J. Hyg., Camb. (1984), 93, 621–628
Printed in Great Britain

Isolation of bluetongue and related viruses from *Culicoides* spp. in the Sudan

By P. S. MELLOR, R. OSBORNE and D. M. JENNINGS
Animal Virus Research Institute, Pirbright, Surrey GU24 0NF

(*Received* 3 *July* 1984; *accepted* 26 *July* 1984)

SUMMARY

Infection of domestic ruminants with bluetongue virus (BTV) is widespread in the Sudan but there are no records of vector species of *Culicoides* in that country. Therefore, light-trap collections of *Culicoides* for virus isolation procedures were made in the Khartoum and Um Benein areas of the Sudan during September–October 1982.

Two virus isolates were made from pools of unengorged, female *Culicoides*. An isolate from a pool of *C. kingi* (*schultzei* gp) is a member of the Epizootic Haemorrhagic Disease (EHD) serogroup. The other isolate from a pool of *C. imicola*, a known BTV vector in other parts of Africa, is type-5 BTV.

In laboratory experiments, the North American vector of BTV, *C. variipennis*, supported replication of both Sudanese isolates to a high titre and transmission occurred after 10 days' incubation.

This paper records the first isolation in the Sudan of arboviruses from *Culicoides*, with the identification of a BTV serotype and the presence of a member of the EHD (genus orbivirus, family *Reoviridae*) serogroup.

INTRODUCTION

Bluetongue virus (BTV) is widespread in the Sudan (Eisa, Karrar & Abu Elrahim, 1979; Eisa *et al.* 1980; W. P. Taylor (personal communication) but so far there have been no isolates of this or any other virus from *Culicoides* midges in that country (Boorman & Mellor, 1982). Previous work has shown that *C. kingi* (*schultzei* gp) and *C. imicola* are the only *Culicoides* species collected around cattle and sheep pens in the Khartoum area in sufficient numbers to be efficient vectors of BTV. However, in order to assess vector competence, a necessary preliminary is to isolate the virus from unengorged midges. Simultaneous recovery of the same virus from sheep or cattle in the area would be significant additional evidence.

MATERIALS AND METHODS

Insects

Insects were collected at the University Farm, Shambat, Khartoum North, and at Um Benein, 250 miles south of Khartoum on the Blue Nile. Two Monks Wood light-traps (Service, 1970) operated from the mains supply via a transformer were used from dusk until dawn. Collection was made into a solution of PDAM (8%

calcium lactobionate, 2% bovine plasma albumen, 90% Eagles's basal medium) containing 0·1% detergent as a wetting agent, 0·075 mg/ml neomycin, 0·05 mg/ml streptomycin, 25 i.u./ml penicillin, 50 i.u./ml polymyxin and 25 i.u./ml mycostatin. *Culicoides* species were sorted under a binocular microscope within 12 h of capture and were distributed into the following groups: *C. kingi* (female, parous and nulliparous, non-engorged); *C. imicola* (female, parous and nulliparous, non-engorged); other *Culicoides* (female, parous and nulliparous, non-engorged); and mixed blood-engorged *Culicoides*. All *Culicoides* were kept at 4 °C until required for processing.

Virus isolation

Pools of midges numbering up to 200 were ground in Griffiths tubes containing 3·5 ml of 2% bovine albumen in phosphate-buffered saline plus 0·075 mg/ml neomycin, 0·05 mg/ml streptomycin, 25 i.u./ml penicillin and 50 i.u./ml polymyxin. The suspensions were clarified by centrifugation at 2000 rev/min for 5 min and 10-fold dilution series were prepared to 10^{-2}. Virus isolation was attempted from each pool by i.v. inoculation into 12-day-old embryonating chick eggs (Herniman *et al.* 1980) and inoculation of BHK 21 cells (Mellor & Boorman, 1980). Eggs dying between 2 and 7 days post infection and tissue cultures showing cytopathic effects (c.p.e.) within the same time were harvested and stored at −70 °C until required. Apparently negative pools were blind-passaged in eggs and tissue culture at least twice to avoid missing virus isolates of low pathogenicity.

Virus isolates were adapted to BHK 21 cells and were then plaque-purified, using the method of Lake (1974). Preliminary virus identification was attempted as follows.

