THE USE OF A MEMBRANE FEEDING TECHNIQUE TO DETERMINE THE INFECTION RATE OF *CULICOIDES IMICOLA* (DIPTERA, CERATOPOGONIDAE) FOR 2 BLUETONGUE VIRUS SEROTYPES IN SOUTH AFRICA

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ABSTRACT

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Culicoides spp. in the Lowveld of the northern Transvaal, Republic of South Africa, were fed bluetongue virus serotypes 3 and 6 and African horsesickness virus serotype 1 through latex and chicken skin membranes. After an incubation period of 10 days at 25–27 °C, the infection rate of *C. imicola* for bluetongue virus serotypes 3 and 6 was established at 31 % and 24 % respectively. No African horsesickness virus could be recovered. The membrane feeding technique and handling procedures proved to be suitable for field studies.

INTRODUCTION

To estimate infection rates in *Culicoides* it is necessary to infect them artificially with virus and, after a varying period of multiplication of the virus within their bodies (incubation period), determine the percentage of *Culicoides* still infected.

There are many different methods to artificially infect *Culicoides* with virus. These include the use of infected hosts (Du Toit, 1944; Foster, Jones & McCrory, 1963; Luedke, Jones & Jochim, 1967; Foster, Jones & Luedke, 1968; Luedke, Jones & Jochim, 1976; Standfast, St. George, Cybinski, Dyce, McCaughan, 1978; Muller, 1979; Muller, 1985; Standfast, Dyce & Muller, 1985; Jennings & Mellor, 1988), the use of embryonated chicken eggs (Foster & Jones, 1973; Boorman, Mellor, Penn & Jennings, 1975), intrathoracic inoculation (Jochim & Jones, 1966; Jones & Foster, 1966; Boorman, 1975; Jennings & Boorman, 1984; Muller, 1987; Jennings & Mellor, 1988), oral infection of *Culicoides* with virus using fine needles (Mellor, Jennings & Boorman, 1985), the use of cotton wool pledgets (Jennings & Mellor, 1987; Jennings & Mellor, 1988) and membrane feeding techniques, briefly reviewed below.

There is extensive literature on the artificial feeding of fluids to biting flies through membranes. Jones & Potter (1972) used chicken skin membranes in a six-position feeding apparatus for *C. variipennis*. In Britain, bluetongue virus was fed to *Culicoides nubeculosus* and *C. variipennis* through chicken skin membranes (Mellor & Boorman, 1980; Jennings & Mellor, 1987). Owens (1981) successfully fed 3 Australian species of *Culicoides* on heated bovine blood through a stretched parafilm membrane. Davis, Butler, Roberts, Reinert & Kline (1983) produced a silicone membrane using gauze and silicone sealant. Standfast & Muller (CSIRO, Long Pocket Lab., Brisbane, personal communication, 1984) transferred *Culicoides* to a perspex feeding chamber where the midges fed through a silicone membrane on a shallow pool of heated blood/virus mixture. This technique imitated the natural situation in that blood heated in a water-bath was then circulated through thin tubing.

To determine the vector status of a *Culicoides* species for a specific virus, it is important to compare the infection rates of all the possible candidate species. A comparison of the infection rates of various Australian *Culicoides* species for bluetongue virus demonstrated that the most abundant and most widespread species is not necessarily the most effective vector (Standfast *et al.*, 1985).

In South Africa, there are about 10 *Culicoides* species which are important in stock-associated situations; of these *C. imicola* is the most abundant species (Venter, Nevill & Meiswinkel, 1987). As far back as 1944, Du Toit demonstrated that in South Africa *C. imicola* (=*C. pallidipennis*) was a biological vector of bluetongue and could be one of African horsesickness. In the last ten years various workers have isolated a number of bluetongue virus serotypes and African horsesickness virus from *C. imicola* throughout the length of Africa and into the eastern Mediterranean. Bluetongue virus serotypes 4, 6, 11, 13, 14, 18, 19 and 24 and African horsesickness virus serotypes 2, 4 and 7 have been isolated from field-collected *C. imicola* in South Africa (B. J. Erasmus, Veterinary Research Institute, Onderstepoort, personal communication, 1985) while bluetongue virus serotype 11 and African horsesickness virus serotype 4 have also been isolated from *C. imicola* in Zimbabwe (Blackburn, Searle & Phelps, 1985). Further north in Kenya, Davies, Walker, Ochieng & Shaw (1979) isolated bluetongue virus serotypes 1 and 4 from *C. imicola*, while in Sudan serotype 5 was isolated from *C. imicola* (Mellor, Osborne & Jennings, 1984). Finally, in Israel, Braverman, Barzilai, Frish & Rubina (1985) isolated bluetongue virus serotypes 2, 4, 6 and 10 from *C. imicola*.

