on the 31st day. The fifteen ticks consisted of ten females, two males and three mature nymphs. The six groups of ticks which failed to transmit in the same experiment (3) while feeding on 5.5 ml aliquots were six males, eight females, sixteen females, seventeen females, nineteen mature nymphs and a mixture of twenty males and mature nymphs. Assay for antigens in all the ticks after they had been killed the same day or 1–3 days after the transmission attempt showed they were all HBsAg-positive but HBeAg-negative.

The results of four attempts to transmit to hamsters by the bites of HBsAg-positive adults (5 females, 15 males) and thirteen mature nymphs which had been infected 36 or 63 days previously, in some cases at an earlier developmental stage, were unsuccessful.

### *Infectivity of coxal and rectal fluids*

Tests for antigens in coxal and rectal fluids collected from the ticks after feeding revealed

TABLE 5. Tests for HBsAg and HBeAg in samples of coxal and rectal fluids collected from groups of mature nymphs/adult ticks directly after the infecting meal (1st) or the uninfected meal (2nd).

| Fluid | Expt no. | Meal | Days after infective meal | No. samples | |
|---|---|---|---|---|---|
| | | | | HBsAg-pos. | HBeAg-pos. |
| Coxal | 3 | 2nd | 31 | 4/5 | 0/5 |
| | 3 | 2nd | 36 | 2/2 | 0/2 |
| | 3 | 2nd | 63 | 1/2 | ND |
| | 4 | 1st | 0 | 2/2 | 1/2* |
| Rectal | 3 | 2nd | 31 | 4/6 | 0/6 |
| | 4 | 1st | 0 | 2/2 | 0/2 |

* Marginal.

that all samples of these fluids from the first (infecting) feed were HBsAg-positive while one of the two samples was marginaliy HBeAg-positive. The majority of samples of each kind of fluid collected after the second feed were HBsAg-positive but none were HBeAg-positive (Table 5).

*Transovarial transmission*

In experiments 1, 2 and 3, egg masses deposited by some of the HBsAg-positive female ticks were allowed to hatch and the first nymphs tested for HBsAg. More than 1000 tampans were assayed in nineteen pools but all were negative.

*Interval between blood-meals*

In experiment 5, after an infected blood-meal, the individual ticks in batches of females, males and mature nymphs were held in separate tubes so that developmental times could be recorded. Out of twenty adult females which deposited eggs, only four had started oviposition by day 16. Those refed were offered a second feed on day 17 and would almost certainly have done so on day 15 or 16 if they had been offered a feed then. Among thirty-seven males none moulted after their feed but two out of six individuals refed when offered blood on day 17. In the case of mature nymphs twenty-three out of twenty-six which had moulted between days 11 and 14 refed on day 14.

## Discussion

There have been no previous laboratory studies on *O.moubata* and HBV. Our findings are as follows: (i) HBsAg persisted in nymphal and adult ticks for up to 779 days after the infective blood-meal; (ii) HBeAg persisted in mature nymphs up to 13 days, in adult males up to 11 days and in adult females up to 16 days; (iii) HBsAg persisted through two moults (trans-stadial transmission); (iv) HBsAg was not transmitted transovarially; (v) HBsAg was transmitted in two instances by single ticks (a female and a mature nymph) into HBV-negative blood, although the transmission rate was low in the experiments overall; (vi) this antigen was not transmitted to hamsters; (vii) HBsAg was detected in samples of both coxal and rectal fluid secretions always at the infecting feed and usually at the second feed but HBeAg was only detected in one of the two samples of coxal fluid collected at the infecting feed.

Studies on HBV infection in humans (Alter *et al.*, 1976; Grady *et al.*, 1976; Sherlock & Thomas, 1983) and in chimpanzees (Shikata *et al.*, 1977) show that the presence of both HBsAg and HBeAg in the blood indicates a higher level of infectivity of HBV than if HBsAg is present alone, although the latter still indicates infectivity (Burrel, 1980; Hadziyannis *et al.*, 1983). Extrapolation of these results over to the tick indicates that the tampan would be potentially more infectious when both the antigens can still be detected. This situation exists with 11–16 days of the tick's engorgement on the infective blood-meal, depending on its life stage, at which time a crushed tick could prove a source of infection or a tick could refeed and possibly transmit HBV. Refeeding within this period of HBeAg positivity would seem a possibility for some ticks since our experiments showed that at 26°C a proportion of individuals would be ready to refeed from day 12–16. This period could be shorter under natural conditions in the Kavango when higher temperatures would prevail. Further transmission tests require to be carried out from day 12–16 to see whether the e antigen would be transmitted into HBV-negative blood in addition to the surface antigen after this short incubation period.

