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STUDIES ON THE TRANSMISSION OF AFRICAN HORSESICKNESS

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ABSTRACT

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Laboratory-reared *Aedes aegypti* (Linnaeus, 1762), *Culex pipiens fatigans* Wiedemann, 1828, and trap-caught *Culicoides* biting midges were fed on African horsesickness (AHS) virus solutions and on horses infected with AHS. Attempts to isolate AHS virus from these insects from 1 to 40 days after feeding by intracerebral inoculation of suckling mice were unsuccessful. The successful artificial infection of mosquitoes with AHS virus and biological transmission of AHS virus by *Culicoides* spp. recorded by other workers could not be repeated. Multiplication of AHS virus in these insects will have to be shown before existing claims of successful biological transmission can be accepted completely.

INTRODUCTION

One of the reasons for the establishment of the Veterinary Laboratory at Onderstepoort in 1908 by Sir Arnold Theiler was the prevalence of African horsesickness (AHS) in this area. This is still an important disease of equines in Africa and its spread in recent years as far as India has justified it being considered an emerging disease (Howell, 1963).

Theiler (1915) investigated this problem and concluded that the disease was transmitted by insects. Schuberg & Kuhn (1912) had already shown *Stomoxys calcitrans* (Linnaeus, 1758) to be capable of transmitting AHS mechanically. Nieschulz, Bedford & du Toit (1934) and Nieschulz & du Toit (1937) made a thorough study of the mosquito fauna at Onderstepoort and after numerous unsuccessful transmission attempts concluded that "mosquitoes are not vectors of horsesickness".

In 1944 du Toit published findings which proved that wild-caught *Culicoides* spp. were infected with AHS virus from March to May 1943. This was shown by injecting emulsions of *Culicoides* caught in a modified New Jersey light trap into susceptible horses. He then attempted to transmit AHS by *Culicoides* bite from an infected to susceptible animal and succeeded in April 1945. Wild-caught *Culicoides* were fed on an infected horse. Twelve days later they were refed on a susceptible horse, seven engorged, and the horse died of AHS after 12 days (R. M. du Toit, formerly of the Veterinary Research Institute, Onderstepoort, personal communication, 1969).

Ozawa & Nakata (1965) transmitted AHS by means of the bites of *Anopheles stephensi* Liston, 1901 and *Culex pipiens* Linnaeus, 1758 which had fed on infected horse blood 15 to 22 days previously. The two test horses died after unusually long periods of 35 and 48 days respectively after exposure to bites. Ozawa, Nakata, Shad-Del & Navai (1966) succeeded in transmitting AHS with *Aedes aegypti* (Linnaeus, 1762). These were fed a virus suspension with a titre of $10^{6.2}$ $TCID_{50}$/ml. Nineteen days later they were refed on a horse which in turn died 18 days after exposure. It is doubtful whether biological transmission occurred since the virus titre in these mosquitoes dropped to $10^{2.7}$ $TCID_{50}$/ml 18 days after the infective feed. These workers reasoned, however, that since AHS virus was recovered from *A. aegypti* up to 36 days after artificial infection it was unlikely that mechanical transmission could have occurred.

It was felt that the transmission of AHS had still to be explained satisfactorily. Further attempts were therefore made to transmit AHS virus with *Culex pipiens fatigans* Wiedemann, 1828, *A. aegypti*, and *Culicoides* spp. as potential vectors.

MATERIALS AND METHODS

Mosquitoes

A. aegypti were from a two-year old colony kept at Onderstepoort. *C. p. fatigans* were originally collected at Komatipoort, Transvaal in April 1967 and a colony was established at Onderstepoort. Both species were raised at temperatures between 20°C and 24°C with a 12 hour larval daylight period.

Culicoides

These were caught nightly in front of an open horse stable at Onderstepoort in a suction-type light-trap described by Nevill (1967). The most prevalent species were:
C. pallidipennis Carter, Ingram & Macfie, 1920; *C. pycnostictus* Ingram & Macfie, 1925; *C. distinctipennis* Austen, 1912; *C. schultzei* Enderlein, 1908; and *C. milnei* Austen, 1909. *C. pallidipennis* accounted for more than 80 per cent of the catches.

