Onderstepoort J. vet. Res. 43 (3), 123–128 (1976)

LIGHT AND ELECTRON MICROSCOPIC OBSERVATIONS ON THE DEVELOPMENT OF SMALL MEROZOITES OF *BABESIA BOVIS* IN *BOOPHILUS MICROPLUS* LARVAE

F. T. POTGIETER and H. J. ELS, Veterinary Research Institute, Onderstepoort

ABSTRACT

POTGIETER, F. T. & ELS, H. J., 1976. Light and electron microscopic observations on the development of small merozoites of *Babesia bovis* in *Boophilus microplus* larvae. *Onderstepoort J. vet. Res.*, 43 (3), 123–128 (1976).

The development of small pyriform merozoites of *B. bovis* in the granule-secreting cells of the salivary glands of *B. microplus* larvae, studied with a light microscope, showed a close resemblance to that of *B. argentina* described by Riek (1966) in the same tick vector. Development took place through a process of schizogony and resulted in the formation of many merozoites. A study of the ultrastructure of developing merozoites in the schizont revealed the following: a poorly defined outer membrane; a granular osmiophilic inner membrane; anterior and posterior polar rings; rhoptries; micronemes; microtubules; a nucleus; spherical bodies of varying size. The schizonts were membrane-bound but no parasitophorous vacuoles were seen.

Résumé

LE DÉVELOPPEMENT DES PETITS MÉROZOÏTES DE BABESIA BOVIS *DANS LES LARVES DE* BOOPHILUS MICROPLUS *EN MICROSCOPIE ÉLECTRONIQUE ET OPTIQUE*

Au microscope optique le développement des petis mérozoïtes piriformes de B. bovis *dans les cellules sécrétrices de granules des glandes salivaires des larves de* B. microplus, *ressemble assez étroitement, d'après Riek (1966), à celui de* B. argentina *dans le même tique. Le développement passe par un processus de schizogonie aboutisant à la formation de plusieurs mérozoïtes. Une étude en microscopie électronique, portante sur les mérozoïtes au cours de développement a fournie les données suivantes: une membrane extérieure mal définie; une membrane intérieure osmiophylique et granulaire; des anneax polaires antérieurs et postérieurs; des rhoptries; des micronèmes; des microtubules; un noyau; des corps sphériques aux tailles différentes. Les schizonts enveloppés ne révéllent aucunes vacuoles parasitophores.*

INTRODUCTION

Developmental stages of a variety of *Babesia* spp. have been described in the salivary glands of some of their vector ticks. These stages are thought to represent the terminal or infective forms and have been found in the case of *B. bigemina* (Riek, 1964), *B. argentina* (Riek, 1966), *B. caballi* (Holbrook, Anthony & Johnson, 1968), *B. equi* (Young & Purnell, 1973) and *B. canis* (Shortt, 1973) with the aid of the light microscope.

There is still some confusion regarding the exact identity of the so-called infective forms in the salivary glands of the ticks. Shortt (1973) refers to them as possible sporozoites, whereas Young & Purnell (1973) believe that they correspond to similar stages in the life cycle of *Theileria parva*, described as "sporozoites" by Cowdry & Ham (1932) and as "infective forms" by Martin, Barnett & Vidler (1964). On the strength of their studies on the fine structure of the spindle-shaped forms of *B. bovis* in the salivary glands of *R. bursa*, Friedhoff, Scholtyseck & Weber (1972) came to the conclusion that they are produced by schizogony and are therefore merozoites.

Potgieter & Van Vuuren (1974) recorded the successful transmission of *B. bovis* with a stabilate prepared from infected larvae of *B. microplus*. Since infective forms of the parasite were obviously present in these larval ticks, this study was initiated to identify them and to study their morphology and fine structure.

MATERIALS AND METHODS

Larvae of a laboratory-maintained strain of *B. microplus* infected with *B. bovis* were used to infest a splenectomized calf susceptible to *Babesia* infection. As a control for the morphological studies, a batch of non-infected larvae of the same tick strain was fed on a 2nd splenectomized calf.