Our results indicate that there was no biological multiplication of HBV in *O.moubata* and no biological transmission by the tampan. Rather it seems that HBV persists in these ticks and can be transmitted mechanically. Evidence in favour of this is the disappearance of HBeAg after 16 days and the paucity of *in vitro* transmissions of HBsAg. Contrary evidence is the long survival of HBsAg in tampans including its trans-stadial transmission through two successive moults which may be compared to the result of similar experiments in bedbugs where HBsAg was undetectable after 127 days and only survived after one moult (Jupp & McElligott, 1979). However, the much larger blood-meal size in tampans as compared to bedbugs and the absence of defecation may explain this. In this connection it is noteworthy that the HBeAg remained detectable longer in females than in

males and nymphs which take a considerably smaller blood-meal. Coxal fluid and occasionally rectal fluid is discharged by the tick while it is still attached to the host. An infecting tick taking its second feed on a human, although it failed to transmit virus by bite, might still cause an infection due to the infectious (HBsAg-positive) coxal or sometimes rectal fluid which is secreted onto the skin. Such fluid could be inoculated by the host scratching at the site of the bite. Rectal fluid would be less important than coxal fluid in this respect as it is more usually secreted after the tick has finished feeding and left the host.

The human host upon which a tampan is feeding or attempting to feed may crush the tick and scratch the area of the bite or mucosal surfaces causing self-inoculation with infectious material. There is also the possibility of a crushed tick releasing an infective aerosol. This kind of transmission would be most likely during the period that a proportion of the ticks are still HBeAg-positive.

In conclusion, the results of our study indicate an absence of biological replication of the virus but possible mechanical transmission. This could occur by three possible routes: (i) infection by tick bites, (ii) contamination with infected coxal fluid, especially by scratching bites; (iii) contamination of a person who crushes infected ticks. These routes are similar to those suggested for *Cimex lectularius* (Jupp *et al.*, 1983), except that the tampan does not produce faeces, but coxal fluid discharged in large volumes on the host may be as important for infection. No evidence is available as to whether transmission by bite would be by regurgitation of material from the gut on refeeding. Regurgitation is thought more likely to occur than secretion from infected salivary glands. Interrupted feeding with a tick completing its meal on a second host is thought unlikely.

## Acknowledgments

We wish to thank Sister S. Bloem of the Johannesburg General Hospital for a supply of HBV-positive specimens and the donors for their willing co-operation. Grants towards the study were received by P. G. Jupp from the Poliomyelitis Research Foundation and by J. J. Joubert from the Medical Research Council. These are both gratefully acknowledged. Assistance in the field by Mr E. Steyn is greatly appreciated.

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Accepted 20 May 1987

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Reprinted from "South African Medical Journal" Vol. 79, 16 March 1991, pp 320-322

# Attempts to transmit hepatitis B virus to chimpanzees by arthropods

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## Summary

**Bedbugs (*Cimex lectularius* L.) were fed on an infective blood-hepatitis B virus (HBV) mixture. Further bedbugs and tampan ticks (*Ornithodoros moubata* [Murray]) were fed on HBV-carrier chimpanzees. After a 10 - 13 day interval for oviposition, tests done on samples of individual arthropods showed that 53 - 85% of the bugs were HBsAg-positive and none HBeAg-positive, while 100% of the ticks were HBsAg-positive and 88% HBeAg-positive. The remaining arthropods were fed on 3 susceptible chimpanzees, which had failed to develop HBV infection after 11 months, indicating no transmission had occurred. Subsequently the presence of viable virus in the original infective meals was confirmed by inoculation of the relevant donor sera directly into the 3 still susceptible chimpanzees. HBV infections quickly followed in each animal. It is concluded that, while mechanical transmission of HBV is most unlikely after a 10 - 13-day interval between feedings in bedbugs and tampans, it is still possible that mechanical transmission between humans might occur during interrupted feeds.**

*S Afr Med J* 1991; 79: 320-322.

It has been shown that human populations in the northern Transvaal, the northern territories of Namibia and in West Africa have a high positivity rate for hepatitis B virus (HBV).[1-4] None of the usual transmission methods for HBV seemed adequate to account for these high positivity rates and there was strong epidemiological evidence favouring transmission by bedbugs or argasid ticks.[5-7] Tests on such arthropods collected from huts showed high infection rates, based on the presence of HBsAg and HBeAg in the common bedbug (*Cimex lectularius* L.) in South Africa,[5,8] the tropical bedbug (*Cimex hemipterus* [Fabr]) in West Africa,[6,9] and the tampan tick (*Ornithodoros moubata* [Murray]) in Namibia.[7] Laboratory vector experiments provided further evidence that these arthropods could be capable of transmitting the virus.[8,10-12] Among such *in vitro* experiments HBsAg was transmitted by bite from infected to uninfected blood by *C. lectularius* and *O. moubata*, although other experiments suggested two additional potential routes for mechanical transmission.[8,13] Since it was not possible to know with certainty that viable virus was indeed transferred by bite in these *in vitro* tests, it was decided to determine whether HBV could be transmitted to chimpanzees and so cause hepatitis B infection in recipient animals.

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Accepted 26 Mar 1990.

## Materials and methods

Three transmission experiments were conducted using 3 HBV-susceptible chimpanzees. In experiment 1, adult bedbugs were given an infective feed in the Johannesburg laboratory on a blood-virus mixture, which contained serum from a human donor, and on the 10th day were allowed to refeed on a non-immune chimpanzee (No. 1296). The 10th day was chosen because 3 *in vitro* transmissions of HBsAg had previously been accomplished with small numbers of bugs after that interval of time.[10] Furthermore, such a period would permit the completion of oviposition by the bugs so that they would then be ready to feed again. In experiment 2, 2 HBV-carrier chimpanzees (No. 1164 and No. 1241) were exposed to adult bugs and on the 10th day the bugs were refed on a non-immune chimpanzee (No. 72). In experiment 3, adult and late nymphal ticks were given their infective feed on the 2 carrier chimpanzees and on the 13th day were refed on a non-immune chimpanzee (No. 73). The 13-day period allowed the nymphal ticks to moult and some of the adults to oviposit.