Techniques for feeding mosquitoes and Culicoides *on horses*

In the experiments with mosquitoes the feeding technique used by Nieschulz & du Toit (1937) was adopted. Small cages (12.7 × 7.6 × 6.4 cm) covered with cotton mosquito netting were held in position in holes in a special saddle fitted to a horse's shaven back.

Culicoides midges were initially placed in similar cages covered with nylon chiffon. It was found that the shape of the saddle did not fit all horses equally well so another method was adopted. Three 150 cm lengths of latex tubing were attached to a metal box which fitted snugly over the feeding cage and were tied tightly around the horse's body so that the cage was in close contact with its shaven back allowing the midges to feed through the nylon chiffon.

The entire cage, except its base and sleeve, was covered with strong polythene, and a pledget of wet cotton wool was placed between the chiffon and polythene to maintain a high humidity.

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Technique for holding mosquitoes and Culicoides *during the incubation period*

After being allowed to feed overnight, engorged specimens were placed in 500 ml white unwaxed cardboard cups covered with nylon chiffon on which rested a cotton wool wad saturated with 5 per cent sugar solution. The cups were kept in the dark at 22°C for the duration of the incubation period (the period necessary for virus multiplication to take place in the insect body).

Horses: The horses used were between 1 and 2 years old, fully susceptible to AHS, and were kept in an insect-free stable. Before and after exposure they were tested for AHS antibodies by means of the complement fixation test. Temperatures were taken twice a day for up to 3 months.

Virus: In the tests with mosquitoes Type 4 Isolate No. 32/62 third passage in suckling mouse brain (32/62 generation 3 Smb) was used, whereas Type 5 Isolate No. 30/62 third passage in suckling mouse brain (30/62 gen. 3 Smb) was employed with *Culicoides* spp. Both isolates were previously stored at −76°C.

Titrations: Virus titrations were conducted in 2-day old mice and end-points (LD_{50}) were calculated by the method of Reed & Muench (1938). Mosquitoes or *Culicoides* spp. were ground up with alundum, suspended in 0.03 ml of stabilizing solution prepared from 0.066 molar phosphate buffer (pH 7.4) with 2 per cent peptone and 10 per cent lactose and centrifuged at 3000 rpm for 10 min. The supernatant fluid was tested in suckling mice by intracerebral inoculation of 0.03 ml of serial tenfold dilutions. The mice were counted until the 7th day after inoculation. The brains of the dead or dying mice were retested for AHS virus.

AHS Transmission attempts with *C. P. fatigans* and *A. aegypti*

Infection of mosquitoes in vitro

Type 4 (32/62 gen. 3 Smb) virus was diluted with haemolyzed blood of a healthy horse as described by Ozawa *et al.* (1966). Blood and phosphate buffered saline (pH 7.4), were mixed in equal parts and centrifuged for 10 min at 3000 rpm. The sediment was resuspended in 5 volumes distilled water and again centrifuged. The supernatant was diluted with an equal volume of virus suspension containing 5 per cent glucose, titrated and diluted to contain $10^{2.9}$ LD_{50}.

Mosquitoes that were 3 to 5 days old and had only received water during the previous 36 hours, were allowed to feed overnight on a cotton wool wad soaked with the virus-haemoglobin mixture. The wad was placed in a 9 cm diameter petri dish which projected down through a hole in the floor of a 30 × 30 × 30 cm cage into water kept at 38°C. About 85 per cent of the mosquitoes engorged.

Both individual mosquitoes and pools of five were tested for AHS virus almost daily over a period of 40 days. No AHS virus was recovered from either *C.p. fatigans* or *A. aegypti* (Table 1).