Thick and thin blood smears were prepared daily from both calves and examined for the presence of *B. bovis*. Partly engorged larvae were removed from the experimental animals at 12 h intervals, starting immediately after infestation and continuing for 5 days. Squash and smear preparations were prepared from each collection, fixed in methanol and stained with a 10% Giemsa solution for 30 min. All preparations were examined under 1 000 × magnification with a Zeiss photomicroscope.

Other larvae were dissected as follows with the aid of a Wild stereoscopic microscope: Each tick was covered with a drop of cold 2,5% glutaraldehyde. The mouthparts and approximately 1/3 of the scutum and anterior part of the exoskeleton were then removed with the aid of an iris knife. In this way the viscera of the larva were forced posteriorly and remained undamaged. The legs were subsequently severed. This procedure allowed for the rapid penetration of the fixative and eventually also facilitated penetration of the epon-embedding medium.

The tick tissues were processed for examination with a Philips 300 and an Elmiskop 1A electron microscope, as described by Potgieter, Els & Van Vuuren (1976), the sections ranging from 60–90 nm in thickness. The dimensions of the parasites were determined with a Nikon profile projector, model 6C, from negatives obtained by means of both light and electron microscopy.

RESULTS

The batch of non-infected ticks had been obtained from a previous, unpublished experiment. Briefly, this entailed the manual removal of newly moulted

Received 4 May 1976—Editor.

nymphal instars of a *B. bovis*-infected strain of *B. microplus* from an infested calf and their transfer to another calf 5–7 days after the initial larval infestation. More than 60% of the 200 nymphs which had been transferred reattached themselves and completed their life-cycle without transmitting the *B. bovis* infection. The progeny of these ticks were subsequently maintained in the laboratory as a non-infected strain.

In the calf infested with *B. bovis*-infected ticks, parasites were first seen in thick blood smears on the 8th day after infestation. The animal was subsequently treated with diminazene* to terminate the reaction. Developmental stages of *B. bovis* were most frequently found in smears prepared from ticks removed on the 3rd day after infestation. Only 6% of the ticks were infected. Because of the low incidence of the infection in the ticks, the amount of material suitable for detailed microscopic examination was extremely limited, especially for the electron microscopic study.

The calf infested with non-infected ticks did not contract babesiosis and nothing resembling developmental stages of *B. bovis* could be demonstrated in smears prepared from the larvae.

Light microscopy

Two types of merozoites of *B. bovis* were observed in larvae which had fed on a calf for approximately 72 h. The larger club-shaped merozoites (Fig. 1) occur extracellularly and average 9,4 μm in length and 4,1 μm in width. A relatively large single nucleus is situated in the middle third of the body of the merozoite, in most cases closer to the anterior pole. The nucleus stains dark red and the cytoplasm pale blue with Giemsa's stain.

These merozoites apparently enter the cells of the salivary glands where they lose their club shape and become spherical bodies with centrally placed nuclei (Fig. 2). The round forms increase in size, the nucleus breaks up and the chromatin material spreads through the cytoplasm (Fig. 3). As the schizont grows, the chromatin material becomes diffuse and disintegrates into small particles (Fig. 4). Eventually a massive schizont 60 μm in diameter develops, causing a distension of the host cell with almost total displacement of the host cell cytoplasm (Fig. 5). Differentiation commences as the cytoplasm appears to condense around chromatin particles (Fig. 5). The differentiation continues and large numbers of small pyriform merozoites are formed and are subsequently released from the mature schizont (Fig. 6).

The small pyriform merozoites, the average measurements of which are 2,5 μm in length and 1,3 μm in width, show a red staining nucleus lying towards the tapering posterior end of the body, and a pale blue-staining cytoplasm. The larger spherical forms illustrated in Fig. 6 are approximately twice the size of the small merozoites and contain more than 1 red staining dot.

