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VIRUSES ISOLATED FROM *CULICOIDES* MIDGES IN SOUTH AFRICA DURING UNSUCCESSFUL ATTEMPTS TO ISOLATE BOVINE EPHEMERAL FEVER VIRUS

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ABSTRACT

THEODORIDIS, A., NEVILL, E. M., ELS, H. J. & BOSHOFF, S. T., 1979. Viruses isolated from *Culicoides* midges in South Africa during unsuccessful attempts to isolate bovine ephemeral fever virus. *Onderstepoort Journal of Veterinary Research*, 46, 191–198 (1979).

Five viruses, unrelated to bovine ephemeral fever virus (BEFV), were isolated from *Culicoides* biting-midges collected during the summer months of the years 1968–69 and 1969–70 near a cattle herd in which cases of BEF occurred and at an open horse stable at Onderstepoort. These viruses were investigated by means of serological, electron-microscopical and physicochemical tests. It was established that 2 isolates, Cul. 1/69 and Cul. 2/69, were related to each other and belonged to the Palyam subgroup of the genus Orbivirus, that isolate Cul. 3/69 belonged to the equine encephalosis subgroup of the genus Orbivirus, while Cul. 1/70 was related to Akabane virus, which belongs to the Simbu subgroup of the family Bunyaviridae. One isolate, Cul. 5/69, though prevalent in the cattle population, could not be identified at this point.

A brief serological survey indicated that the cattle in the nearby herd possessed antibodies against all the isolates except Cul. 3/69.

BEFV could not be isolated in mice or in cultured cells from the wild-caught *Culicoides.*

Résumé

VIRUS TROUVÉS CHEZ DES COUSINS CULICOIDES *EN AFRIQUE DU SUD AU COURS D'ESSAIS INFRUCTUEUX POUR ISOLER LE VIRUS DE LA FIÈVRE ÉPHÉMÈRE BOVINE*

Cinq virus non apparentés à celui de la fièvre éphémère bovine (BEFV) ont été isolés à partir de cousins piqueurs du genre Culicoides *que l'on avait récoltés pendant les mois d'été des années* 1968–69 *et* 1969–70 *près d'un troupeau de gros bétail où s'étaient produits des cas de BEF, ainsi que dans une écurie ouverte à Onderstepoort. On a examiné ces virus au moyen de tests sérologiques et physicochimiques ainsi que par la microscopie électronique. Il s'est avéré que 2 isolats, Cul. 1/69 et Cul. 2/69, sont apparentés entre eux et se rattachent au sous-groupe Palyam du genre Orbivirus; que l'isolat Cul. 3/69 appartient au sous-groupe de l'encéphalite équine du genre Orbivirus et que l'isolat Cul. 1/70 est apparenté au virus Akabane, lequel appartient au sous-groupe Simbu de la famille des Bunyaviridae. Malgré son abondance dans la population bovine, un autre isolat, Cul. 5/69, n'a pu être identifié jusqu'ici.*

Une brêve enquête sérologique dans le troupeau voisin a révélé que les bovins de ce troupeau possédaient des anticorps contre tous les isolats sauf Cul. 3/69.

Il n'a pas été possible d'isoler le BEFV en souris ou dans des cellules en culture à partir des Culicoides *capturés en nature.*

INTRODUCTION

Many attempts have been made during the past 40 years to find the vectors of bovine ephemeral fever virus (BEFV). In Australia, Seddon (1938) and Mackerras, Mackerras & Burnet (1940) speculated that this virus was possibly transmitted by biting-midges. Standfast, Murray, Dyce & St. George (1973) reviewed the ephemeral fever situation in Australia and cited unpublished work of Standfast in which he stated that he was able to infect *Culex annulirostris*, *Culicoides brevitarsis* and *Culicoides marksi* in the laboratory after feeding them a mixture of infected mouse brain and sucrose. St. George, Standfast & Dyce (1976) isolated 2 strains of BEFV from 2 collections of mosquitoes from the Northern Territory and Queensland, but did not consider the species involved likely to be important as vectors.

In Kenya, Davies & Walker (1974) in 1972–73 succeeded in isolating BEFV from a pool of *Culicoides* midges collected with portable suction-light traps baited with carbon dioxide. Walker (1977) found that large populations of *Culicoides* midges could be expected at any time of the year, and that *C. imicola* (=*C. pallidipennis*) and *C. schultzei* were able to persist in large numbers to the end of long dry spells at some sites and should therefore have a high potential as vectors.

