

## Further studies on *Haematoxenus separatus* (Sporozoa, Theileriidae) of sheep in Tanzania

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### RÉSUMÉ

Acquisitions nouvelles dans la connaissance d'*Haematoxenus separatus*  
(Sporozoa, Theileriidae) du mouton en Tanzanie

L'évolution des rechutes d'*Haematoxenus separatus*, après splénectomie de deux moutons porteurs de parasites, est décrite. Le parasite a été trouvé pour la première fois chez un mouton non splénectomisé.

Deux autres moutons, indemnes du parasite, ont été utilisés après splénectomie pour des expériences de transmission de stade à stade par les tiques. Quatre essais avec *Amblyomma variegatum* et un avec *Rhipicephalus appendiculatus* ont donné des résultats négatifs. Par contre, *Rhipicephalus evertsi* a transmis le parasite deux fois sur deux. Aussi bien les *Haematoxenus* typiques que les organismes sans voile, présents chez tous les moutons porteurs, ont été transmis par *R. evertsi*, et on ne sait toujours pas s'il s'agit de deux espèces différentes ou non ; les proportions des organismes avec et sans voile sont variables de mouton à mouton et, chez un même mouton, dans le temps. Bien que les deux moutons splénectomisés, auxquels le parasite a été transmis par *R. evertsi*, aient montrée une anémie marquée, ils ont guéri, et il est peu probable que la pathogénicité d'*H. separatus* pour les moutons intacts soit importante.

Utilisant de l'antigène préparé à partir de sang contenant un mélange d'*Haematoxenus* typiques et d'organismes sans voile, il a été possible de démontrer, au moyen de la technique d'immunofluorescence indirecte, l'apparition d'anticorps après transmission du parasite par *R. evertsi*.

Il n'a pas été possible de transmettre le parasite à une chèvre splénectomisée par injection de sang infecté.

### INTRODUCTION

*Haematoxenus separatus* UILENBERG and ANDREASEN, 1974 (*Theileriidae*), was described from a sheep in Tanzania (5), in which it appeared after splenectomy. Artificial transmission, by subcutaneous injection of infected

blood, was successful, but the natural vector remained unknown, as did the pathogenicity (\*), and the significance of unveiled theilerial piroplasms, which were present at the same time as the typical veiled *Haematoxenus*.

In this paper, we describe the results of some experiments on transmission by ticks, and some further observations on the course of the infection in splenectomized sheep, as well as attempts to transmit it to a goat.

(\*) Tanzania F. A. O./U. N. D. P. Project « Improvement of Tick Control », c/o Central Veterinary Laboratory, P. O. Box 9254, Dar-es-Salaam, Tanzania ; a project of the Government of Tanzania, financed by the United Nations Development Programme and executed by the Food and Agriculture Organization of the United Nations.

(\*) In the first paper (5), it is stated (p. 460), that *H. separatus* is a pathogenic parasite. The word « pathogenic » had been deleted in the final manuscript submitted to the Editor, and should not have been printed.

## MATERIAL AND METHODS

### Animals

The sheep, local breed, blackheaded Persians, and crosses, were obtained from the experimental herd at the Central Veterinary Laboratory at Dar es Salaam. Some tick control is practised in this herd, but this does not prevent *Rhipicephalus evertsi evertsi* NEUMANN, 1897, being present in the herd. The tick *Boophilus microplus* (CANESTRINI, 1887) has also been observed in the compound of the laboratory, and low numbers of other species may be present.

One adult goat of local breed, born and raised at the Central Veterinary Laboratory, was used in attempts to transmit *H. separatus* from sheep to goat by the injection of infected blood.

The animals used in our experiments were isolated in pens, and sprayed twice a week with acaricides (\*\*).

### Ticks

Strains of ticks (*Amblyomma variegatum* (FABRICIUS, 1794), *Rhipicephalus appendiculatus* NEUMANN, 1901, and *R. evertsi*) were maintained in the laboratory by breeding on the ears of animals. *R. appendiculatus* and *R. evertsi* could be completely bred on rabbits, while larvae and nymphs of *A. variegatum* could be fed on rabbits, but cattle were used for the adults. The strain of *R. appendiculatus* had been obtained from the East African Veterinary Research Organization at Muguga, while strains of the other species originated from engorged females collected on domestic animals near Dar es Salaam.