### Arthropods

The arthropods were reared in an insectary at the National Institute for Virology in Johannesburg. Newly moulted *C. lectularius* adults and *O. moubata* adults and late nymphs were taken by the senior author to the National Institutes of Health (NIH) in Washington, DC. They included one group of *C. lectularius*, which had already taken an infective blood meal (experiment 1). The bedbugs originated from a laboratory colony described previously[10] and were maintained in plastic tubes, 6 cm high and 4,5 cm in diameter, closed with organdie mesh. The tampan ticks used were the $F_1$ generation of an HBV-free and *Borrelia duttoni*-free colony established from ticks collected at Rundu, Kavango region, Namibia. Ticks were maintained in fine sand inside the same kind of plastic tubes as were used for the bugs. Except during their flight from South Africa to the USA and while feeding on the chimpanzees, the arthropods were housed in insectaries where the temperature was maintained at 25 - 27°C, relative humidity at 75 - 80% and the illumination regimen was 12 hours light-12 hours darkness.

### Arthropod feeding

For their infective feed in experiment 1, bugs were fed through a membrane on a defibrinated blood-virus mixture as described previously except that stretched parafilm was used for membranes.[10] For the feedings on chimpanzees, either donor or recipient, each animal was anaesthetised and an open PVC ring, 7 cm deep and 10,5 cm in total diameter, was tightly applied and secured to the shaven abdomen; 120 - 200 bugs or ticks were then placed within a ring whose top was closed with Terylene mesh. The age of the chimpanzees was 4 - 5 years.

## Virological techiques

**Infective donor blood.** In experiment 1, the infective meal consisted of a mixture of defibrinated blood and serum in the proportion 2:1. The blood was drawn from an HBV-negative (negative for HBsAg, anti-HBs and anti-HBc) donor less than 2 hours before the bugs were fed, while the serum was obtained 3 days earlier from the blood of a carrier-donor. Until used, the serum was kept at -20°C except for a sample which was tested to confirm the donor was still an HBV carrier. Tests done on this sample were for HBsAg, HBeAg, anti-HBs, anti-HBe and anti-HBc by solid phase radio-immunoassay (RIA) (AUSRIA II, $HB_e$, AUSAB, $HB_c$ and CORAB; Abbott, Chicago) and HBV DNA was detected by DNA probe as described by Bowyer *et al.*[14] All tests were positive except anti-HBs and anti-HBe. The donor chimpanzees were bled and a liver biopsy taken just before they were exposed to arthropods (experiments 2 and 3) so that tests could be done to confirm that they were still HBV carriers and probably highly infectious. Serological RIA tests already mentioned (except anti-HBe) and tests for the HBV DNA and HBV DNA polymerase were performed. The latter was done as described previously.[15] The serum levels of three enzymes were determined in liver function tests: alanine aminotransferase (ALT), isocitrate dehydrogenase (ICD) and γ-glutamyltranspeptidase (GGTP). The recipient chimpanzees were assessed just before exposure to the potentially infected arthropods and after exposure weekly for 9 months. The same three liver function tests were done but only tests for HBsAg, anti-HBs and anti-HBc.

**Arthropods.** Samples of arthropods were killed immediately after their second feed by placing them in a -70°C freezer where they were stored until extracts could be prepared from them for antigen assay. For this, each individual bug or tick was homogenised in 1,0 ml bovine phosphate albumin (0,75% bovine serum albumin in phosphate-buffered saline (BPA)) centrifuged at 3 000 rpm for 30 minutes and the supernatants frozen immediately at -70°C. Subsequently, supernatants were thawed and tested for HBsAg. Those found HBsAg-positive were also tested for HBeAg. Samples of arthropods which had not engorged on an infecting meal and samples of the BPA were also tested individually as controls.

## Results

The bedbug feeding rates on chimpanzees were consistently high at both the first and second feeds (81 - 97%) after the insects had been allowed ample time (20 - 80 min). The number of bugs, an equal number of adult males and females, which fed a second time in the transmission attempts were 102 (experiment 1) and 99 (experiment 2). With the ticks, however, although feeding rates were high (88% and 92%) at the first feed (adult females and mature nymphs), at the second feed it fell to 32 out of 149 adult females (21%). This was probably because only a proportion of the female ticks had completed oviposition before the second feed.

The antigen positivity rates for both bedbugs and ticks on the day of transmission attempts are given in Table I. These rates were very high for both HBsAg and HBeAg in the ticks, while HBsAg positivity varied from 53% to 85% in bedbugs and no HBeAg was detected. No arthropods in samples of 6 - 10 individuals that had not taken infective meals were found positive for HBsAg in each experiment and 4 aliquots of BPA were also HBsAg-negative. In experiment 3, a sample of coxal fluid was collected from the ticks at the second feed and diluted in 0,5 ml BPA. This was HBsAg-positive but was not tested for HBeAg.