The following report records unsuccessful attempts to isolate BEFV from *Culicoides* midges during the 1968–69 and 1969–70 outbreaks of ephemeral fever and the isolation and characterization of 5 viruses new to South Africa.

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One of these viruses is related to Akabane virus which was first isolated from *Aedes vexans niponnii* in Japan (Oya, Okumo, Ogata, Kobayashi & Matsuyama, 1961). Akabane virus can cause outbreaks of abortions and epidemics of congenital arthrogryposis-hydranencephaly (AH) syndrome in cattle (Kurogi, Inaba, Goto, Miura, Takahashi, Sato, Omori & Matumoto, 1975). In a serological survey, Akabane virus was shown to be prevalent in northern Australia, and in New South Wales (Della-Porta, Murray & Cybinski, 1976), and the virus was isolated from the blood of a normal bull by St. George, Cybinski & Paull (1977). In Kenya, a virus related to Akabane virus was isolated from a pool of *Anopheles funestus* by Metselaar (1975), and in South Africa on one occasion from the organs of a new-born lamb (B. J. Barnard, unpublished results, 1977). Another of these viruses, characterized by Erasmus, Boshoff & Pieterse (1978), belongs to the equine encephalosis subgroup of the genus Orbivirus, causing encephalosis in horses in South Africa. Two more belong to the Palyam subgroup of the Orbiviruses, a group which consists of the Abadina virus isolated from *Culicoides* spp. in Nigeria (Lee, Causey & Moore, 1974), the D'Aguilar virus isolated from *Culicoides brevitarsis* in Australia (Doherty, 1972) and the Nyabira virus isolated from an aborted calf in Rhodesia (Swanepoel & Blackburn, 1976).

MATERIALS AND METHODS

Virus isolation

The prevalence of BEFV in the Onderstepoort area during the summers of 1968–69 and 1969–70 prompted the investigation of a survey of *Culicoides* midges for the presence of BEFV.

Midges were collected during the above periods with 220 volt suction-light traps set at Onderstepoort and at the nearby farm belonging to the Institute. The traps were similar in design to that used by Du Toit (1944), insects being sucked into a cage without passing between the fan blades.

Live *Culicoides* were aspirated from collecting cages, anaesthetized with CO_2, transferred to 10 mℓ screw-top bottles and stored at −80 °C. From each night's catch ±500 *Culicoides* were macerated with mortar and pestle, suspended in phosphate-buffered saline (PBS) containing 500 IU penicillin and 500 μg streptomycin per mℓ. The suspensions were kept at 4 °C for not less than 4 h, centrifuged at 1 500 rpm to sediment the large debris, the supernatant being inoculated on to BHK21 cells and into mice. Monolayers of BHK21 line cells were cultivated in tubes with modified Eagle's medium (Macpherson & Stoker, 1962) containing 10% bovine serum, 200 IU penicillin, 200 μg streptomycin, 200 IU colistin sulphate and 2,5 μg amphotericin (Fungizone) per mℓ. Each tube received 0,2 mℓ of the insect suspension and was incubated for 1 h at 37 °C. The monolayers were then rinsed, washed twice with medium and maintenance medium without serum being added. The cultures were incubated at 37 °C for 8 days while being examined for cytopathic effect (CPE).

Day-old albino mice were inoculated intracerebrally with 0,025 mℓ of the insect suspensions. The brain tissue of sick or dead mice was harvested and a 10% suspension prepared in buffered lactose peptone (BLP). This material was further passaged in mice to shorten the incubation period of the virus and increase its titre.

Murine ascitic fluid

Immune globulins were prepared in adult Swiss albino mice by the intraperitoneal inoculation of the brain suspensions described above and according to the schedule described by Sommerville (1967). Ascitic fluid was collected with a large-bore sterile needle from the mice that showed distended abdomens. The fluid collected from the first 2 harvests was allowed to clot overnight at 4 °C and then centrifiged at 2 000 rpm for 15 min to remove fibrin. The supernatant fluid was aspirated aseptically and tested for neutralizing and complement fixing antibodies against the homologous virus. The harvest with the highest titre was used for the subsequent tests.

Virus assay and serological tests

Virus titrations were carried out in tube cultures (BHK cells) and in day-old albino mice, using tenfold dilutions of the antigen concerned. The murine ascitic fluid and the bovine sera were inactivated at 56 °C for 30 min and diluted 1:4 in PBS. The neutralization test was carried out as described by Theodoridis, Boshoff & Botha (1973). The end-points were calculated by the method of Reed & Muench (1938).