Electron microscopy. Droplets of the supernatants (Eagle's growth medium) from BHK Roux bottles individually infected with each virus isolate were allowed to absorb on to formvar carbon grids for 10 s and were then stained with 2% phosphotungstic acid. Examination for virus particles was carried out at magnifications up to 183000 times.

Double immunodiffusion (*DID*). Virus isolates were tested against standard antisera, using the method of Jeggo, Gumm & Taylor (1983). The group-specific sheep antiserum to BTV was placed in the central well and the test virus and positive control antigens were placed in alternate peripheral wells.

Microneutralization tests

The isolates were checked against all 22 known serotypes of BTV and against Akabane virus, Ibaraki virus and two unidentified viruses from Sudanese cattle, using the method of Herniman, Boorman & Taylor (1983).

Where a relationship between viruses became apparent, a chequerboard system was set up using dilutions of each specific antiserum (1/5, 1/10, 1/20). The reduction in titre of the test viruses against each antiserum compared with their mean titre against three dilutions of normal guinea pig serum gave a measure of the relationship between the viruses.

Infection of colonized C. variipennis *and* C. nubeculosus

Virus isolates that had been plaque-purified at least twice and adapted to grow in BHK 21 cells were fed separately to colonized *C. variipennis* and *C. nubeculosus*,

Table 1. Culicoides *collected for virus isolation in the Sudan during September 1982*

Species of *Culicoides*	Location	No. of non-engorged female *Culicoides*	No. of groups	Virus isolations
C. kingi	Shambat	12049	66	1 (Sudan 48)
	Um Benein	1651	8	0
C. imicola	Shambat	577	15	1 (Sudan 11)
	Um Benein	49	1	0
Others*	Shambat	23	3	0
	Um Benein	0	0	0

* *C. distinctipennis*, *C. milnei*, *C. circumscriptus*, *C. neavei* and C. clear-winged spp.

using the membrane feeding technique described by Mellor, Boorman & Loke (1974). After feeding to repletion on the virus suspensions, the engorged midges were incubated at 27 °C until required. Groups of midges were then assayed for virus (see Mellor 1974). The method of Spearman-Kärber (Finney, 1964) was used to calculate the virus titre in pools of infected midges.

Confirmation and further clarification of the identity of the Sudanese viral isolates was carried out by the World Reference Centre for Bluetongue at Onderstepoort, South Africa.

RESULTS

A total of 14349 unengorged female *Culicoides* were collected for virus isolation over a period of 21 nights in September 1982 (Table 1). More than 95% were *C. kingi* (*schultzei* gp), while *C. imicola* comprised 4·36% of the catch. At least five other species of midge were collected: *C. distinctipennis*, *C. milnei*, *C. circumscriptus* and *C. neavei*, with one or more clear-winged species; these formed 0·16% of the total catch.

Of the midges collected at Shambat, 3·18% of the *C. kingi* and 3·88% of the *C. imicola* were blood-engorged. Such midges were not included in virus isolation procedures, since a positive isolation result may refer only to virus in the blood meal which has no correlation with biological transmission.

Two virus isolates were made from *Culicoides* collected at Shambat (code numbers Sudan 11 and Sudan 48).

Sudan 11 virus was isolated from a group of 85 *C. imicola*. The virus first caused c.p.e. during the second passage in BHK 21 cells after 6 days at 37 °C. However, on subsequent passage, cell sheets were stripped after 24–36 h. The virus also caused haemorrhage and death in chick embryos 2–5 days after infection.

Sudan 48 virus was isolated from a pool of 100 *C. kingi*. C.p.e. was observed during its second passage in BHK 21 cells after 5 days and subsequent passages showed c.p.e. after 3–4 days. Sudan 48 virus was not pathogenic to chick embryos.

Both viruses were re-isolated from the residues of the original pools of *Culicoides*, which had been kept at −70 °C for 3 months.