During experiments, blood smears were made daily (except Sundays), and rectal temperature was taken at the same time. During transmission experiments with ticks, lymphnode biopsy smears were also regularly made. Blood smears were made at least twice a week before and after experiments. Smears were fixed in methanol and stained with Giemsa stain.

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(\*\*) Carbaryl (Sevin <sup>®</sup>) at 0.2 p. 100 was used at first, but its long residual activity made it difficult to eliminate it completely from the skin and hair before transmission experiments with ticks, and we later changed to chlorfenvinphos (Supona <sup>®</sup>) at 0.05 p. 100.

## Serological test

A few attempts were made to demonstrate the appearance of antibodies in the indirect fluorescent antibody (IFA) test. Piroplasm antigen was prepared and tested according to the technique described for cattle *Theileriae* by Burridge (1), from a sheep with a high parasitaemia of a mixture of typical veiled *H. separatus* and unveiled theilerial piroplasms, unveiled organisms being more numerous. (Sheep 1553, 31 days after infection by ticks, when parasitaemia was over 5 p. 100 of erythrocytes infested; see below.) Commercial rabbit anti-sheep globulin, conjugated with fluorescein isothiocyanate, was used. Sera of sheep 1553 and 1, taken prior to infection, were used as negative control sera.

## EXPERIMENTS AND RESULTS

### A. OBSERVATIONS AFTER SPLENECTOMY

#### *Sheep no. 1549*

The animal in which the parasite was first observed. After splenectomy *H. separatus*, theilerial organisms without a veil, and *Anaplasma ovis* LESTOQUARD, 1924, appeared. The anaplasms disappeared after one treatment with a tetracycline (Reverin<sup>®</sup>) and have not reappeared since. (For details see UILENBERG and ANDREASEN (5).) We may add that both *H. separatus* and unveiled theilerial organisms are still regularly found in its blood, nearly 2 years after its splenectomy.

#### *Sheep no. 1547. Adult male*

Unveiled theilerial organisms appeared 6 days after splenectomy and increased gradually in number; the parasitaemia never exceeded 0,3 p. 100 of erythrocytes infested. These parasites had become very scanty 7 weeks after the operation; they were still present, although not found every day, until day 110, when observations on the animal were stopped.

Typical *H. separatus* were seen from day 10 onwards, but always remained scanty. A maximum of less than 0,05 p. 100 infested red cells was seen on day 16. After this maximum, the parasites were only found occasionally, and none were seen after day 72.

The ratio between typical *H. separatus* and unveiled organisms was variable; sometimes no

*Haematoxenus* could be found while unveiled organisms were fairly numerous, sometimes both were present in approximately equal numbers.

No other blood parasites appeared.

No clinical symptoms of disease were observed.

*Sheep no. 1552. Adult female*

Scanty unveiled theilerial organisms were regularly seen prior to its splenectomy, and a typical *H. separatus* was seen on one occasion. *H. separatus* was again observed 5 days after splenectomy and increased in number until a maximum of approximately 0,5 p. 100 of erythrocytes infested was seen 16 to 17 days after the operation; parasitaemia then declined to a very low level, but scanty *H. separatus* were usually to be found until observations were stopped, three and a half months after splenectomy. The unveiled organisms started to increase from 6 days after the operation and reached a maximum of nearly 1,5 p. 100 of red cells infested between days 13 to 21; the level of parasitaemia then declined; the parasites remained present, in numbers varying between very scanty to approximately 0,1 p. 100, until observations were stopped.

*Anaplasma ovis* also appeared 19 days after the operation; the anaplasms became numerous and then declined, without treatment, after a maximum on day 27. Very scanty anaplasms reappeared occasionally.

The number of typical *H. separatus* was usually lower than that of the unveiled organisms, but at times it was practically equal.

No clinical symptoms of disease were observed.

*Sheep no. 1553. Adult female*

No blood parasites were observed before or after splenectomy.

*Sheep no. 1. Male lamb of two months old*

No blood parasites were observed before or after splenectomy.