The complement fixation test (CF) was conducted with the murine ascitic fluid and sucrose-acetone-extracted mouse brain antigen of the isolates (Clarke & Casals, 1958). The 6-volume test, a modified technique of McIntosh (1956), was employed, using 1 volume serum, 1 volume antigen, 2 volumes complement and 2 units sensitized sheep red blood cells. The primary incubation was done at 37 °C for 90 min and the secondary for 30 minutes.

The haemagglutination-inhibition test (HAI) (Clarke & Casals, 1958) was carried out with the murine ascitic fluid, using 0,2 mℓ of twofold dilutions in a diluent of pH 9,0. To the serum dilutions 0,2 mℓ antigen containing 4 units haemagglutinin was added and incubated at 4 °C overnight. The following day 0,4 mℓ of appropriate cell suspension was added, incubated, and the inhibition recorded.

Physicochemical properties

Sodium-desoxycholate (SDOC).—For comparative purposes ECBO SA-1 virus (Oellermann, Els & Verwoerd, 1967) was incorporated in these tests. Virus suspension containing 10% normal bovine serum was centrifuged at 5 000 rpm for 1 h (Theiler, 1957). Aliquots of SDOC diluted 1:500 were mixed with an equal volume of each virus suspension and incubated at 37 °C for 1 h. The mixtures were then titrated as described above.

Chloroform.—The chloroform test was carried out according to the method of Bögel & Mayr (1961). Each virus suspension was centrifuged at 2 000 rpm for 30 min and the supernatant divided into 2 equal volumes. To one of the volumes 10% chloroform was added, while the other was kept as an untreated control. Both bottles were under continuous agitation for 60 min at 4 °C. Both samples were then centrifiged at 3 000 rpm for 45 min at 4 °C. The supernatant phase containing the virus was removed aseptically and titrated simultaneously with the control sample.

5-Iodo-2-deoxy-uridine (IUDR).—Eagle's medium containing 30 μg/mℓ IUDR was prepared to test the viruses isolated for inhibition by IUDR. The cultures were pretreated for 24 h with this medium, then inoculated with the virus and kept at 37 °C for 1 h. The maintenance medium also contained 30 μg/mℓ IUDR.

The virus of infectious pustular vulvo-vaginitis (IPV) was included as a known DNA virus and bovine ephemeral fever (BEF) virus as a known RNA virus.

Effect of pH 3 *and* 5.—For this test the virus suspensions were centrifuged at 2 000 rpm for 15 min and the supernatant fluids were removed aseptically. The virus suspensions were adjusted to pH 3,0 and 5,0 and kept at 4 °C for 1 h before titration in tissue culture tubes.

Heat sensitivity.—Harvested virus suspensions were centrifuged at 2 000 rpm for 15 min and the supernatant fluid divided into 2 volumes. One part was incubated in a water-bath at 56 °C for 10 min, while the control sample was kept at 4 °C. Both samples were diluted serially and titrated as described.

Electron microscopy

BHK21 cell cultures infected with each of the 5 isolates (*Culicoides* 1, 2, 3 & 5/69 and *Culicoides* 1/70) were prepared for electron microscopy as follows: Cells were detached from the glass with a long needle attached to a 5 mℓ syringe and centrifuged at 1 000 rpm for 5 minutes. The pelleted cells were prefixed in 3% glutaraldehyde for 1 h, washed 3 times in phosphate buffer and post-fixed in 1% osmium tetroxide at pH 7,2–7,4 (Milloning, 1961). After dehydration with alcohol, the cells were embedded in Epon (Luft, 1961), sectioned, double-stained with uranyl acetate and lead citrate and examined in a Siemens Elmiskop 1A electron microscope.

RESULTS

Virus isolation

Large numbers of *Culicoides* midges ranging from 60–20 000 were caught nightly during the summer months of 1968–69 and 1969–70. The predominant species, *C. imicola* (=*C. pallidipennis*), accounted for up to 97,4% of the catch (Nevill, 1971). A total of 45 collections of *Culicoides* were tested in BHK cells and in mice. From the 1969 harvests, 5 virus strains could be grown in tissue culture (TC) and were designated Cul. 1/69, Cul. 2/69, Cul. 3/69, Cul. 4/69 and Cul. 5/69 (Table 1). Isolate Cul. 4/69 was lost while it was being kept in lyophilized form at −20 °C. Cul. 1/70 was isolated in mice from a pool of about 100 *Culicoides* collected on 13th April 1970 and held in a constant temperature room for 7 days at 26–27 °C. The CPE in BHK cells caused by the isolates of 1969 appeared during the 2nd subculture and the cultures were destroyed by the 6th day. The TC isolates were subsequently adapted to the brain tissue of day-old albino mice and Cul. 1/70 to BHK cells, causing cell destruction after the 2nd sub-inoculation.