Although the 3 recipient chimpanzees were monitored for almost a year after the transmission attempts, there was no indication of infection by HBV as measured by serological tests and liver function tests.

In the light of this, we decided to confirm the presence of viable virus in the original infective blood meals. For this, 0,1 ml of the original human serum used on 4 June 1987 in the infective blood-virus mixture in experiment 1 was inoculated intravenously into chimpanzee No. 1296. Similarly, 0,1 ml of serum from each of the carrier chimpanzees, No. 1164 and No. 1241, bled on 8 and 9 June 1987 was pooled and 0,2 ml inoculated into chimpanzees No. 72 and No. 73, respectively. After 2 - 3 weeks, the 3 previously non-immune chimpanzees became highly HBsAg-positive, HBsAg disappeared by weeks 9 and 8 in chimpanzees No. 72 and No. 73, respectively, followed by conversion to anti-HBs-positive and anti-HBc-positive status. ALT values became elevated 7 and 5 weeks after inoculation in chimpanzees No. 72 and No. 73 and both animals developed anti-HBc. Chimpanzee No. 1296 demonstrated *de novo* synthesis of HBsAg by 2 weeks after inoculation, developed elevated serum ALT values during week 10 and has proved to be strongly HBsAg positive, with markedly elevated results of liver function tests for over 2 years.

## Discussion

The direct inoculation of sera corresponding to those of the original infective blood meals into the still susceptible recipient chimpanzees clearly produced HBV infection in all 3 of these animals. Thus it was confirmed that the blood meals were infectious and contained viable HBV. It must therefore be concluded that adult *C. lectularius* and adult *O. moubata* will not transmit HBV mechanically by bite if an interval elapses between the infecting and transmission feeds sufficient to permit digestion of blood, egg development and oviposition. Such an interval would be expected in the normal course of events before bedbugs or ticks would refeed.

**TABLE I. TESTS FOR HBsAg AND HBeAg IN ARTHROPODS ON DAY OF TRANSMISSION ATTEMPT**

| Exp. No. | Infective bloodmeal* | Arthropod | Days after infective meal | No. arthropods tested HBsAg+ | No. arthropods tested HBeAg+ |
|---|---|---|---|---|---|
| 1 | Human | Bedbugs | 10 | 16/30 (53%)† | 0/16 |
| 2 | Chimpanzees | Bedbugs | 10 | 13/20 (65%) | 0/13 |
| | | | | 17/20 (85%) | 0/17 |
| 3 | Chimpanzees | Ticks | 13 | 40/40 (100%) | 28/32 (88%) |

*Human and chimpanzee donors were positive for HBsAg, HBeAg, Anti-HBc, HBV-DNA and HBV-DNA polymerase activity and negative for anti-HBe and anti-HBs. Both chimpanzees had elevated levels of serum ALT, ICD and GGTP.
†An equal number of adult male and female bugs but only adult female ticks were tested.

With bedbugs, however, it is still possible that mechanical transmission between humans could occur by interrupted feeding. Unfortunately, a shortage of chimpanzees prevented the inclusion of an experiment to test this in the present study. In such instances there would be an interval lasting only a few minutes between the start of feeding on an HBV carrier and the completion of the blood meal on a susceptible person. Although there is some evidence that interrupted feeding may occur in *C. lectularius* in the laboratory,[16] whether it occurs in natural human bedbug infestations is not known. One previous experiment has been reported in which the mechanical transmission of HBV was attempted by interrupted feeding of mosquitoes on chimpanzees.[17] Two attempts to transmit virus by 100 *Aedes aegypti* from a carrier to 2 different susceptible chimpanzees failed. It would seem unlikely that bedbugs would succeed where mosquitoes failed unless bedbugs differ from mosquitoes in the anticoagulatory enzymes introduced at the time of biting. Perhaps enzymes secreted onto the mouthparts of *C. lectularius* would not inactivate the virus present, whereas this would occur in mosquitoes as suggested by Berquist *et al.*[17] In the case of *O. moubata* it is unlikely that interrupted feeding would occur because once a tick has attached itself to its host it is not easily dislodged.

The two other possible routes for mechanical transmission previously suggested for bedbugs and tampan ticks cannot be ruled out on the basis of the results of the present experiments.[6,13] These are, firstly, the infection of a susceptible person by means of bedbug faeces or by tick coxal or rectal fluid, which could occur by scratching of skin lesions or mucosal surfaces that had been contaminated by these products. Secondly, a person may become infected because he crushes the bug or tick and then scratches the area of the bite or mucosal surfaces causing self-inoculation with infectious material. However, these routes now appear less likely. Serological studies to determine the prevalence of HBV among blacks with viral hepatitis admitted to Johannesburg hospitals,[18] and among residents of Kangwane (South Africa) and Ovamboland (Namibia),[2,19] showed a rise in the HBsAg and HBeAg positivity between the age of 2 years and 4 years accompanied by a fall in anti-HBe positivity. This paucity of perinatal infection but horizontal spread among young children is difficult to explain other than by arthropod transmission. However, the experimental data described here do not support the concept of anthropod transmission as a major factor in the horizontal spread of HBV among young children and other modes of transmission must be considered. These might include contamination from open sores caused by impetigo or by scratches and cuts incurred during rough play.