Sheep nos. 1553 and 1 were used for transmission experiments with ticks.

*Goat no. 4814. Adult female*

*Anaplasma ovis* appeared after splenectomy, and the relapse was treated three times with

oxytetracycline, at approximately 10 mg/kg intramuscularly, on days 15, 26 and 27 after the operation; this treatment did not eliminate *A. ovis*, which is persisting at a fairly high level up till now, over 4 months after splenectomy.

## B. TRANSMISSION EXPERIMENTS WITH TICKS

### a) Experiments with *Amblyomma variegatum*

#### 1. From sheep 1549 to sheep 1553 (see fig. 1)

Larvae of *A. variegatum* were fed on the ears of 1549 and collected engorged from 15 to 21.11.73, during the maximum of its initial parasitaemia of *H. veliferus* after splenectomy, when over 1 p. 100 of the red cells were infested (and over 5 p. 100 with unveiled organisms), and before 1549 was treated with a tetracycline against *A. ovis* (see 5).

Over 250 of the resulting nymphs were applied in earbags to sheep 1553 on 1.6.74, and 40 more were added on 7.6.74. Most attached, but only 3 engorged fully, 6 to 7 days after attachment; all others died without fully engorging.

Neither *H. separatus*, unveiled theilerial organisms nor *A. ovis* appeared in 1553 (\*).

#### 2. From sheep 1549 to sheep 1553 (see fig. 1)

Larvae of *A. variegatum*, fed on the ears of 1549, were collected engorged from 9 to 10.10.74. *H. separatus* was very scanty and fairly numerous unveiled organisms (up to 0,1 p. 100 infested red cells) were present.

200 of the resulting nymphs were applied in earbags to sheep 1553 on 4.11.74. 86 engorged nymphs were collected from 1553, from 5 to 7 days after attachment.

No blood parasites appeared in 1553.

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(\*) Dr M. P. ANDREASEN has informed us (correspondence) that he has transmitted a pure infection of *H. separatus* by feeding 100 nymphs of *A. variegatum*, of the same batch as those used in experiment 1, on a non-splenectomized sheep in Denmark, approximately 2 months after the ticks had moulted. There was however no evidence of this in the bloodsmears we received from him for confirmation.

A first version of the manuscript describing *H. separatus* incorporated this, before we had seen the bloodsmears, and although the final summary in French (p. 5, 459) is correct (except for its title), the summary no. 74-148 on p. 518 of the same issue has unfortunately not been corrected.