Electron microscopy

No mature schizonts or large merozoites were observed. Fig. 7 illustrates the only parasitic structure found, namely, a large immature schizont lying in a granule-secreting type of alveolus of the salivary gland. The schizont is tightly packed with small immature merozoites with average measurements of 1,4 × 1 μm. The schizont illustrated in Fig. 7 & 8 appears to be at approximately the same stage of differentiation as the one observed under the light microscope (Fig. 5). The host cell cytoplasm remains as a peripheral layer, presumably displaced by a massive number of developing merozoites (Fig. 8). Although individual merozoites can be distinguished, well-defined outer membranes are lacking. Furthermore, the merozoites are so closely arranged that no intercellular spaces can be identified (Fig. 7 & 8). The schizont appears to be separated from host cell cytoplasm by a well-defined double plasma membrane (Fig. 8a & b). Some of the developing merozoites show an osmiophilic inner membrane (Fig. 10, 11a & b).

Both anterior and posterior polar rings are present (Fig. 10, 11a & b), and it appears as though the posterior polar ring is a thickening of the inner membrane. The latter is clearly illustrated in Fig. 11a as well as in a merozoite which has presumably completed the fission process (Fig. 11b).

Relatively large rhoptries are seen directed towards the "opening" of the anterior polar ring (Fig. 7, 8 & 10), while similar but smaller osmiophilic structures or micronemes are concentrated only at the anterior polar area (Fig. 7 & 11).

Spherical membrane-bound organelles ("spherical bodies") of varying diameter occur in positions lateral and anterior to the nucleus (Fig. 10) but no structural details can be identified in them, though some appear to be vacuolated (Fig. 8).

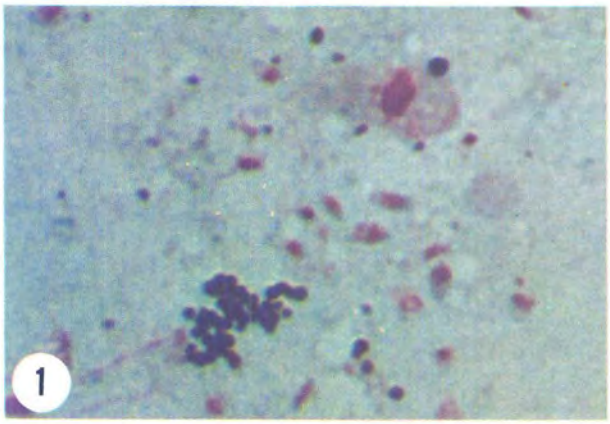
An undetermined number of microtubules can be seen in cross and longitudinal sections in the apical regions of the merozoites only (Fig. 7 & 8).

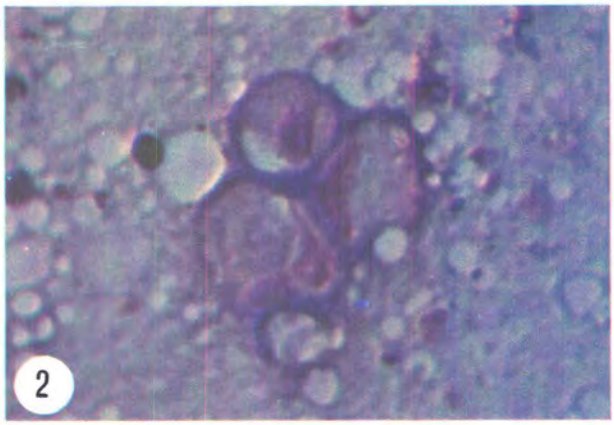
A membranous endoplasmic reticulum is absent and, in the rest of the cytoplasm, densely packed ribosomes occur, especially posterior to the nucleus (Fig. 7, 8 & 10). A single large nucleus, often surrounded by perinuclear spaces, is present (Fig. 10).