We wish to thank Mrs Doris Wong for technical assistance and Dr Bob Gwadz for allowing the use of his insectary, both of the National Institute of Allergy and Infectious Diseases (NIAID), Bethesda, Maryland, USA. We are also indebted to the animal attendants at SEMA for much help. Dr Geoff Dusheiko, recently of the University of the Witwatersrand Hepatology Unit, provided the human donor and certain technical assistance and Professor Jennifer Alexander of the Department of Microbiology at the same university kindly reviewed our manuscript, for which we are most grateful. Our thanks are due to Dr Ron Eagle of Georgetown University Medical Center for assistance with serological tests. One of us (P.G.J.) gratefully acknowledges grants towards the study from the Poliomyelitis Research Foundation, Johannesburg, and the South African Medical Research Council.

This work was performed in part under NIAID contracts No. 1-A1-52567 and No. 1-A1-72623.

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pressure levels in the rural and urban areas are higher than in The Gambia and Senegal, but comparable with levels in the so-called salt-deprived Yoruba ethnic group.[6] If Wilson's hypothesis were correct, no differences in blood pressure should be observed between Benin City,[7] The Gambia, and Senegal.

The role of sodium in salt-induced hypertension, itself controversial, is central to Wilson's hypothesis. However, there seems to be little or no difference in the sodium intake or excretion between whites and the US blacks who are more prone to hypertension.[8]

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## SURVIVAL OF HIV IN THE COMMON BEDBUG

SIR,—Transmission of human immunodeficiency virus (HIV) by insects has been postulated as one mechanism of transmission of infection in Africa,[1] although it is thought that insects are not involved in "western" AIDS.[2] The known routes of transmission of AIDS are also common to those hepatitis B virus, with which HIV shares several epidemiological features. There is strong evidence for mechanical transmission of hepatitis B virus by the common bedbug (*Cimex lectularius*).[3] Similar transmission of HIV by bedbugs may be a cause of infection in African children. In Africa 15-22%[4] of AIDS cases have been in children whereas in the USA this proportion is only 1-4%,[5] suggesting that there are modes of transmission other than those recognised in the USA. Since bedbug infestation is common throughout Africa, children are continually exposed to the bites of these insects. Bedbugs would probably transmit low levels of infection but a combination of factors could enhance the susceptibility of African children—ie, repeated exposure to the insects, a possibly lower threshold to infection in young children, immunosuppression due to malnutrition, and excessive activation of T4 lymphocytes by recurrent infections.

Mechanical transmission depends upon an insect being interrupted while feeding on an infected host and then moving to a susceptible host to complete its blood meal. Transmission would occur via contaminated mouthparts and/or regurgitation. Interrupted feeding has been observed in laboratory bedbugs,[6] and transmission of virus by this means is a possibility.[6] Regurgitation has been observed in the related bug *Rhodnius prolixus*.[7]

In preliminary experiments to assess whether insects can transmit HIV, adult *C lectularius* and *Aedes aegypti* mosquitoes from laboratory colonies were allowed to engorge on defibrinated blood to which had been added tissue culture supernatant from HIV infected cells. 60 min after feeding the insects were homogenised in a very small amount of RPMI 1640 and the fluid was assayed for reverse transcriptase (RT) activity after clarification by centrifugation. The fluids were all negative for RT activity at this stage so they were sterile filtered and added to the permissive H9 cell line (HIV negative, kindly supplied by Dr Robert C. Gallo, NIH). Supernatant fluids from the cultures were tested for RT activity every 3-4 days. From the 23rd day of culture, RT activity was recorded in the supernatant from cells inoculated with fluid from

RT ACTIVITY (AS MULTIPLE OF NEGATIVE CONTROL) RECORDED IN SUPERNATANTS FROM H9 CULTURES INOCULATED WITH EXTRACTS FROM POOLS OF 5 BEDBUGS OR 5 MOSQUITOES

| Day | RT activity | |
|---|---|---|
| | Bugs | Mosquitoes |
| 20 | 1·0 | 1·0 |
| 23 | 62 | 1·5 |
| 30 | NT | 1·4 |
| 34 | 121 | 1·0 |
| 44 | NT | 1·4 |

ground bedbugs. This activity increased to over 120 times more than the background activity by day 34 (table). In contrast cultures inoculated with fluid from mosquitoes remained negative.

The survival of HIV for 1 h in *C lectularius* following the feed on a blood-virus mixture suggests that mechanical transmission of the virus between human beings could be carried out by bedbugs.