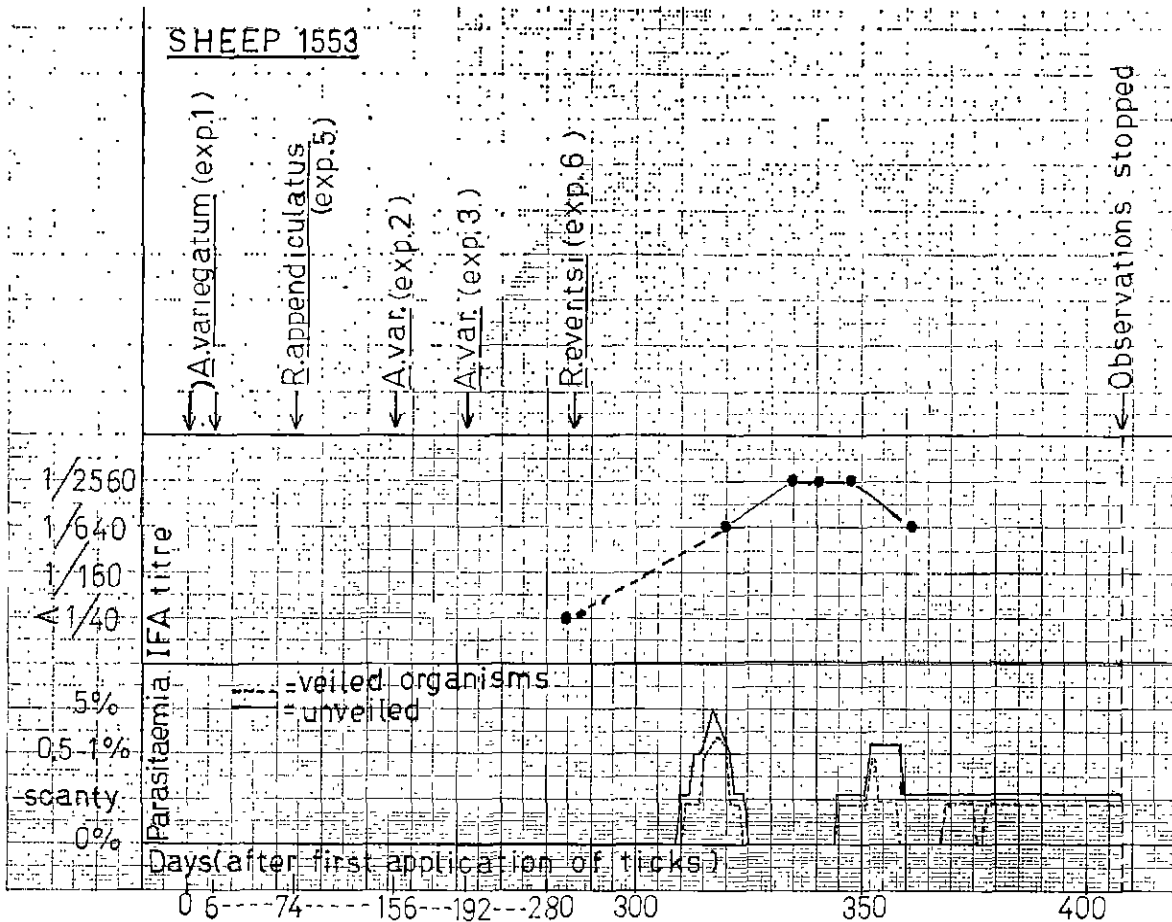


Fig. 1. — Sheep 1553. Summary of experiments and results.

Fig. 1. — Mouton 1553. Résumé des expériences et résultats.

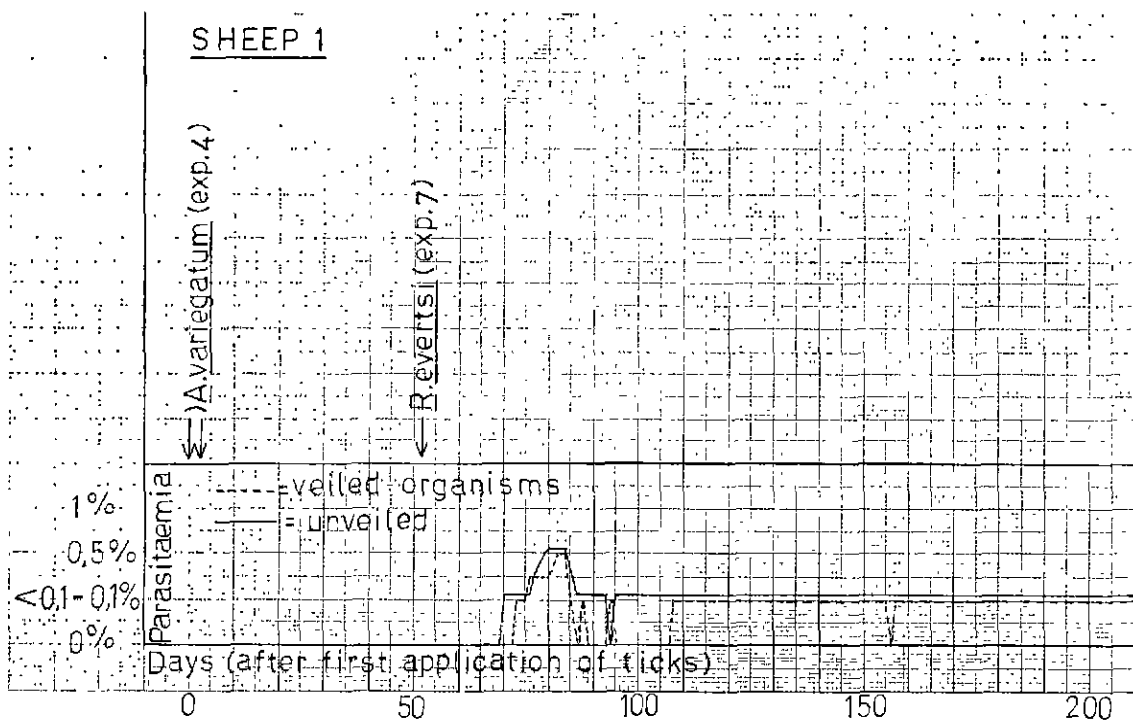


Fig. 2. — Sheep no. 1. Summary of experiments and results.