Infection of mosquitoes in vivo

Horse 6550 was infected by intravenous inoculation of 5 ml AHS virus suspension (32/62 gen. 3 Smb, titre $10^{6.1}$ LD_{50}) and after its temperature had risen above 39.4°C [3rd day post inoculation (p.i.)], one cage containing *A. aegypti* and one of *C.p. fatigans* were placed nightly on its shaven back. The horse died 7 days p.i. [Fig. 1(a)]. A post mortem examination showed that death was due to AHS and a spleen suspension in

Table 1 Mosquitoes tested in suckling mice for presence of AHS virus after feeding on an infective cotton wool wad

No. of days after infective feed	*C.p. fatigans*		*A. aegypti*	
	Numbers tested	Result	Numbers tested	Result
1	5 × 1*	Neg.	5 × 1	Neg.
2	5 × 1	Neg.	5 × 1	Neg.
3	5 × 1	Neg.	5 × 1	Neg.
4	5 × 1	Neg.	5 × 1	Neg.
5	5 × 1	Neg.	5 × 1	Neg.
6	5 × 1	Neg.	5 × 1	Neg.
7	5 × 1	Neg.	5 × 1	Neg.
8	5 × 1	Neg.	5 × 1	Neg.
9	5 × 1 + 5 pooled	Neg.	5 × 1	Neg.
10	5 × 1 + 5 pooled	Neg.	5 × 1	Neg.
11	5 × 1 + 5 pooled	Neg.	5 × 1	Neg.
12	5 × 1 + 5 pooled	Neg.	5 × 1	Neg.
13	5 × 1 + 5 pooled	Neg.	5 × 1	Neg.
14	5 × 1 + 5 pooled	Neg.	5 × 1	Neg.
15	5 × 1 + 5 pooled	Neg.	5 × 1	Neg.
16	5 × 1 + 5 pooled	Neg.	5 × 1	Neg.
17	5 × 1 + 5 pooled	Neg.	5 × 1	Neg.
18	5 × 1 + 5 pooled	Neg.	5 × 1	Neg.
19	5 × 1 + 5 pooled	Neg.	5 × 1	Neg.
20	5 × 1 + 5 pooled	Neg.	5 × 1	Neg.
21	5 pooled	Neg.		
22	5 pooled	Neg.		
23	5 pooled	Neg.		
24	5 pooled	Neg.		
25	5 pooled	Neg.		
26	5 pooled	Neg.		
27	5 pooled	Neg.		
28	5 pooled	Neg.		
29	5 pooled	Neg.		
30	5 pooled	Neg.		
31-39	None tested			
40	5 pooled	Neg.		