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## RIBAVIRIN ENTERS CEREBROSPINAL FLUID

SIR,—During a phase I trial of oral ribavirin for the treatment of patients with AIDS and AIDS-related complex (ARC) the level of ribavirin in the CSF of four patients after several weeks of ribavirin was measured. Patients received a loading dose of 1200 mg ribavirin orally two times a day for three days and then 300 mg orally twice a day for 8 weeks. Ribavirin levels were measured when the patient had been on the drug for 4-7 weeks. Three patients had CSF taken during investigations of neurological symptoms and one patient was a volunteer. Ribavirin levels in the CSF and in plasma from a simultaneous blood sample were measured in three patients. In one patient, ribavirin was measured in CSF and in plasma from a blood sample obtained 3 days later. The ribavirin level was measured by sensitive radioimmunoassay on specimens obtained in Boston and sent to the antiviral laboratory of the University of California, San Diego.[1] Significant ribavirin levels were present in the spinal fluid:

| Patient | Diagnosis | Duration of ribavirin therapy (wk) | Ribavirin levels (μmol/l) in: Plasma | CSF | CSF level as % of blood level |
|---|---|---|---|---|---|
| 1 (ARC) | Thrush | 7 | 5·6 | 7·8 | 73 |
| 2 (AIDS) | CNS confusion | 7 | 7·7 | 11·3 | 67 |
| 3 (ARC) | Numbness in hand | 6 | 6·2 | 5·4 | 115 |
| 4 (AIDS) | CNS confusion, post-cryptococcal meningitis | 4 | 8·6 | 7·0[a] | ... |

[a]Blood drawn 3 days after lumbar puncture. Patient did not receive additional ribavirin after lumbar puncture.

The pharmacokinetics of ribavirin uptake in CSF were not evaluated. These results indicate that with at least 4 weeks of oral RBV, more than 67% of a simultaneous plasma level is found in the

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# Experimental Assessment of Bedbugs (*Cimex lectularius* and *Cimex hemipterus*) and Mosquitoes (*Aedes aegypti formosus*) as Vectors of Human Immunodeficiency Virus

Peter G. Jupp and Susan F. Lyons

*In vitro* experiments were conducted to assess whether bedbugs (*Cimex lectularius* and *Cimex hemipterus*) and mosquitoes (*Aedes aegypti formosus*) could act as vectors of HIV. These insects engorged through a membrane on a blood–virus mixture. Female bedbugs were larger than males and took larger blood-meals when fed to repletion. It was determined that the full blood-meal of a female bedbug contained $0.09 \times 10^5$ tissue culture infectious doses (TCID) of virus and a male $0.07 \times 10^5$ TCID, while partial meals taken when feeding was interrupted contained $0.013 \times 10^5$ TCID and $0.015 \times 10^5$ TCID for female and male bugs, respectively. Reverse transcriptase activity was assayed after culture of insect extracts in H9 cells: this showed survival of virus in *C. lectularius* for up to 4 h, in *C. hemipterus* for up to 1, possibly 2 h, but no survival in *Ae. aegypti formosus*. Four attempts to transmit the virus by interrupted feeding by *C. lectularius* from a blood–virus mixture to uninfected blood failed. It is concluded that *Ae. aegypti formosus* and probably other mosquitoes are not mechanical vectors of HIV and that such transmission is also unlikely to occur in bedbugs under natural conditions.

AIDS 1987, 1:171–174

**Keywords: HIV, insect vector experiments, *Cimex lectularius*, *Aedes aegypti*.**

## Introduction

The epidemiological pattern of 'Western' AIDS in the USA does not suggest the involvement of arthropods in its transmission [1]. However, there are aspects in the epidemiology of 'African' AIDS in Central Africa which suggest that arthropods could be involved in this region [2,3]. Arthropod transmission could account for the equal sex distribution of AIDS cases in Central Africa, the high prevalence of antibodies to the virus in children there [4] and the clustering of HIV infection both in a family in Rwanda [5] and in households in Zaire [6]. In a preliminary study our laboratory reported the results of experiments which showed that HIV survived in the bedbug, *C. lectularius*, for at least 1 h after feeding on a blood–virus mixture [7]. These *in vitro* experiments were continued to assess whether bedbugs and mosquitoes possess the ability to act as mechanical vectors of HIV.

## Materials and methods

### Insects

*C. lectularius* Linnaeus, the common bedbug, came from a long-established colony maintained at the laboratory of the South African Bureau of Standards. *C. hemipterus* Fabr, the tropical bedbug, was collected in northern Kwazulu and these field bugs were used directly in the experiment. *Ae. aegypti formosus* (Walker) came from a colony which originated from mosquitoes collected at Richards Bay, Natal.

### Insect feeding

For all infective meals, insects were fed through a membrane on a blood–virus mixture. The membrane feeding was done as described previously [8,9], except that mixtures were heated to a higher temperature (34°C) in the water bath and the membranes were different.

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Sponsorship: Financial assistance was received from the Medical Research Council and the Poliomyelitis Research Foundation

Date of receipt: 3 February 1987, revised: 10 August 1987

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Bedbugs fed through Parafilm (American Can Company, Greenwich, CT, USA) which had been tightly stretched over mosquito netting, while mosquitoes fed through Badruche membrane which fitted tightly over netting. The feeding took place inside an insect-proof laminar flow hood. Afterwards the insects were held at 22–25°C and 75% relative humidity until killed by being placed in a −70°C freezer, where they were stored until processed. For attempted bedbug transmission of the virus by interrupted feeding, a canister with 100 adult *C. lectularius* was placed in contact with the blood–virus mixture for 3 min to permit partial engorgement. The bugs were then transferred to a clean canister with a new membrane before being allowed to complete their feed on uninfected defibrinated blood.