Virus assay and serology

The original insect pools were not titrated except for Cul. 1/70 which had a titre of $10^{6,0}$ MLD_{50}/0,025 mℓ. Satisfactory virus concentrations were obtained in the infected BHK cells and in mice exceeding $10^{5,0}$ $TCID_{50}$/0,1 mℓ and mouse LD_{50}/0,025 mℓ, respectively.

The CF and serum-virus neutralization tests showed that Cul. 1/69 and Cul. 2/69 are antigenically closely related, though the titres of the homologous antisera were higher than those of the heterologous (Table 1). Table 1 shows that Cul. 3/69, Cul. 5/69 and Cul. 1/70 react only with their homologous specific antisera, a fact which was confirmed by the serum-virus neutralization test. In an attempt to classify these isolates on a serological basis, they were tested against a number of insect-transmitted viruses by means of CF and HAI tests (Table 2). This study demonstrated that Cul. 1/69 and Cul. 2/69 belong to the Palyam subgroup of the genus Orbivirus.

Cul. 3/69 specific ascitic fluid reacted with the equine encephalosis virus subgroup of the genus Orbivirus.

Cul. 5/69 was not related to any of the viruses incorporated in this study (Table 2) and requires further investigation.

Cul. 1/70 specific antiserum reacted with Akabane virus of the Simbu group, family Bunyaviridae (Table 2). In serum-virus neutralization tests of Akabane and Cul. 1/70 viruses there was neutralization both ways with the homologous serum, but the titres were higher than with the heterologous serum.

Sera taken at random from adult cows at the Institute's farm where the majority of the *Culicoides* were collected, revealed neutralizing antibodies to Cul. 1/69, Cul. 2/69, Cul. 5/69 and Cul. 1/70 (Table 4). The highest titres were against Cul. 5/69. No antibodies were demonstrated to Cul. 3/69 (Table 4).

TABLE 1 Complement fixation test of the 5 *Culicoides* isolates against homologous and heterologous antisera

Virus strain	Murine ascitic fluid				
	Cul. 1/69	Cul. 2/69	Cul. 3/69	Cul. 5/69	Cul. 1/70
Cul. 1/69	*≥1:192	1:85	< 1:4	< 1:4	< 1:4
Cul. 2/69	1:128	1:341	< 1:4	< 1:4	< 1:4
Cul. 3/69	**< 1:4	< 1:4	1:214	< 1:4	< 1:4
Cul. 5/69	< 1:4	< 1:4	< 1:4	1:128	< 1:4
Cul. 1/70	< 1:4	< 1:4	< 1:4	< 1:4	1:512

* equal to or more than

** less than

TABLE 2 Complement fixation test of viruses isolated from *Culicoides* against viruses representing various viral groups

Marine ascitic fluid	Virus strain						
	Akabane virus	Bluetongue virus	African horsesickness virus	Wesselsbron virus	Encephalosis virus group	Palyam species	Rift Valley fever
Cul. 1/69	*< 1:4	< 1:4	< 1:4	< 1:10	< 1:4	1:69	< 1:10
Cul. 2/69	< 1:4	< 1:4	< 1:4	< 1:10	< 1:4	1:54	< 1:10
Cul. 3/69	< 1:4	< 1:4	< 1:4	< 1:10	1:14	< 1:4	< 1:10
Cul. 5/69	< 1:4	< 1:4	< 1:4	< 1:10	< 1:4	< 1:4	< 1:10
Cul. 1/70	**≥1:96	< 1:4	< 1:4	< 1:10	< 1:4	< 1:4	< 1:10

* less than

** equal to or more than

TABLE 3 Physicochemical properties of viruses isolated from *Culicoides*

Virus strain	Virus titre prior to treatment	Titres after treatment with chemicals			Titres after exposure to pH 3,0 & 5,0	Titres after head treatment 56 °C 10 min
		S.D.O.C.	Chloroform	IUDR		
Cul. 1/69	6,5*	3,5	2,5	6,5	< 0,5 < 0,5	< 0,5
Cul. 2/69	6,5	4,0	2,0	6,5	< 0,5 < 0,5	< 0,5
Cul. 3/69	5,5	4,0	2,5	5,5	< 0,5 < 0,5	< 0,5
Cul. 5/69	6,0	3,5	< 0,5	6,5	< 0,5 < 0,5	< 0,5
Cul. 1/70	5,5	1,0	< 0,5	5,5	< 0,5 < 0,5	< 0,5
EFI	4,0	N.T.**	N.T.	4,0	N.T. N.T.	N.T.
FH 335	6,5	N.T.	N.T.	< 0,5	N.T. N.T.	N.T.
ECBO	5,5	5,5	6,0	5,5	5,5 6,0	< 0,5