Despite these numerous isolations of bluetongue and African horsesickness viruses from *C. imicola*, the infection rate of *C. imicola* for these viruses has never been determined. The infection rate will not only show the effectiveness of *C. imicola* as a vector for bluetongue and African horsesickness but will also indicate what numbers of *Culicoides* are needed for successful transmission of virus. This, coupled to data on seasonal prevalence, parity rates, longevity, distribution and host preference will eventually lead to determination of the vector competence of *C. imicola* for these viruses in different parts of South Africa.

The aim of this study was to explore the value of an artificial membrane feeding technique and other

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procedures for the determination of the infection rate of *C. imicola* for 2 of the 21 bluetongue virus serotypes known to be present in South Africa.

MATERIALS AND METHODS

Source of test insects

Trials were conducted from 4–10 September 1985 at Eiland (23° 40′ S; 30° 45′ E), a mineral spring in the Hans Merensky Nature Reserve which is situated near Letsitele in the subtropical Lowveld of the northern Transvaal, Republic of South Africa. This site was chosen because high numbers of *Culicoides* midges (especially *C. imicola*) occur there throughout the year (G. J. Venter, unpublished data, 1985).

Light-trap catches were made at the horse stable at Eiland as well as near cattle on adjacent farms. A commercially available 220 volt down-draught suction light-trap, equipped with an 8-watt black-light tube, was used to collect live *Culicoides* midges into a 1 000 mℓ plastic beaker. A piece of crumpled paper towel was placed into the beaker prior to catching to provide shelter for the trapped midges against the down-draught of the trap. This increased midge survival (Jennings & Mellor, 1988).

At an improvised field laboratory the catches were emptied into a gauze cage. Active female *Culicoides* were aspirated from this cage and immobilized in a freezer. Once movement of the midges ceased after 3–5 min they were removed from the freezer and transferred to the feeding chamber described below. Exposure of midges to a low temperature prior to blood feeding was found to stimulate feeding in *C. furens* and *C. barbosai* (Linley, 1966).
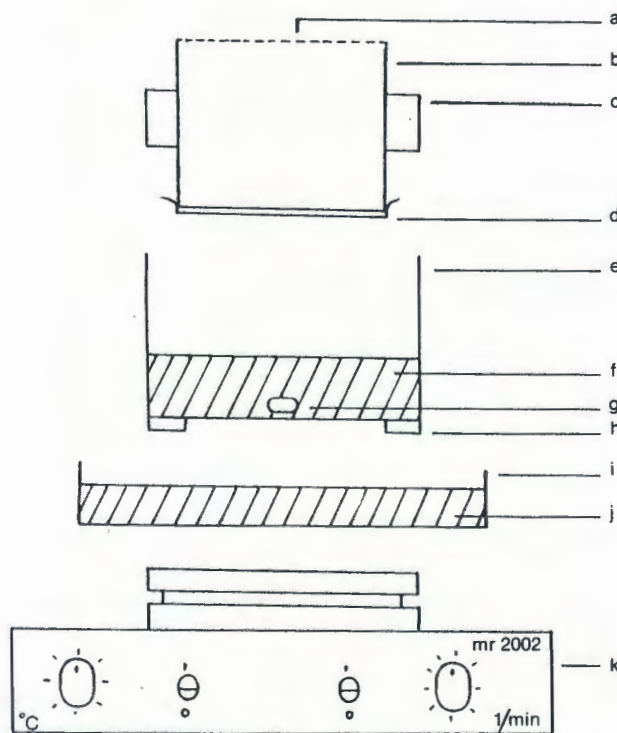