Fig. 2. — Mouton n° 1. Résumé des expériences et résultats.

### 3. From sheep 1549 to sheep 1553 (see fig. 1)

Larvae of *A. variegatum*, of the same batch as those used in experiment 2, were collected on 1549 from 8 to 11.10.74.

450 of the resulting nymphs were applied in earbags to sheep 1553 on 10.12.74. 153 engorged nymphs were collected from 1553, from 6 to 8 days after attachment.

No blood parasites appeared in 1553.

### 4. From sheep 1547 to sheep no. 1 (see fig. 2)

Larvae of *A. variegatum*, fed on the scrotum of 1547, were collected engorged from 17 to 18.1.75, during the initial parasitaemia after splenectomy of 1547, when *H. separatus* was very scanty and unveiled organisms were fairly numerous (but not over 0,1 p. 100).

Some 400 of the resulting nymphs were applied in earbags to sheep 1, partly on 21.2.75, partly on 24.2.75. 271 engorged nymphs were collected on no. 1, from 26.2. to 3.3.75.

No blood parasites appeared in sheep no. 1.

### b) Experiment with *Rhipicephalus appendiculatus*

#### 5. From sheep 1549 to sheep 1553 (see fig. 1)

Nymphs of *R. appendiculatus*, fed on the ears of 1549, were collected engorged from 27 to 29.11.73, after the high initial parasitaemia following splenectomy; both *H. separatus* and unveiled organisms were scanty, as was *A. ovis* following tetracyclin treatment on 23.11.73.

The batch used had contained originally after moulting over 170 adults; they were fed on the ears of 1553 on 14.8.74, but viability was low and only 17 females engorged, and a corresponding number of males also fed.

No blood parasites appeared in 1553.

### c) Experiments with *Rhipicephalus evertsi*

#### 6. From sheep 1547 and 1549 to sheep 1553 (see fig. 1)

*R. evertsi*, fed on the ears of 1547 and 1549 as larvae and nymphs. They were collected as engorged nymphs on 1547 from 22 to 30.1.75 and from 1549 from 3 to 8.2.75; *H. separatus* was very scanty in both sheep and unveiled organisms fairly numerous (not over 0,3 p. 100).

Over 50 of the resulting adults from 1547 and over 100 from 1549 were applied in earbags to 1553 on 14.3.75. Many attached, but none succeeded in engorging fully, and all were dead a week after attachment.

Unveiled theilerial organisms appeared in the blood of 1553 24 days after tick attachment, typical *H. separatus* were seen with certainty one day later. The parasitaemia of both types of organisms increased rapidly, until on day 31 (after tick attachment) as many as 5 p. 100 of the red cells were infested with unveiled organisms and on day 32 typical *H. separatus* reached a maximum of over 1,5 p. 100. The numbers of both types of organisms then declined rapidly, while bloodsmears showed very important anaemic changes (anisocytosis, basophilic punctations, polychromatophilia, Jolly bodies, normoblasts) from day 36 onwards. No parasites were found from day 39 to 55, and both types then reappeared, and were present in low numbers, until observations were stopped, 4 months after tick attachment. The rectal temperature remained normal throughout, and there were no clinical symptoms of disease, apart from the anaemia after the high parasitaemia. Superficial lymphnodes did not swell significantly, and we did not succeed in finding schizontal stages in lymphnode biopsy smears.

The ratio of typical *H. separatus* to unveiled organisms was variable, unveiled ones often being the most numerous, but on several occasions numbers were nearly equal.