Samples of male and female bugs, respectively, were weighed before and after feeding on defibrinated blood through a membrane, either to repletion or after a partial 3 min feed. This enabled weights of individual blood-meals to be calculated.

### Tissue culture

HIV-infected H9 cells and uninfected control H9 cells were maintained in RPMI 1640 supplemented with 10% fetal calf serum (FCS) and diluted to a concentration of $0.5 \times 10^6$ viable cells/ml twice weekly. Culture supernatants of the HIV-infected cells, prepared by centrifuging the cell suspension at 900 g for 20 min at 4°C, were used as the source of HIV for the infective blood-meals.

### Virological techniques

Three experiments were carried out. For each, a blood–virus mixture was prepared for the infective feed by mixing human defibrinated blood with HIV-infected tissue culture supernatant in the proportion 2:1. The amount of virus in the HIV-infected tissue culture supernatant was determined using the Dupont HIV p24 enzyme-linked immunosorbent assay test (Boston, MA, USA) for antigen detection. From this, the concentration of virus in the blood–virus mixture as well as in blood-meals imbibed by single bedbugs was calculated. An HIV-antibody-negative donor was bled and the blood–virus mixture made just before the insects were fed. The mixture was assayed for reverse transcriptase (RT) activity before and immediately after the insects had completed their feed. While the assay for RT activity was done directly on the blood–virus mixture or uninfected defibrinated blood, extracts made from the insect pools and blood into which transmission was attempted were first cultured in H9 control cells. Supernatants from these cultures were assayed as direct assay of insect pools gave uniformly negative results, presumably because of the low levels of virus present.

To determine the RT activity, the blood–virus mixture, defibrinated blood and cell culture fluids were clarified by centrifugation at 900 g for 20 min at 4°C and then ultra-centrifuged at 66 000 g. The pellets thus obtained were resuspended in pH 8.3 buffer (20 mmol/l Tris-HCl and 0.33 mmol/l EDTA) and disrupted with 0.24% triton X-100 for measurement of enzyme activity. The polymerase reaction mixture (55 µl) contained 14.7 mmol/l Tris-HCl, 0.243 mmol/l EDTA, 5.0 mmol/l $MgCl_2$, 18.0 mmol/l KCl, 0.3 mmol/l GTP, 14.5 mmol/l DTT, 1.52 µmol/l $^3$H-TTP (40–60 Ci/mmol) and 35 µg/ml of poly-rA, oligo-dT. Fivefold or greater increases in RT activity over background were considered as indicative of the presence of HIV.

Each pool of five bedbugs or mosquitoes was processed as follows: the pools were homogenized in 1 ml of RPMI 1640 and centrifuged at 900 g for 20 min at 4°C, and the supernatant was sterile filtered. The filtered supernatant was then added to the H9 control cell line: 1 ml filtrate and 2.5 ml RPMI 1640 supplemented with 10% FCS were added to 1.5 ml cells to give a final concentration of $0.5 \times 10^6$ viable cells/ml. These cell cultures were maintained by dilution to a concentration of $0.5 \times 10^6$ viable cells/ml with RPMI 1640 plus 10% FCS twice a week. Supernatants from these cultures were assayed for RT activity weekly from week 3 until week 6.

## Results

Table 1 shows the weights of unengorged bedbugs and the weights and volumes of their full and partial blood-meals. To determine the size of the partial meal a batch of partially fed bugs were weighed, their mean weight calculated and the mean unfed weight previously found subtracted from this. It can be seen that female bugs were larger than males and took considerably more blood when they fed to repletion. However, the male bugs took slightly more blood during a 3 min partial feed than the females because they started feeding sooner. The mean amount of virus present in the infective feed was $4.4 \times 10^5$ TCID per ml. From this figure and the mean blood-meal volumes it was calculated that a full female meal contained $0.09 \times 10^5$ TCID, a full male meal $0.07 \times 10^5$ TCID. Partial meals contained $0.013 \times 10^5$ TCID and $0.015 \times 10^5$ TCID for females and males, respectively.

Table 2 shows the results obtained in tests for HIV

**Table 1.** Weight (g) of the common bedbug, *Cimex lectularius* and its blood-meal

| | Unengorged bugs | | | Full blood-meal | | | Partial blood-meal* | |
|---|---|---|---|---|---|---|---|---|
| | No. | Range | Mean | No | Range | Mean | No | Mean |
| Females | 50 | 0.0033–0.0088 | 0.0055 | 28 | 0.0120–0.0261 | 0.0188 (0.0185) | 18 | 0.0025 (0.0028) |
| Males | 49 | 0.0021–0.0059 | 0.0036 | 27 | 0.0096–0.0206 | 0.0154 (0.0151) | 10 | 0.0032 (0.0031) |

*This was determined by weighing the sample of partially fed bugs together, calculating their mean weight and subtracting from this the mean unengorged weight. Figures in parentheses are volumes (ml) of defibrinated blood imbibed