After two passages in BHK 21 cells the isolates were plaque-purified (Lake, 1974). Sudan-11 virus was given two cycles, and Sudan 48 three cycles, of plaque purification, viruses from plaques being passed once through a monolayer culture

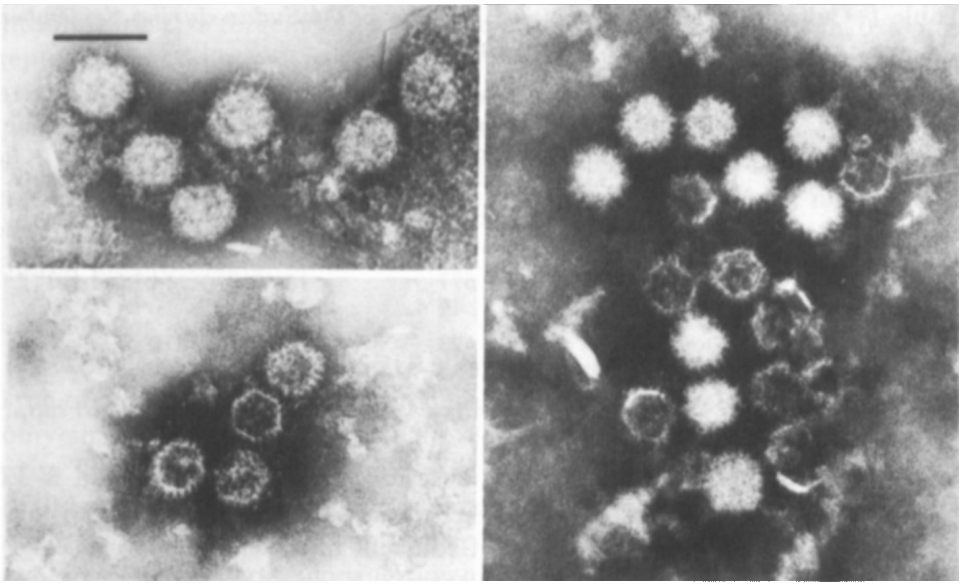

Fig. 1. Electron micrograph of Sudan 11 virus particles. Barline 100 nm. Particle size 70 nm.

of BHK 21 cells subsequent to each cycle. Plaques of Sudan 11 virus were clear and distinct and about 1 mm in diameter, whereas those of Sudan 48 virus were indistinct and difficult to see.

Electron microscopy

Figs 1 and 2 show electron micrographs of Sudan 11 and Sudan 48 virus, respectively. The virus particles are 70 nm in diameter and show the diffuse appearance typical of orbivuruses under the electron microscope.

Double immunodiffusion

Sudan 11 virus at three dilutions gave lines of complete identity with BTV group antigen raised in sheep (Fig. 3), while Sudan 48 virus did not. Antisera to Sudan 11 virus and to Sudan 48 virus raised in both rabbits and guinea pigs recognized group antigens to BTV, epizootic haemorrhagic disease virus (EHD) and Eubenangee virus, with lines of complete identity.

Microneutralization tests

Sudan 11 virus showed no reduction in virus infectivity against specific antisera to Akabane virus, Ibaraki virus, Su 3863 virus (isolated from Sudanese cattle – K. A. J. Herniman, personal communication) and BTV types 1–4, 6–8 and 10–22. However, antiserum to type 5 BTV inhibited virus growth by 10000-fold (4·0 $\log_{10}$ $TCID_{50}$/0·1 ml) while antiserum to type 9 BTV inhibited virus growth by over 6000-fold (3·8 $\log_{10}$ $TCID_{50}$/0·1 ml). Further, antiserum to Sudan 11 virus raised in guinea-pigs reduced the infectivity of BTV types 5 and 9 by 45000-fold (4·7 $\log_{10}$ $TCID_{50}$/0·1 ml) and 13000-fold (4·1 $\log_{10}$ $TCID_{50}$/0·1 ml) respectively. Conversely, antiserum to Sudan 11 virus raised in rabbits had only a marginal effect on type

https://doi.org/10.1017/S0022172400065190 Published online by Cambridge University Press