* Virus titres expressed as log 10 $TCID_{50}$/0,1 mℓ
** Not tested

TABLE 4 Neutralizing antibodies in cattle to viruses isolated from *Culicoides*

Serum No.	Virus strains				
	Cul 1/69	Cul. 2/69	Cul. 3/69	Cul. 5/69	Cul. 1/70
5594	1,6*	2,5	**< 0,5	***≥3,6	1,7
6358	3,1	2,6	< 0,5	≥3,6	2,2
2434	4,2	2,5	< 0,5	≥3,6	≥4,4
5180	1,7	2,0	< 0,5	≥3,6	1,2
6063	4,2	3,1	< 0,5	≥3,6	1,7
2392	2,8	2,6	< 0,9	≥3,6	—
5934	3,0	1,8	< 0,5	≥3,6	1,4
5448	≥4,2	2,2	< 0,5	≥3,6	1,5
1146	2,5	2,9	< 0,5	≥3,6	3,1
2282	2,1	≥4,6	< 0,5	2,3	1,8
5603	2,7	3,3	< 0,5	2,5	1,9
6865	2,7	1,8	< 0,6	≥3,6	1,5
946	2,1	2,9	< 0,5	≥3,6	1,8
5881	≥4,2	3,5	1,1	≥3,6	2,2
1220	1,4	2,9	< 0,5	≥3,6	1,9

* Neutralization indices expressed as log. 10 MLD_{50}/0,025 mℓ
** Less than
*** Equal to or more than

Physicochemical properties

The results of the SDOC, chloroform, IUDR treatment of the viruses and pH and heat sensitivity tests are presented in Table 3. Cul. 1, 2, 3 and 5/69 were relatively sensitive to SDOC, while Cul. 1/70 was very sensitive. Chloroform greatly reduced the titre of Cul. 1, 2 and 3/69, while Cul. 5/69 and Cul. 1/70 were completely inactivated. IUDR had no effect on any of the virus isolates. All the virus isolates were inactivated at pH 3 and 5 and at 56 °C. The control viruses reacted as expected, thus confirming the specificity of the tests.

Electron microscopy

Uniform particles in varying amounts were observed in section of BHK cells infected with Cul. 1, 2, 3 and 5/69 (Fig. 1–4). Repeated examinations of Cul. 1/70 infected TC cells and brain tissue of baby mice were negative for virus particles.

The size of the virus particles (about 70 nm in sections) observed in the cytoplasm of infected cells correlates with the diameters of known orbiviruses (Fenner, 1976).