*5 × 1 = 5 mosquitoes were tested individually

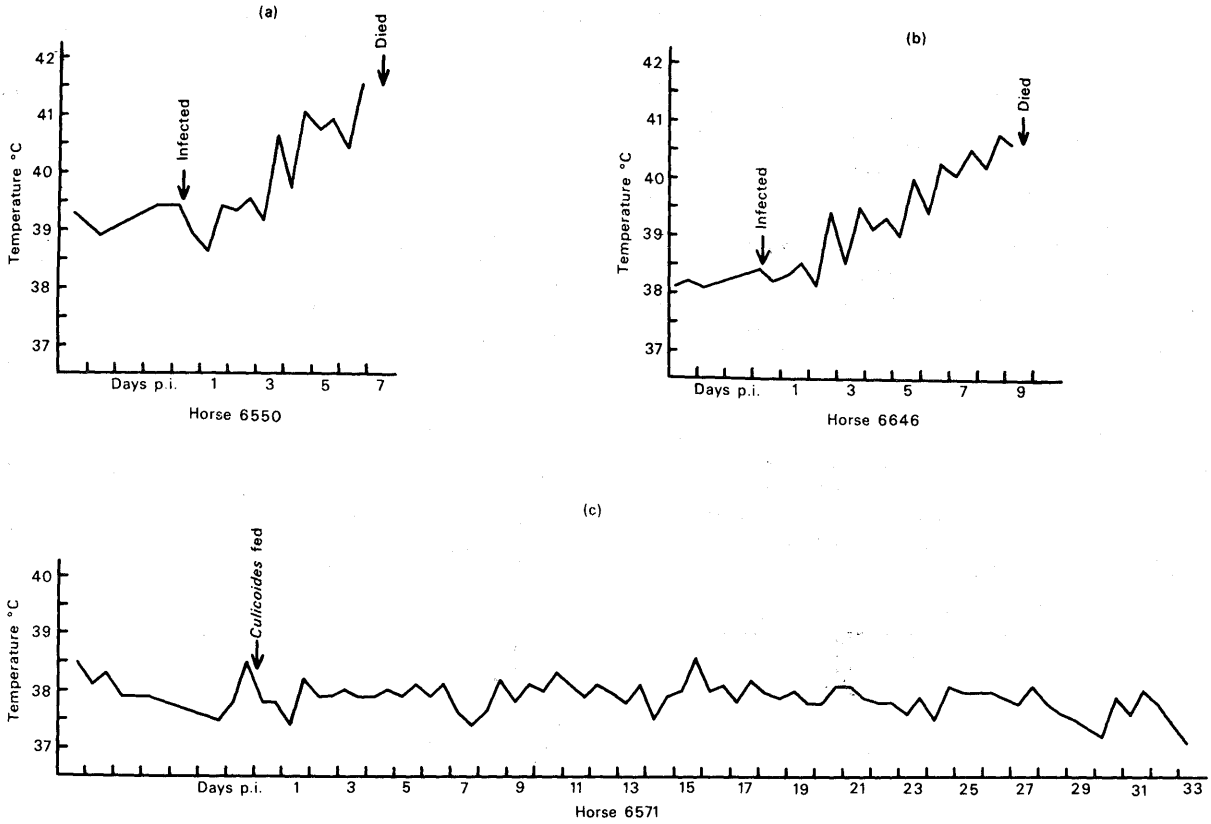

FIG. 1 Daily temperature charts of experimental horses
(a) and (b) Horses artificially infected with AHS virus
(c) Horse on which "infected" *Culicoides* midges were allowed to feed
(p.i. = post infection)

stabilizing solution was shown to have a virus titre of $10^{2.2}$ LD_{50}.

Pools of five mosquitoes were tested in mice from the 4th until the 20th day after feeding. Six *A. aegypti* and 15 *C.p. fatigans* which fed on the AHS infected horse on the 5th, 6th or 7th nights p.i. were fed on Horse 6535 from 15 to 17 days later and subsequently tested for AHS virus in suckling mice.

AHS virus was not recovered from mosquitoes nor did the horse react to AHS clinically or serologically.

TABLE 2 Triturated *Culicoides* tested in suckling mice for presence of AHS virus after feeding on an infective cotton wool wad +

No of days after infective feed	*Culicoides* Groups		
	A Infected 28.1.69	B Infected 29.1.69	C Infected 31.1.69
1		10 *C. pallidipennis*	
2	10 *C. pallidipennis*	10 *C. pallidipennis*	10 *C. pallidipennis*
3		10 *C. pallidipennis*	10 *C. pallidipennis*
4	10 *C. pallidipennis*	10 *C. pallidipennis*	10 *C. pallidipennis*
5		10 *C. pallidipennis*	10 *C. pallidipennis*
6	10 *C. pallidipennis*	10 *C. pallidipennis*	10 *C. pallidipennis*
7		10 *C. pallidipennis*	10, 40 *C. pallidipennis**
8	10 *C. pallidipennis*	10, 30 *C. pallidipennis**	10, 30 *C. pallidipennis**
9		10, 30 *C. pallidipennis**	10, 13 *C. pallidipennis** 8 *C. pycnostictus*
10	10 *C. pallidipennis*		
11		10, 230 *C. pallidipennis** 4 *C. schultzei*	
12	10 *C. pallidipennis*		
13			
14	10, 20 *C. pallidipennis**		
15			
16	10 *C. pallidipennis*		
17			
18	10, 248 *C. pallidipennis**		

+ = No AHS virus was recovered
* = Two batches of *C. pallidipennis* were tested separately.
In addition to those shown, 13 *Culicoides* midges from Groups A, B, and C were pooled and tested at the termination of the experiment (15.2.69)

AHS TRANSMISSION ATTEMPTS WITH *Culicoides* SPP.

Infection of Culicoides *spp.* in vitro

A cotton wool wad was saturated with a virus-haemoglobin suspension prepared as described for mosquitoes (Type 5,30/62 gen. 3 Smb, titre $10^{2.8}$ LD_{50}). This was placed overnight on a cage of newly caught *Culicoides* spp. In this way three groups of *Culicoides*, totalling about 5 000, were fed on three different days.