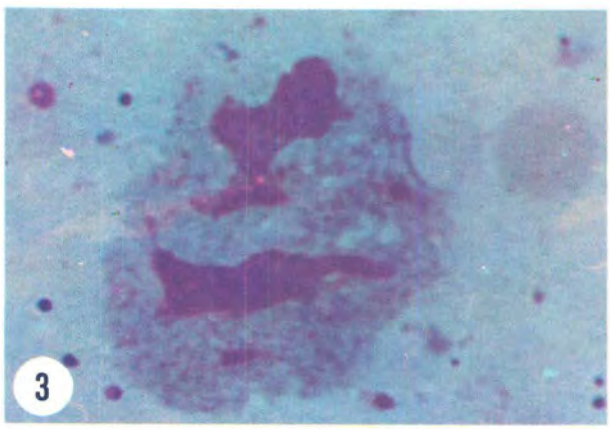
Small numbers of binucleate forms are present in the schizont illustrated in Fig. 11a, and invagination of the cytoplasm can be seen taking place between the 2 nuclei of one of them. A fissure forms in the cytoplasm between the nuclei and it leads to the posterior polar rings of what are presumably the future daughter cells. These rings appear to develop from the inner membrane and the areas in which they occur seem to be the last point of attachment between the dividing cells (Fig. 11b).

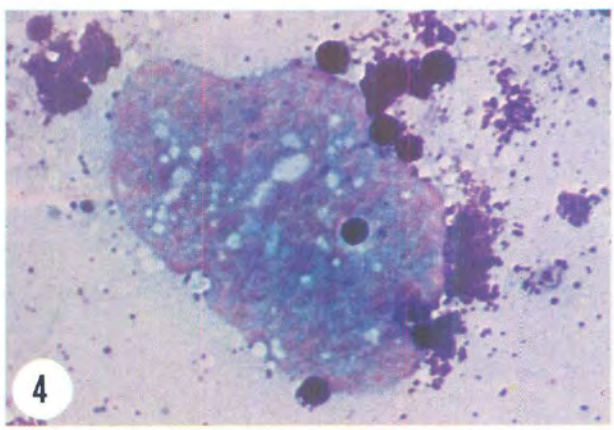
Also present in the schizont are unidentified multinucleated bodies of parasitic origin which show a considerable variation in size (Fig. 7 & 9). They appear spherical to ovoidal in shape and contain nuclei, some of which are twice the size of individual nuclei of developing merozoites. These structures are in direct contact with the merozoites, and are separated only by a poorly defined membrane (Fig. 9). Their electron-lucid cytoplasm contains large irregular vacuoles without lining membranes. Ribosomes occur throughout the cytoplasm, but, apart from occasional spherical bodies, no other organelles can be identified in them.