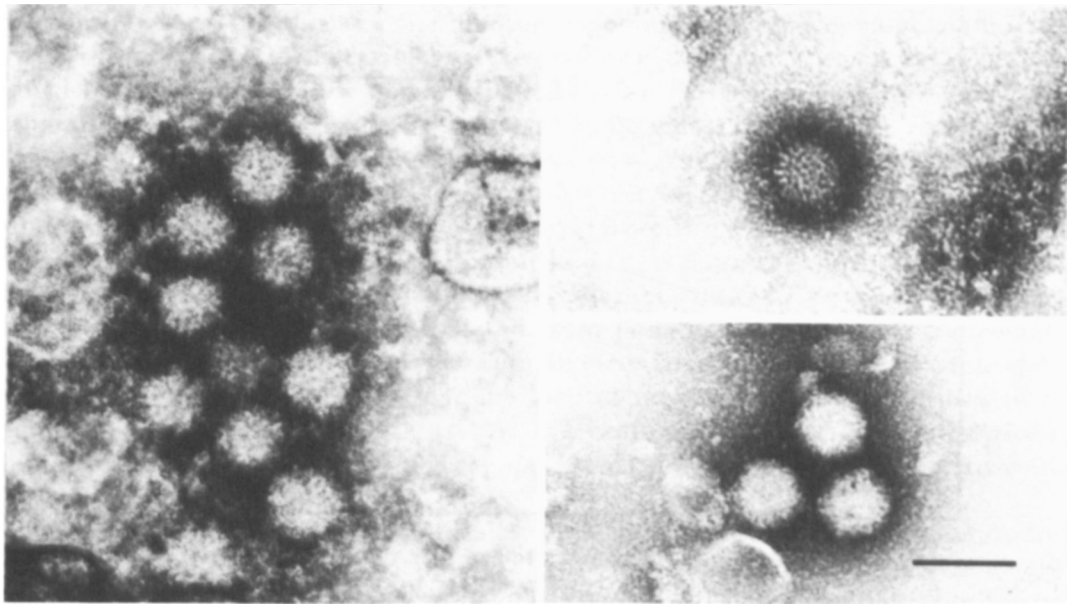

Fig. 2. Electron micrograph of Sudan 48 virus particles. Barline 100 nm. Particle size 70 nm.

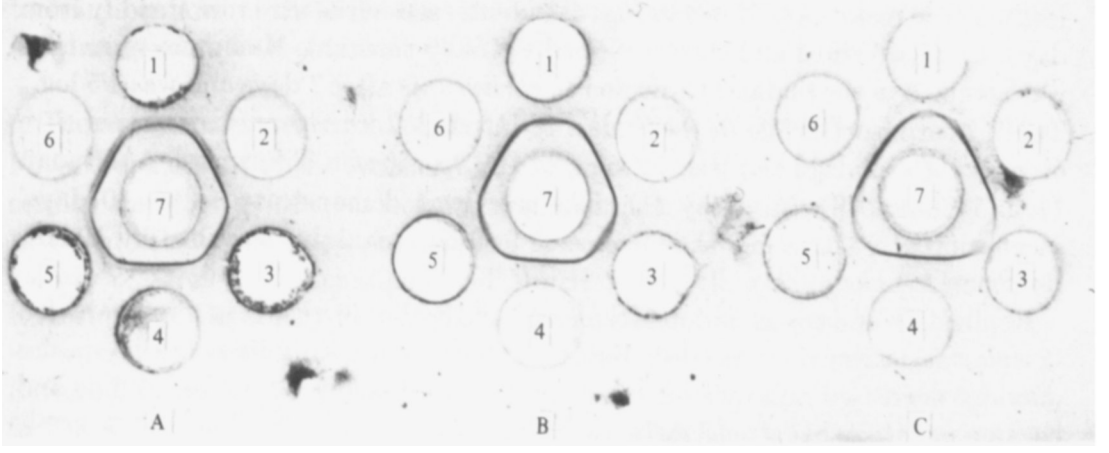

Fig. 3. Double immunodiffusion pattern of Sudan 11 virus (wells 1, 3 and 5) and a bluetongue group antigen from sheep (wells 2, 4 and 6) against a standard bluetongue virus antiserum raised in sheep (well 7). (A–C) neat, 1 in 2 and 1 in 4 dilutions respectively.

9 BTV but it inhibited the growth of type 5 BTV by a factor of over 2500. Antiserum to Sudan 11 virus failed to neutralize any of the other 22 BTV types.

Sudan 48 virus failed to show any specific relationships with Akabane virus, Ibaraki virus, BTV types 1–22 and Su 3905 virus (from Sudanese cattle – K. A. J. Herniman, personal communication). However, it was neutralized by an antiserum to Su 3863 virus raised in guinea-pigs, infectivity being reduced by a factor of 10000 (K. A. J. Herniman, personal communication).

https://doi.org/10.1017/S0022172400065190 Published online by Cambridge University Press