FIG. 1 Blood-feeding device for *Culicoides* consisting of: a = gauze top; b = feeding chamber (plastic 40 mm diameter "pill-bottle"); c = foam rubber; d=membrane; e = blood container (plastic 45 mm diameter "pill-bottle"); f = blood/virus mixture; g = magnetic stirrer bar; h = rubber stopper; i = water- bath; j = water; k = magnetic heater/stirrer

Feeding technique

In this study, we used the blood feeding technique described by Davis *et al.* (1983), but replaced the silicone sealant membrane they used with either chicken skin or latex membrane.

The feeding chamber consisted of a plastic "pill bottle" 40 mm in diameter and 50 mm high (Fig. 1b). The bottom of the pill bottle was replaced by the membrane (Fig. 1d). The membranes used were either stretched "Tahiti"[1] condoms or day-old chicken skin membranes. The condoms had to be thoroughly washed with a soap solution before they could be successfully used in the feeding trials. The chicken skin membrane was prepared as described by Jones & Potter (1972). The membrane was held in position with elastic bands and sealed with silicone sealant. The top of the pill bottle lid was cut out and replaced with gauze netting (Fig. 1a).

The feeding chamber fitted tightly into a second, wider, plastic pill bottle (45 mm in diameter and 40 mm high) (Fig. 1e). A tight fit was accomplished by gluing pieces of foam rubber (Fig. 1c) onto the sides of the feeding chamber. Into the larger container ± 5 mℓ of a blood/virus mixture (Fig. 1f) and a 5 mm long magnetic stirrer bar (Fig. 1g) were placed. The feeding chamber containing the *Culicoides* was placed into the blood in such a way that no air was trapped between the membrane and the blood. The 2 containers were then placed in a water bath (an aluminium foil "pie dish") (Fig. 1i) on a magnetic heater/stirrer (Fig. 1k). The blood container rested on 3 rubber feet (height 10 mm) (Fig. 1h) to prevent contact between the blood and the hot base of the pie dish. The blood was stirred slowly with the magnetic stirrer, taking care that the stirrer bar did not bump against the sides and membrane of the feeding chamber and thereby disturb the *Culicoides* while feeding. The entire feeding device was placed under a foil cover to ensure that feeding could take place in the dark.

The *Culicoides* were offered defibrinated blood of cattle or horses, free of bluetongue and horsesickness antibodies, mixed with either bluetongue virus serotype 3 with a titre of 1,5 × 10⁵ p.f.u./mℓ, bluetongue virus serotype 6 (3 × 10⁶ p.f.u./mℓ), both assayed in cell cultures, or African horsesickness virus serotype 1 (1 × 10⁶ p.f.u./mℓ) determined in Vero cell cultures. These antigens were prepared at the Veterinary Research Institute, Onderstepoort shortly before the field trial. The bluetongue antigens were prepared on CER cell culture (hamster-epithelium), the horsesickness antigen on BHK cell culture, and both were stored in liquid form with 10 % BLP at 4 °C until used. For the infection attempts, the blood/virus mixture was heated to 37 °C and the room temperature was kept at about 25 °C and 50–70 % RH by an air-conditioner. A feeding time of 60–90 min was allowed.

After feeding, the *Culicoides* were again immobilized in a freezer, and engorged specimens were sorted out on a refrigerated chill-table. These midges were then kept in the dark in disposable 500 mℓ unwaxed cardboard cups at 25–27 °C and fed a 10 % sucrose solution. At the end of the trial the cardboard cups with these *Culicoides* were placed into styrofoam boxes and kept cool by icepacks during the 5 h trip back to the Veterinary Research Institute. There they were retained at 25 °C for the remainder of the incubation period.