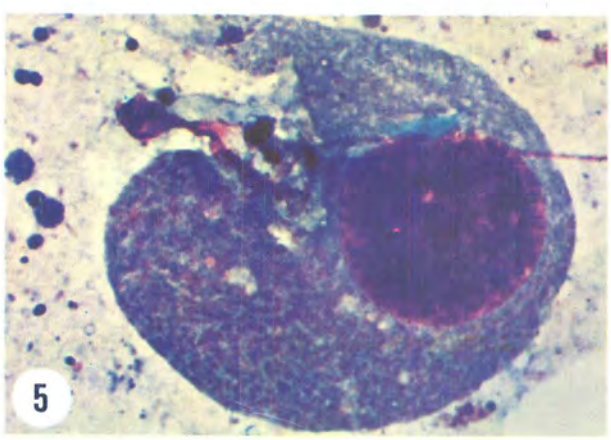
* Berenil, Hoechst











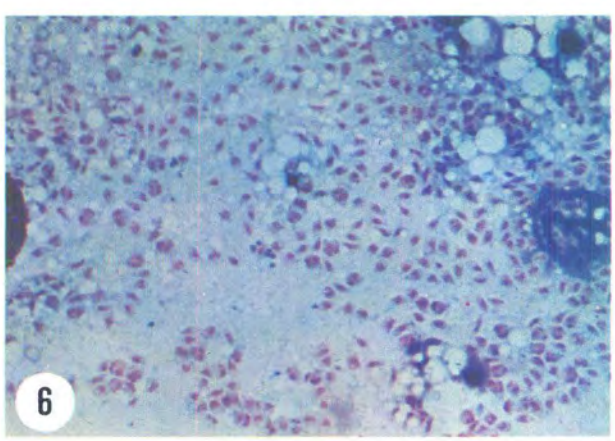
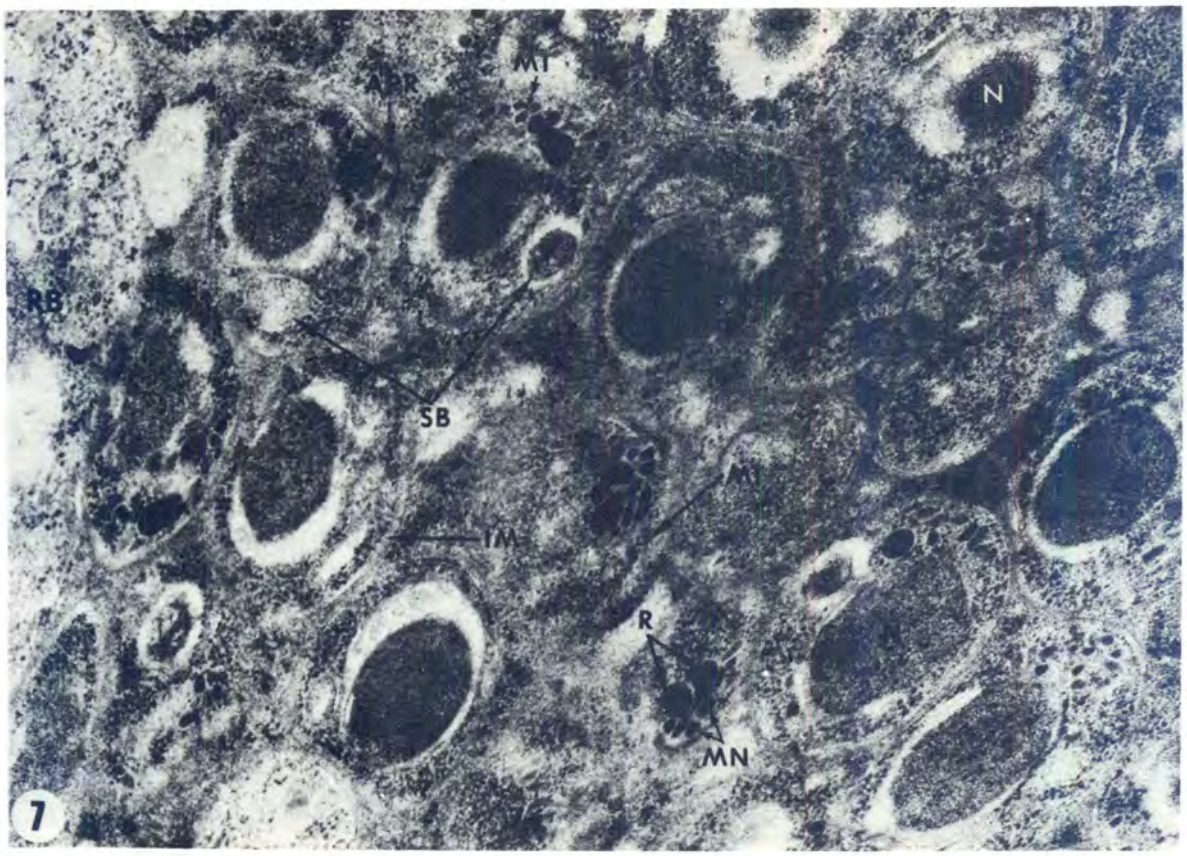


FIG. 1–6 Photomicrographs of developmental stages of *B. bovis* in smears of *B. microplus* larvae after 72 h of feeding