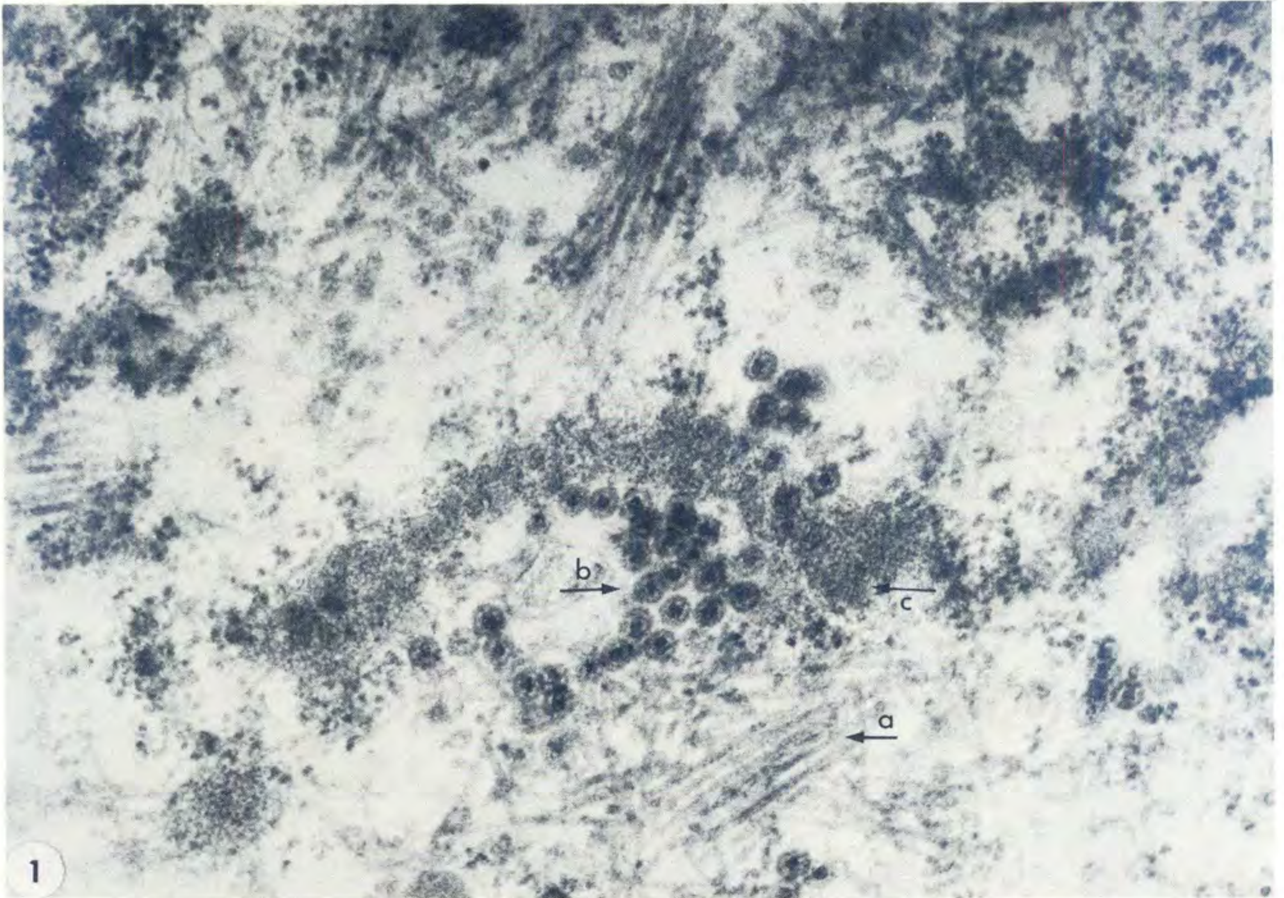
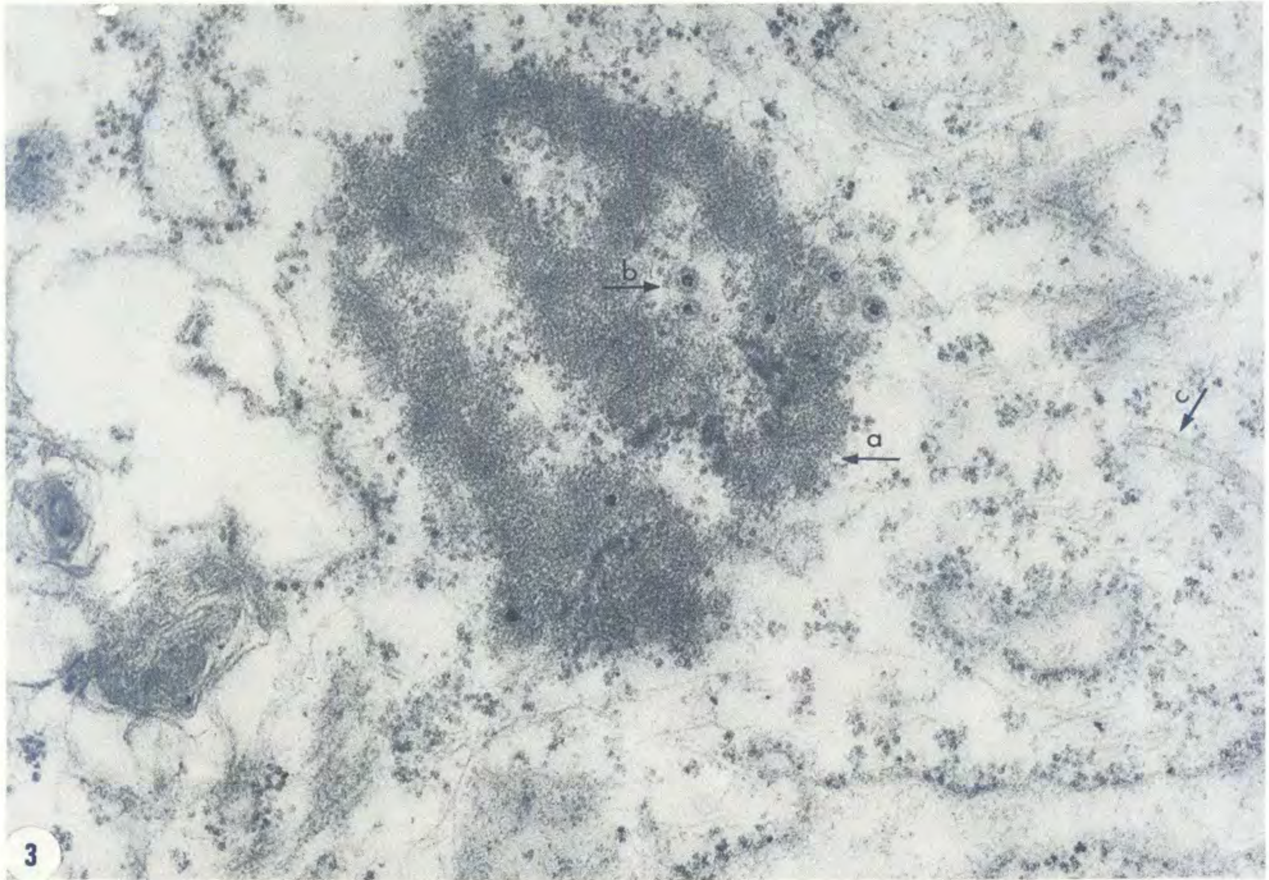