Antibodies to antigen prepared as described in « Material and methods » could be demonstrated in the IFA test after transmission; the titre reached 1/2 560; serum taken before the ticks were applied gave negative results (see fig. 1).

#### 7. From sheep 1549 to sheep no. 1 (see fig. 2)

*R. evertsi*, fed on the ears of 1549 as larvae and nymphs, collected from 6 to 11.2.75, when *H. separatus* was very scanty and unveiled organisms fairly numerous (not over 0,3 p. 100).

Over 300 of the resulting adults were applied to the ears of sheep no. 1 on 14.4.75. Many attached and remained alive for over a week, but none succeeded in engorging fully, and all were dead two weeks after attachment.

Unveiled theilerial organisms in the blood of sheep no. 1 were first seen 18 days after tick



attachment, while typical *H. separatus* appeared 3 days later. Both types of parasites increased rapidly in number, until a maximum of approximately 0,5 p. 100 of red cells infested with unveiled organisms was reached from day 28 to 32, and a maximum of also approximately 0,5 p. 100 infested with typical *H. separatus* from day 30 to 32. Both types then diminished in number, while important anaemic changes in the blood picture (anisocytosis, basophilic punctations and polychromatophilia) appeared from day 33 onwards. The number of parasites decreased rapidly after day 33. Scanty unveiled organisms, as well as typical *H. separatus*, are still present up to now (over 6 months after tick attachment). The rectal temperature of the animal was slightly higher than normal during the initial high parasitaemia, oscillating around 40 °C as opposed to its normal temperature of about 39 °C. There were no clinical symptoms of disease, other than the anaemia. Superficial lymphnodes did not swell significantly, and we did not succeed in finding with certainty schizontal stages; however there was evidence of activity in the prescapular lymphnodes from 10 to 14 days after tick attachment, as evidenced by an abnormal number of dividing cells and the presence of abnormally high numbers of lymphoblasts.

Numbers of veiled and unveiled organisms were roughly equal most of the time, but on a few occasions there were more typical *H. separatus* than unveiled organisms.

Only a few sera were taken from sheep 1: two of them, one of a month before, one 3 days after the infective ticks were applied, gave negative results in the IFA test, while a low IFA titre (1/160) could be demonstrated in serum taken 45 days after these ticks attached.

### C. EXPERIMENTS WITH A GOAT, NO. 4814

10 ml of blood from sheep 1549 were injected intravenously and a further 10 ml subcutaneously into the goat, 33 days after it was splenectomized. The blood contained both typical veiled *H. veliferus* and unveiled piroplasms in roughly equal numbers, some 0,1 p. 100 of red cells being infested.

76 days after splenectomy, the goat was again injected with blood from sheep 1549, 17 ml

subcutaneously. This blood also contained both types of organisms, approximately 0,3 p. 100 of red cells being infested with unveiled piroplasms and 0,1 p. 100 with typical *H. separatus*.

Up to now, 126 days after splenectomy, no parasites other than *A. ovis*, of which the animal was a carrier, have been seen in the goat.

## DISCUSSION AND CONCLUSIONS

None of the 4 experiments with *A. variegatum* was successful, although one (exp. 1) was admittedly carried out with old nymphs. We had hoped that this tick might at least transmit the unveiled organisms, if they belong to a separate species, which in that case is likely to be *Theileria ovis* RODHAIN, 1916. In Madagascar, where *T. ovis*, or a similar parasite, is widespread none of the proven vectors of *T. ovis* (see 4) occurs, and only *A. variegatum* or *B. microplus* can be its vector. *A. variegatum* appears to be the more likely candidate, as it is a proven vector of Theileriidae (of East African *Theileria mutans* (THEILER, 1906) of cattle (6), and of *Haematoxenus veliferus* (UILENBERG, 1964) of cattle (7)).

The one experiment with *R. appendiculatus* was also negative; the ticks were quite old, and the results cannot be considered as conclusive.

Both experiments with *R. evertsi* were successful, in spite of their poor feeding, establishing this tick as a vector of *H. separatus*.