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TABLE 1 Results of attempts to artificially infect wild-caught *Culicoides* midges with bluetongue and African horsesickness viruses in the field at Eiland (N. Transvaal) September 1985

Virus type & titre	*Culicoides* species	No. alive after 10 days incubation	No. tested	No. of pools of 5 tested	Pools positive	Pools negative	Infection rate
Bluetongue serotype 3 $(1,5 \times 10^5 \text{ pfu/m}\ell)$	C. imicola	420	345	69	58	11	31 %
	C. schultzei grp.	35	25	5	0	5	
	C. bedfordi	5	NT				
	C. nivosus	5	NT				
	C. brucei	8	NT				
Bluetongue serotype 6 $(3 \times 10^6 \text{ pfu/m}\ell)$	C. imicola	440	180	36	27	9	24 %
	C. schultzei grp.	10	NT				
	C. bedfordi	10	NT				
	C. similis	2	NT				
African horsesickness serotype 1 $(1 \times 10^6 \text{ pfu/m}\ell)$	C. imicola	120	115	23	0	23	
	C. nivosus	1	1		0	1	

NT = not tested

It has been shown that *C. variipennis* is capable of orally transmitting bluetongue virus after an incubation period of 10–14 days at a temperature of 23 °C (Foster & Jones, 1979). For the successful transmission of African horsesickness virus 6–13 days at 26 °C was found suitable (Mellor, Boorman & Jennings, 1975; Mellor, Jennings, Braverman & Boorman, 1981). Taking the above studies into consideration, we decided on an incubation period of 10 days. Thereafter the surviving *Culicoides* were again sorted on a chill table into species. Batches of 5 individuals of each species were placed into vials and stored in liquid nitrogen until virus isolation was attempted.

Virus isolation

Bluetongue: Artificially fed and incubated *Culicoides* were homogenized in groups of 5 in 1 mℓ BLP. This suspension was then divided into 2. One part was passaged once in embryonated eggs, via the intravenous route, followed by a passage on CER-cell culture in roller tubes. The remaining part was passaged twice on CER-cell culture in roller tubes with a 14 day interval. Since wild-caught *Culicoides* of unknown viral history were originally used, all positive samples were serotyped by a plaque neutralization test to confirm the identity of the virus.

African horsesickness: The *Culicoides* were homogenized in groups of 5 in 1 mℓ Eagle's medium. This suspension was then divided into 2; 1 half was passaged once on CER-cell culture, followed by 3 passages, at 14 day intervals, in suckling mice. The 2nd half was passaged 3 times in suckling mice only.

Estimation of infection rate

To reduce the volume of work needed to test individual specimens of *Culicoides*, pools consisting of 5 midges each were used. We applied the formula of Chiang & Reeves (1962) to determine the infection rate.

According to this formula:

$$P = 1 - [(n-x)/n]^{1/m} \times 100$$

where P = estimate of the infection rate
  m = pool size. The pool size must be such that there will be both positive and negative pools amongst the pools tested
  n = number of pools tested
  x = number of positive pools

RESULTS AND DISCUSSION

The feeding rate for the different trials varied from 10–70 %. With the chicken skin membrane a higher feeding rate was obtained than with the latex membrane.

Table 1 shows the number of *Culicoides* which fed and survived the 10 day incubation period, the numbers tested for the presence of bluetongue virus serotypes 3 and 6 and African horsesickness virus serotype 1, and, finally, the estimated infection rates.

It has long been known that *C. imicola* can transmit bluetongue (Du Toit, 1944), but now for the 1st time the infection rate for a single population of *C. imicola* has been established, namely, 31 % for bluetongue virus serotype 3 and 24 % for bluetongue virus serotype 6. Even if only 1 *Culicoides* out of every pool of 5 tested positive was infected, the estimated infection rate would still have been high, namely, 15 % for bluetongue serotype 6 and 17 % for bluetongue serotype 3. This infection rate is high enough for *C. imicola* to be an important vector of bluetongue, especially if one takes into account the high populatons of *C. imicola* that can occur and the feeding preference of this species for large mammals (Nevill & Anderson, 1972; Nevill, Venter, Edwardes, Pajor, Meiswinkel & Van Gas, 1988).