FIG. 1 Cul. 1/69. Section through cell containing thin tubular elements (a) and mature virus particles (b) adjacent to remnants of dense, granular material (c) (cytoplasmic matrix). ×62 500

FIG. 2 Cul. 2/69. Scattered thin tubular elements (a) filaments, mature virus particles (b) and dense granular material (c). Some virus particles are in the process of leaving the cell. ×62 500



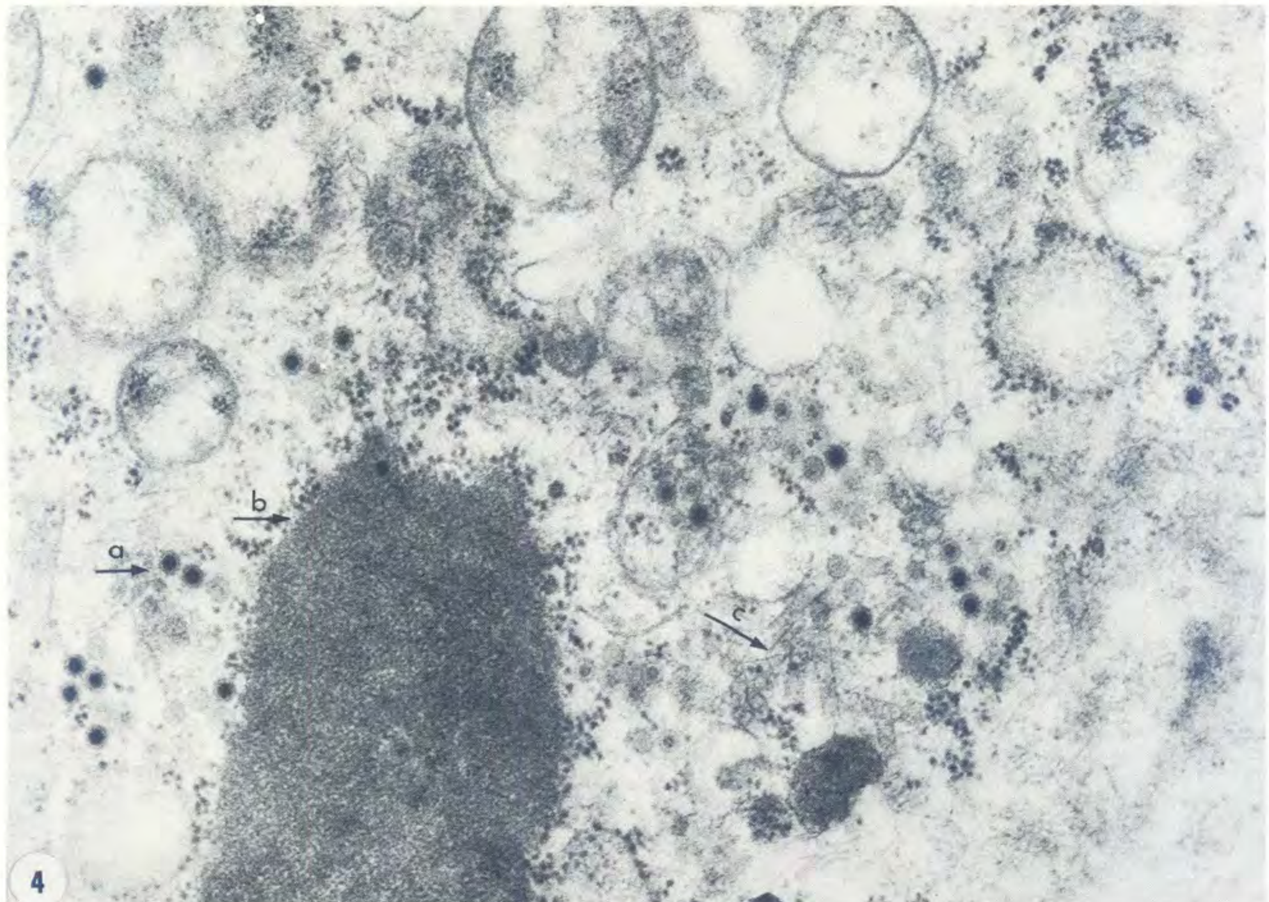


FIG. 3 Cul. 3/69. Dense granular material (a) containing a few virus particles (b) as well as fine filaments and characteristic tubular structures (c) are seen in the cytoplasm. ×50 000

FIG. 4 Cul. 5/69. Mature virus particles (a) near dense granular body (b) (matrix) tubular structures (c) fine filaments and mitochondria are seen in the cytoplasm. ×50 000

Cytopathic changes are very similar for all 4 isolates and include features such as the appearance of dense inclusion bodies, swelling of the endoplasmic reticulum, fine filaments and bundles of tubular elements. The virus particles were often associated with granular matrices of varying density, shape and size.

The isolates Cul. 3/69 and 5/69 produced bundles of tubular structures similar to those found in bluetongue virus (BTV) infected cells (Fig. 3 & 4), whereas the isolates Cul. 1/69 and 2/69 showed bundles of thinner tubules of about 20 nm in diameter similar to those observed in BHK cells infected with African horsesickness virus (AHSV) (Fig. 1 & 2) (Lecatsas & Erasmus, 1967).

As the infected cells were usually observed during a late stage of infection when the cells were in a broken-up condition, the major virus release mechanism seems to be via cell lysis. The released virus particles observed were not enveloped.

Discussion

Attempts to isolate BEFV from *Culicoides* midges failed during 2 consecutive summers, despite the prevalence of the disease in nearby cattle. Since BEFV has been isolated from *Culicoides* spp. (see Introduction), the present results suggest either failure of the isolation techniques or possibly the need for a more sensitive laboratory host system for the isolation of the virus directly from wild-caught BEFV vectors.