It is still not clear whether all or some of the unveiled organisms are *T. ovis* or whether they are unveiled *H. separatus* (see 5). The fact that both were transmitted at the same time by *R. evertsi* does not prove that they are one species, as two subspecies of this tick are proven vectors of *T. ovis* (2, 4). We tend to believe that both *H. separatus* and a *Theileria* sp. are present in our sheep, as the proportion of veiled to unveiled organisms was so variable in the different sheep and at different times, but, as in *H. veliferus*, a certain proportion of *H. separatus* might be without a veil. It should also be remembered that not even the unveiled organisms were transmitted to the splenectomized goat, although it has been generally accepted that both goats and sheep are hosts to *T. ovis*. The IFA test has not been of any help in deciding whether we are dealing with one or two species, as the veil does not fluoresce, just like that of *H. veliferus* (3).

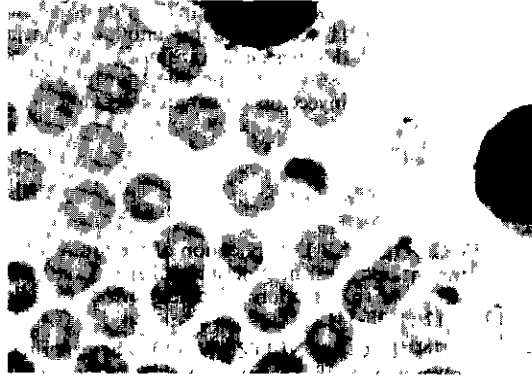
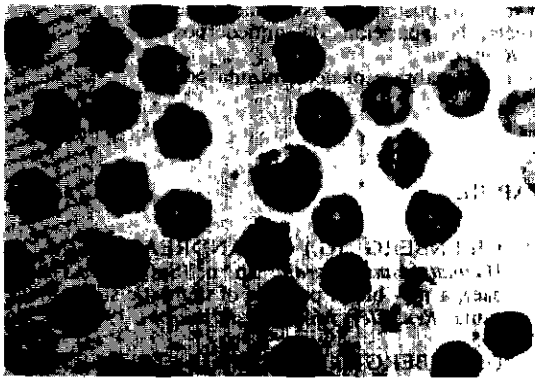
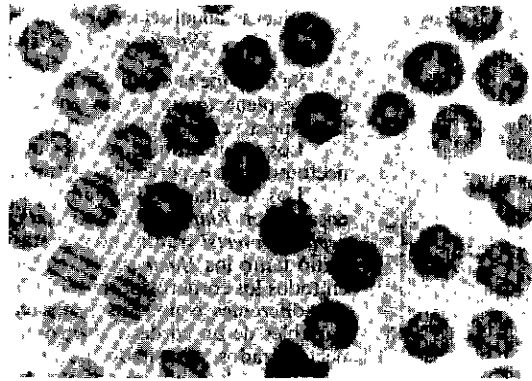
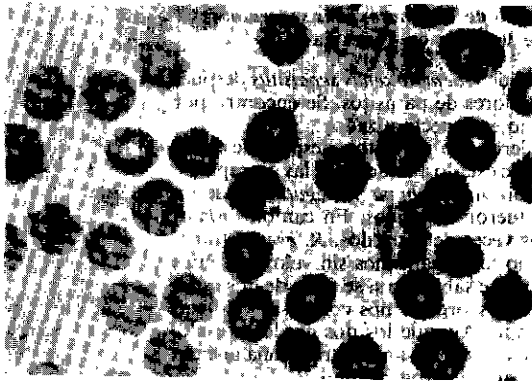
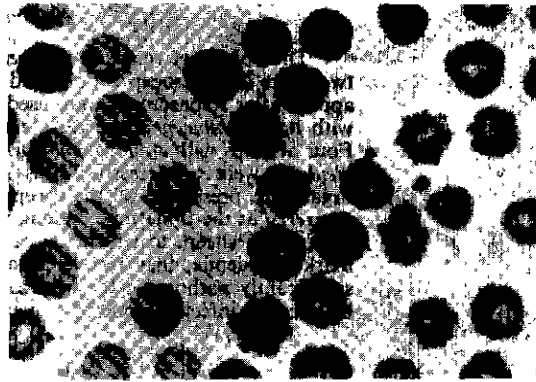
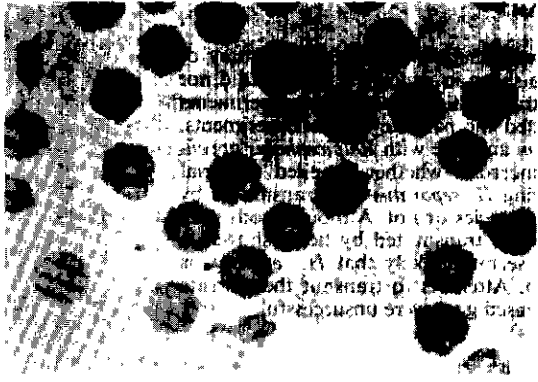