**Table 2.** Tests showing positive (+) or negative (−) reverse transcriptase activity tests in pools of five bedbugs or mosquitoes

| | Interval after infective meal | Reverse transcriptase activity |
|---|---|---|
| *C. lectularius* | 1 h | +, +, +, − |
| | 2 h | +, − |
| | 3 h | +, + |
| | 4 h | +, + |
| | 5 h | −, − |
| | 6 h | −, − |
| | 1–4 days | −, −, −, − |
| *C. hemipterus* | 1 h | +, − |
| | 2 h | ±*, −, − |
| | 3 h | −, − |
| *Ae. aegypti* | 1 h | −, − |
| | 2 h | −, − |
| | 3 h | −, − |
| | 4 h | −, − |
| | 5 h | −, − |
| | 1 day | − |

*Marginally positive.

infectivity by RT assay on *C. lectularius*, *C. hemipterus* and *Ae. aegypti formosus*, respectively. As can be seen, the virus survived for up to 4 h in *C. lectularius*, up to 1 but possibly 2 h in *C. hemipterus* but remained undetectable in *Ae. aegypti formosus*. Four attempts to transmit HIV by interrupted feeding, using groups of *C. lectularius*, from a blood–virus mixture to uninfected blood failed.

## Discussion

Our results showing that full blood-meals taken by *C. lectularius* females were larger than those taken by males are in accord with the results for Senegalese *C. hemipterus* obtained by Yanovski and Ogston [10]. However, our colony of *C. lectularius* took much larger volumes of blood although the unfed bug weights were similar.

Amplification of HIV through passage in a permissive cell line demonstrated that viable virus was present in the bugs. However, as the technique was both time-consuming and complex, it was impractical to apply this test to large numbers of insect pools and to include those of insects killed at long intervals after an infective meal. The possibility cannot, therefore, be excluded that HIV might have multiplied and reached the salivary glands in some or all of the insect species tested if longer incubation periods had been allowed in the experiments. However, this is thought to be unlikely because of the requirement for the T4 receptor for viral attachment. It is considered that the most likely mode of insect transmission of HIV would be mechanical by interrupted feeding without viral replication in the insect. Hence our experiments were designed to investigate this second possibility.

It is unlikely that the RT activity seen in our experiments was caused by an endogenous insect retrovirus as insects fed on uninfected blood gave uniformly negative results even when the same amplification system was employed. Our results indicate that *Ae. aegypti formosus* would not be able to act as a mechanical transmitter since no short-term survival of virus was demonstrated. Mosquitoes as a whole can probably, therefore, be excluded as vectors of HIV, although other species larger than *Ae. aegypti*, which take a correspondingly larger blood-meal, still need to be evaluated. The failure of *C. lectularius* to transmit the virus after interrupted feeding in four attempts, each involving a large number of bugs (100), tends also to discount transmission by bedbugs. However, the survival of virus in both species of bug, especially the 4 h survival in *C. lectularius*, would permit bedbugs to transmit HIV mechanically between humans under natural conditions provided interrupted feeding were to occur with a sufficiently large number of bugs and the virus was sufficiently infectious. Whether the former occurs naturally is unknown but HIV is known to have a low infectivity [11,12]. Both species of bug apparently occur in Central Africa where bedbugs might play a role in HIV transmission [13]. The shorter survival of the virus in *C. hemipterus* compared with *C. lectularius* may be due to the smaller size of individuals of the former species, with their correspondingly smaller blood-meal.

There is strong evidence that hepatitis B virus (HBV) is mechanically transmitted by bedbugs [14] but not by mosquitoes [15], and that bedbugs remain infected for up to 7½ weeks [9,14] after an infective meal. Although data on the levels of HIV circulating in the bloodstream of an AIDS patient are very scarce, it has been reported that there are only $10^4$ particles of HIV per ml of infected blood compared with $10^{13}$ particles of HBV in an individual with chronic hepatitis B infection [12]. This higher concentration of HBV must be part of the reason why HBV is much more infectious than HIV. The transmission of HBV but not HIV by accidental needle-stick to a health worker from a patient infected with both viruses demonstrates the comparative infectivity of the two viruses [11]. Rift Valley fever virus has been mechanically transmitted in the laboratory [16,17], by mosquitoes which were allowed to engorge partially on viraemic hamsters and then allowed to complete their meals on susceptible hamsters. It is noteworthy, however, that the viraemia in the hamsters used for the infecting feed was $10^8$ PFU/ml, which is also much higher than the level of HIV reported in AIDS patients. From these experiments and those of the present study it seems unlikely that HIV is transmitted by insects, given the low infectivity of HIV and the short survival of the virus in bedbugs.

## Acknowledgements

We wish to thank Dr R.C. Gallo of the National Cancer Institute, Bethesda, MD, USA, for supplying the HIV-infected H9 cell line and the H9 control cell line, Mr van der Linde of the South African Bureau of Standards and Mr K. Newberry of the National Institute of Tropical Diseases, Eshowe, Natal, for supplying bedbugs and Miss G.M. McGillivray, Mrs M. Bijsters and Sister J. McAneney for technical assistance.

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**Name of thesis: the ecology and epidemiology of arboviruses in South Africa with reference to their arthtopod vectors**

***PUBLISHER:***
**University of the Witwatersrand, Johannesburg**
**©2015**

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