Since the mosquitoes present in the traps were not tested, the possibility that they were acting as vectors of BEFV cannot be excluded.

Four of the viruses isolated from *Culicoides* spp. appeared to be prevalent in cattle as judged by the presence of neutralizing antibodies in the sera of this host. Two of them, Cul. 1/69 and Cul. 2/69, are related serologically to the Palyam subgroup and to each other.

One isolate, Cul. 5/69, which was strongly inhibited by the cattle sera, has not yet been identified. The antibody titre against this virus was higher than that against the rest of the isolates.

The isolate, Cul. 1/70, was found by means of neutralization and CF tests to be closely related to Akabane virus. The antibody titres against the homologous virus were higher than these against the heterologous. Serological investigations in cattle have already revealed the presence of Akabane virus in South Africa, but this is the first isolation from *Culicoides* spp. of a virus identical with or closely related to Akabane in this country.

Isolate Cul. 3/69, which was not inhibited by cattle sera, was shown by CF tests to be related to the equine encephalosis virus. Its characteristics, such as partial resistance to lipid solvents, support the serological findings. A number of the encephalosis viruses have already been isolated from horses in South Africa (Erasmus *et al.*, 1978), but this is the first isolation of a virus of this group from *Culicoides* spp.

The presence of dense, granular bodies (viral matrices) and the association of mature virus particles with tubular structures and fine filaments are the most characteristic features of Cul. 1, 2, 3 and 5/69 isolates. Similar structures have been seen in BHK cells infected with BTV and AHSV, and their presence is considered to be almost sufficient warrant to classify a virus in the genus Orbivirus. The particle size of all 4 isolates mentioned above is in the range of 70 nm, which, together with the other characteristics, endorses its classification in the genus Orbivirus.

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