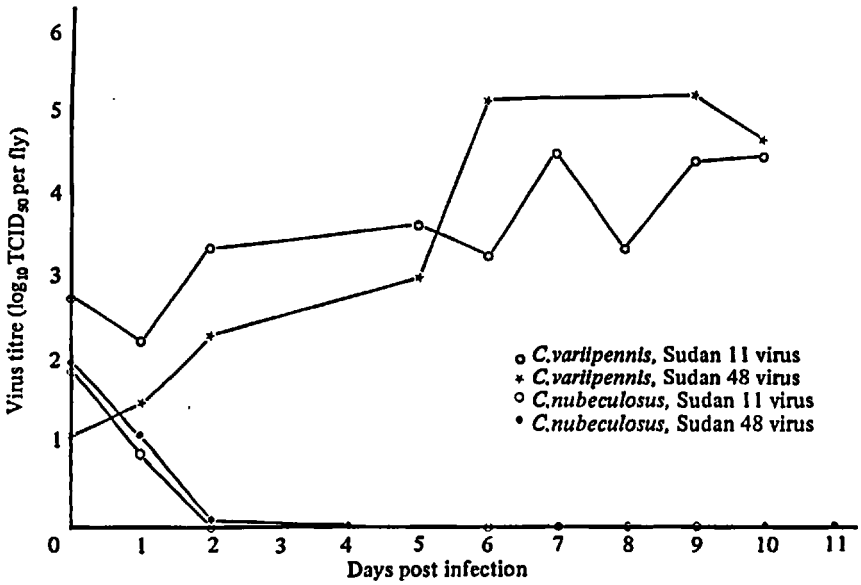

Fig. 4. Replication of Sudan 11 and Sudan 48 viruses in *C. variipennis* and incubation of the viruses in *C. nubeculosus* after oral ingestion.

Replication of Sudan 11 *and* 48 *viruses in* C. variipennis *and* C. nubeculosus

Sudan 11 and 48 viruses both replicated in *C. variipennis* after membrane feeding. The results are shown in Fig. 4. In both cases virus titre rose steadily from day 0 to days 6 and 7 and then remained virtually constant. Maximum virus titre was attained in the Sudan 11-infected *C. variipennis* after 7 days and was 4·5 $\log_{10}$ $TCID_{50}$ per insect, while in the Sudan 48-infected *C. variipennis* maximum titre occurred after 9 days and was 5·2 $\log_{10}$ $TCID_{50}$ per insect. Transmission of Sudan 11 and Sudan 48 viruses by *C. variipennis* was demonstrated after 10 days' incubation at 27 °C, using the membrane feeding technique described by Mellor, Boorman & Loke (1974).

Replication and transmission of Sudan 11 and Sudan 48 viruses in membrane-fed *C. nubeculosus* was not recorded (Fig. 4). The titres of both viruses in this species of midge decreased rapidly from the initial value taken one hour after feeding and became undetectable after 3 days.

DISCUSSION

Epidemiological work on BTV in the Sudan has involved studies on hosts and vectors. Sentinel herds of cattle were established in order to record seroconversion rates throughout the year, together with virus isolation procedures, when appropriate. Studies of insects involved the identification of likely vectors of BTV in the Sudan (Boorman & Mellor, 1982), long-term population studies and virus isolation from non-engorged female *Culicoides*, with a view to determining vector status. Collection of midges for virus isolation usually took place adjacent to sentinel herds, so that circulation of the virus through both the mammals and insects could be followed.

https://doi.org/10.1017/S0022172400065190 Published online by Cambridge University Press

Sudan 11 virus was isolated from a pool of *C. imicola*, a known vector of BTV in Africa. It is an orbivirus of the bluetongue complex, which reacted more with type 5 than type 9 BTV in seriological tests. These two serotypes are related. The World Reference Laboratory has identified Sudan 11 virus as type 5 BTV. Type 5 therefore becomes the first BTV serotype to be identified from the Sudan.

Sudan 48 virus was isolated from a pool of *C. kingi*. It is an orbivirus with relationships to BT, EHD and Eubenangee viruses, as shown by the double immunodiffusion test. The World Reference Laboratory has identified this virus as a member of the EHD serogroup. Sudan 48 virus is therefore the first EHD group virus to be identified from the Sudan. Both Sudan 11 virus (BTV type 5) and Sudan 48 virus (EHD serogroup) were isolated from pools of non-engorged *Culicoides*: Sudan 11 virus from *C. imicola* and Sudan 48 virus from *C. kingi*. At the same time and in the same geographical area, similar but as yet unidentified viruses were isolated from the blood of cattle (K. A. J. Herniman, personal communication), animals which are known hosts of these species of *Culicoides* (El Sinnery & Hussein, 1980; Herniman *et al.* 1983).