Fig. 3. — Microphotographies d'*Haematoxenus separatus* dans le sang de mouton.

Fig. 3. — Microphotographs of *Haematoxenus separatus* in blood of sheep.

*H. separatus* appears to be a common parasite, at least at Dar es Salaam. It has recently also been found at Muguga, Kenya (C. G. D. BROWN, personal communication). It is unlikely to be of pathogenic importance, as it did not kill any of our splenectomized sheep. The anaemia in both sheep, particularly severe in 1553, may have been caused by both types of parasite. Undoubtedly, the veiled organisms destroy the red cell during the veil formation (see fig. 3).

#### ACKNOWLEDGEMENTS

We are grateful to the Director of the Livestock Division of the Ministry of Agriculture in Tanzania and to the Chief of the Animal Health Service of the Food and Agriculture Organization of the United Nations for permission to publish this paper. We are obliged to Mr. C. MPANGALA, laboratory technician in our project, for preparing and testing the antigen in the IFA test.

## SUMMARY

The course of relapses of *Haematoxenus separatus* after splenectomy of two more carrier sheep is described. Two sheep in which the parasite did not appear after splenectomy were used for transstadial transmission experiments with ticks. *Rhipicephalus evertsi* transmitted the parasite in two experiments. Four attempts with *Amblyomma variegatum* and one with *Rhipicephalus appendiculatus* were unsuccessful. It is still uncertain whether unveiled theilerial organisms, present in all the sheep carrying *H. separatus* and transmitted by *R. evertsi* at the same time, belong to this species or not. Although both splenectomized sheep, to which the parasite was transmitted by ticks, showed a marked anaemia, they recovered, and it seems unlikely that *H. separatus* is significantly pathogenic for normal sheep. Attempts to transmit the parasite by injecting infected blood to a splenectomized goat were unsuccessful.

## RESUMEN

Nuevas adquisiciones en el conocimiento de *Haematoxenus separatus* (Sporozoa, Theileriidae) de la oveja en Tanzania

Se describe la evolución de las recaídas de *Haematoxenus separatus* después de la esplenectomía de dos corderos portadores de parásitos. Se encontró por la primera vez el parásito en un cordero no esplenectomizado.

Los autores utilizaron dos otros corderos, sin parásitos, después de esplenectomía para experiencias de transmisión de estado a estado por las garrapatas.

Los resultados de cuatro ensayos con *Amblyomma variegatum* y de un ensayo con *Rhipicephalus appendiculatus* fueron negativos. En cambio, *Rhipicephalus evertsi* transmitió el parásito dos veces de cada dos. *R. evertsi* transmitió tanto los *Haematoxenus* típicos como los organismos sin velo, presentes en todos los corderos portadores. Y no se sigue sabiendo si se trata de dos especies diferentes o no. Las proporciones de los organismos con y sin velo son variables de un cordero al otro en el tiempo. Aunque los dos corderos esplenectomizados, parasitados por medio de *R. evertsi*, hayan mostrado una anemia importante, han curado y es poco probable que la patogenicidad de *H. separatus* para los corderos intactos sea importante.

Utilizando antígeno preparado a partir de sangre cabiendo una mezcla de *Haematoxenus* típicos y de organismos sin velo, fue posible demostrar mediante la técnica de inmunofluorescencia indirecta, la aparición de anticuerpos después de la transmisión del parásito por *R. evertsi*.

No fue posible transmitir el parásito a una cabra esplenectomizada por inyección de sangre infectada.

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