A pooled suspension of 10 *C. pallidipennis* from each group was tested for AHS in suckling mice every 2nd day from the 1st until the 18th day after the infective feed using the method described above while further larger groups of *C. pallidipennis* were tested at irregular intervals. All these groups are shown in Table 2.

At the termination of the experiment on 15 Feb., 1969, 9 *C. distinctipennis* and 4 *C. milnei* from Groups A, B and C were pooled and tested for AHS virus in suckling mice. *Culicoides* survival after an incubation period of 14 days ranged from 25 to 80 per cent.

AHS virus was never recoverd.

Infection of Culicoides *spp.* in vivo

Horse 6646 was inoculated intravenously with 2 ml AHS virus suspension (30/62 gen. 3 Smb, titre $10^{6.7}$ LD_{50}). After its temperature had risen above 39.4°C (3rd day p.i.), *Culicoides* spp. were fed nightly on its shaven back. The horse died 9 days later [Fig. 1(b)]. A post mortem examination and subsequent virus isolation confirmed that death was due to AHS.

Ten days after the infective feed 73 *C. pallidipennis* and 3 *C. milnei* refed on Horse 6571 [Fig. 1(c)], while 87 *C. pallidipennis*, 2 *C. milnei* and 1 *C. schultzei* refed on Horse 6569 12 to 18 days after their infective feed. The following *Culicoides* spp. were still alive 18 days after their infective feed and were tested for AHS virus in baby mice:—

(a) Those which fed a second time on Horse 6569:—
1 *C. schultzei*, 2 *C. milnei*, 20 *C. pallidipennis*.

(b) Those which did not feed again:—
1 *C. schultzei*, 3 *C. pycnostictus*, 1 *C. distinctipennis*, 2 *C. milnei*, 20 *C. pallidipennis*.

Horses 6571 and 6569 were kept under observation in an insect-free stable for 38 days after *Culicoides* spp. had fed on them. Thereafter they were tested by complement fixation for antibodies to AHS and since it was winter and natural infection was unlikely to take place, they were transferred to outside paddocks where their temperatures were taken for a further 2 months.

There was no clinical or serological evidence of AHS transmission by *Culicoides* bites nor could virus be isolated from *Culicoides* midges which had fed upon AHS-infected horses 18 days previously.

DISCUSSION

The most plausible explanation for the failure of these attempts to transmit AHS is that the insects are not vectors of AHS. Successful transmissions have, however, been recorded by other workers with various mosquitoes and with *Culicoides* spp. (see Introduction). It therefore seems likely that one or more of these insects are somehow involved in the transmission of AHS.

The periods over which insects were incubated in this investigation appear to have been long enough to allow for virus multiplication. This is particularly evident if compared with periods used in studies on other viruses. *A. aegypti* and *C. fatigans*, artificially infected with West Nile virus, showed the presence of virus between the 7th and 25th day p.i. (Jupp, Brown & McIntosh, 1966). Jochim & Jones (1966) found that the highest multiplication rate of bluetongue virus in intrathoracically inoculated *Culicoides variipennis* Coquillett, 1901 occurred during the first 7 days p.i. Furthermore, *Culicoides* spp. were incubated for 12 days before they transmitted AHS (R. M. du Toit, personal communication, 1969).

In future studies convincing evidence that AHS virus multiplies in the insect hosts should be sought before incriminating them as biological vectors of the disease. A most useful technique to determine whether virus multiplication is possible in the suspect host is to inoculate it intrathoracically with the virus concerned.

SUMMARY

Laboratory-reared *A. aegypti*, *C.p. fatigans* and trap-caught *Culicoides* biting midges were fed on AHS virus solutions and on horses infected with AHS. Attempts to isolate AHS virus from these insects from 1 to 40 days after feeding by intracerebral inoculation of suckling mice were unsuccessful.

The successful artificial infection of mosquitoes with AHS virus and biological transmission of AHS virus by *Culicoides* spp. recorded by other workers could not be repeated. Multiplication of AHS virus in these insects will have to be shown before existing claims of successful biological transmission can be accepted completely.

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