The results obtained confirm the ability of *C. imicola* to act as a biological vector of BTV and suggest that it is occupying that role in the Sudan. Similarly, it would appear that *C. kingi* is acting as a biological vector of EHD group viruses in the Sudan.

The laboratory experiments with *C. variipennis* confirm the ability of this midge to transmit both BTV and EHD group viruses. It is not yet known whether *C. kingi* is able to transmit BTV in addition to EHD but the ability of *C. variipennis* to transmit both of these closely related viruses means that *C. kingi* should still be considered to be a potential BTV vector.

The authors are grateful to Mr K. A. J. Herniman, Bluetongue/African Swine Fever Department of the Animal Virus Research Institute, for information concerning the serology of virus isolates from Sudanese cattle. Mr I. Gumm and Miss L. Owen of the same department also contributed much valuable advice on a variety of serological techniques and Mrs H. L. Gostling of the Department of Experimental Pathology kindly produced the electron micrographs.

Our thanks are also due to Dr Babiker El Hag Ali, Director, Central Veterinary Research Laboratories, Soba, Sudan, for permission to carry out this work and to Dr S. Kenyon, Veterinary Adviser of the British Overseas Development Administration at Soba for his help and provision of facilities.

This study was undertaken as part of a research programme funded by the Overseas Development Administration, London.

REFERENCES

Boorman, J. P. T. & Mellor, P. (1982). Notes on *Culicoides* (Diptera, Ceratopogonidae) from the Sudan in relation to the epidemiology of bluetongue virus disease. *Revue d'Elevage et de Médecine Vétérinaire des Pays Tropicaux* **35**, 173–178.

Eisa, M., Karrar, A. E. & Abu Elrahim, A. H. (1979). Incidence of bluetongue virus precipitating antibodies in sera of some domestic animals in the Sudan. *Journal of Hygiene* **83**, 539–545.

Eisa, M., Osman, O. M., Karrar, A. E. & Abdel Rahim, A. A. (1980). An outbreak of bluetongue in sheep in the Sudan. *Veterinary Record* **106**, 481–482.

EL SINNARY, K. & HUSSEIN, H. S. (1980). *Culicoides kingi* Austen: a vector of *Onchocerca gutturosa* (Newman 1910) in the Sudan. *Annals of Tropical Medicine & Parasitology* **74**, 655–656.

FINNEY, D. J. (1964). *Statistical Method in Biological Assay*, 2nd ed. London: Griffin.

HERNIMAN, K. A. J., BOORMAN, J. P. T. & TAYLOR, W. P. (1983). Bluetongue virus in a Nigerian dairy cattle herd. 1. Serological studies and correlation of virus activity to vector population. *Journal of Hygiene* **90**, 177–193.

HERNIMAN, K. A. J., GUMM, I. D., OWEN, L., TAYLOR, W. P. & SELLERS, R. F. (1980). Distribution of bluetongue virus antibodies in some countries of the Eastern Hemisphere. *Bulletin de l'Office International des Epizooties* **92**, 581–586.

JEGGO, M. H., GUMM, I. D. & TAYLOR, W. P. (1983). Clinical and serological response of sheep to serial challenge with different bluetongue virus types. *Research in Veterinary Science* **34**, 205–211.

LAKE, J. R. (1974). Studies on the genetic and biological properties of temperature-sensitive mutants of foot-and-mouth disease virus. Ph.D. Thesis, University of London.

MELLOR, P. S., BOORMAN, J. & LOKE, R. (1974). The multiplication of main drain virus in two species of *Culicoides* (Diptera, Ceratopogonidae). *Archiv für die gesamte Virusforschung* **46**, 105–110.

MELLOR, P. S. & BOORMAN, J. (1980). Multiplication of bluetongue virus in *Culicoides nubeculosus* (Meigen) simultaneously infected with the virus and the microfilariae of *Onchocera cervicalis* (Railliet & Henry). *Annals of Tropical Medicine & Parasitology* **74**, 463–469.

SERVICE, M. W. (1970). A battery-operated light trap for sampling mosquito populations. *Bulletin of the World Health Organization* **43**, 635–641.