The method of Chiang & Reeves (1962) for determining infection rates in pools of mosquitoes was originally developed for field populations. In these populations large numbers of mosquitos can usually be collected, and infection rates are generally low. It is therefore impractical to make a separate attempt at virus isolation for each individual mosquito. However, the use of pools places a limit on the maximum infection rate that can be calculated. For example, in the present laboratory study the maximum infection rate that could have been achieved, with a pool size of 5 *Culicoides*, was 51 % (if 35 out of 36 pools tested positive) and 57 % (if 68 out of 69 pools tested positive) for bluetongue serotype 6 and 3 respectively. Although more time consuming, determination of the infection rate will be more reliable if isolations are made from individual specimens. This 'single-specimen' method has been successfully used on *C. variipennis* in the USA (Jones & Foster, 1966; Foster et al., 1968; Foster & Jones, 1973; Jones & Foster, 1974; Jones & Foster, 1978a).

Only 25 specimens of the *C. schultzei* group were

tested in the present study. The fact that no virus could be recovered from them does not necessarily mean that this group of species is not a vector of bluetongue. Its high occurrence near cattle and sheep, especially in the drier parts of South Africa, makes this species group a prime suspect in these areas (Venter *et al.,* 1987). Only 1 specimen of *C. nivosus* was tested and this was negative.

It is also known that *C. imicola* can transmit African horsesickness virus (Du Toit, unpublished data, 1944 cited by Wetzel, Nevill & Erasmus 1970). Outside Africa *C. variipennis* has been infected orally with African horsesickness virus serotype 9 (Mellor *et al.,* 1975). The numerous isolations of African horsesickness virus out of wild-caught *Culicoides* including *C. imicola* (B. J. Erasmus, Veterinary Research Institute, Onderstepoort, personal communication, 1985), support the possibility of *C. imicola* being a vector. However, during the present trial no African horsesickness virus could be recovered from 115 *C. imicola* tested. There could be various reasons for this lack of success. Although the titre of the original antigen was high, no *Culicoides* were tested on Day 0, i.e. 1–24 h after ingestion of blood. We therefore have no proof that virus was actually ingested by the *Culicoides.*

There are a number of factors which can influence the infection rate and the experimental determination thereof. Jones & Foster (1974) demonstrated that oral susceptibility of *C. variipennis* females to infection with bluetongue virus can be controlled by 1 or more genetic mechanisms. They also showed that the infection rate of *C. variipennis* females for bluetongue is, within limits, positively correlated with the viral concentration in the blood meal and to repeated infective feedings, if the blood meal's viral concentration is low (Jones & Foster, 1978b). Different field populations of *C. variipennis* apparently have different levels of susceptibility to the bluetongue serotypes (Jones & Foster, 1978b). This may be influenced by climate, other environmental factors, and geographic distribution (Walton, Barber, Jones & Luedke, 1984). Furthermore, the same *Culicoides* population may also have different levels of susceptibility to different serotypes of bluetongue virus (Jones & Foster, 1978b).

It is therefore inadvisable to define the susceptibility of any vector population, particularly in an apparently refractive one, without extensively testing the infection rate of a number of batches of individuals on several occasions (Jennings & Mellor, 1987). For controlled laboratory studies these authors suggested a sample size of a minimum of 100 individual flies per test.

Therefore our inability to infect *Culicoides* with African horsesickness virus during these trials is not interpreted by us as being proof that the virus does not multiply in *Culicoides* spp. This opinion is supported by recent studies at the Veterinary Research Institute, Onderstepoort, where mousebrain-derived Africanhorse sickness serotype 3 has been reisolated from membrane-fed wild-caught *C. imicola* after 10–11 days' incubation at 23,5 °C (G. J. Venter & B. J. H. Barnard, unpublished data, 1989).

CONCLUSIONS

1. For the 1st time the infection rate for a single population of *C. imicola* has been established, namely, 31 % for bluetongue virus serotype 3 and 24 % for bluetongue virus serotype 6.

2. In the present trial the infection rate was determined using pools each consisting of 5 *Culicoides.* However, the ideal method to determine the infection rate in artificially infected midges is to isolate the virus from individual specimens.

3. The artificial infection method described in this paper proved to be suitable for use under field conditions, thereby enabling one to work *in situ* where the different suspected vector *Culicoides* species are present in sufficient numbers.

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