FIG. 1 Single large merozoite which enters the salivary glands of the larvae. Note smaller merozoites in same field. × 2 200
FIG. 2 Three large merozoites, rounded off after entering a salivary gland cell. × 2 400
FIG. 3 The spherical form seen in Fig. 2 at a later stage (young schizont). × 2 200
FIG. 4 Large schizont released from a ruptured cell of a salivary gland, showing diffuse chromatin breaking up into separate particles. Granules secreted by salivary gland cells can be seen in the same field. × 1 200
FIG. 5 A massive schizont in one of the distended cells of a salivary gland, showing an advanced stage of cytoplasmic differentiation. × 1 000
FIG. 6 Small merozoites released from a ruptured schizont. Note small pyriform merozoites as well as larger spherical bodies with more than one red-staining dot in the cytoplasm. × 1 100



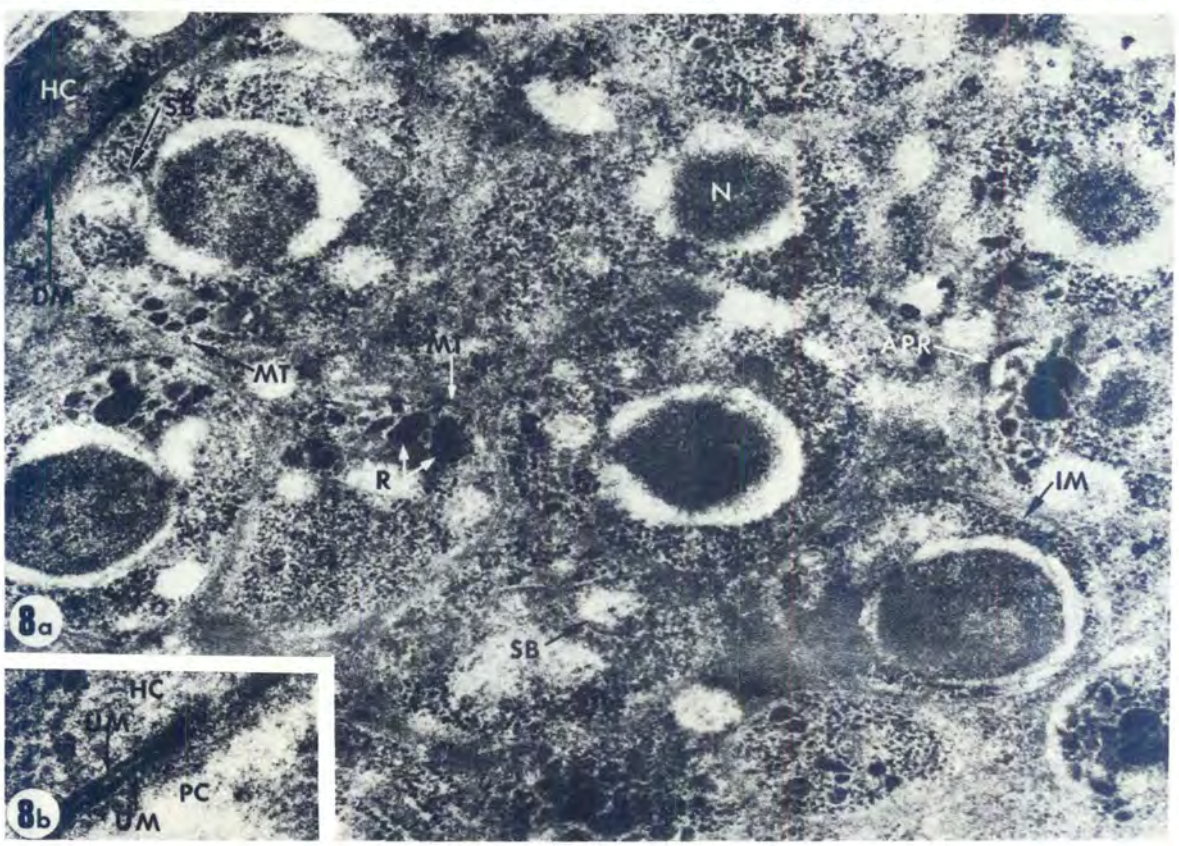
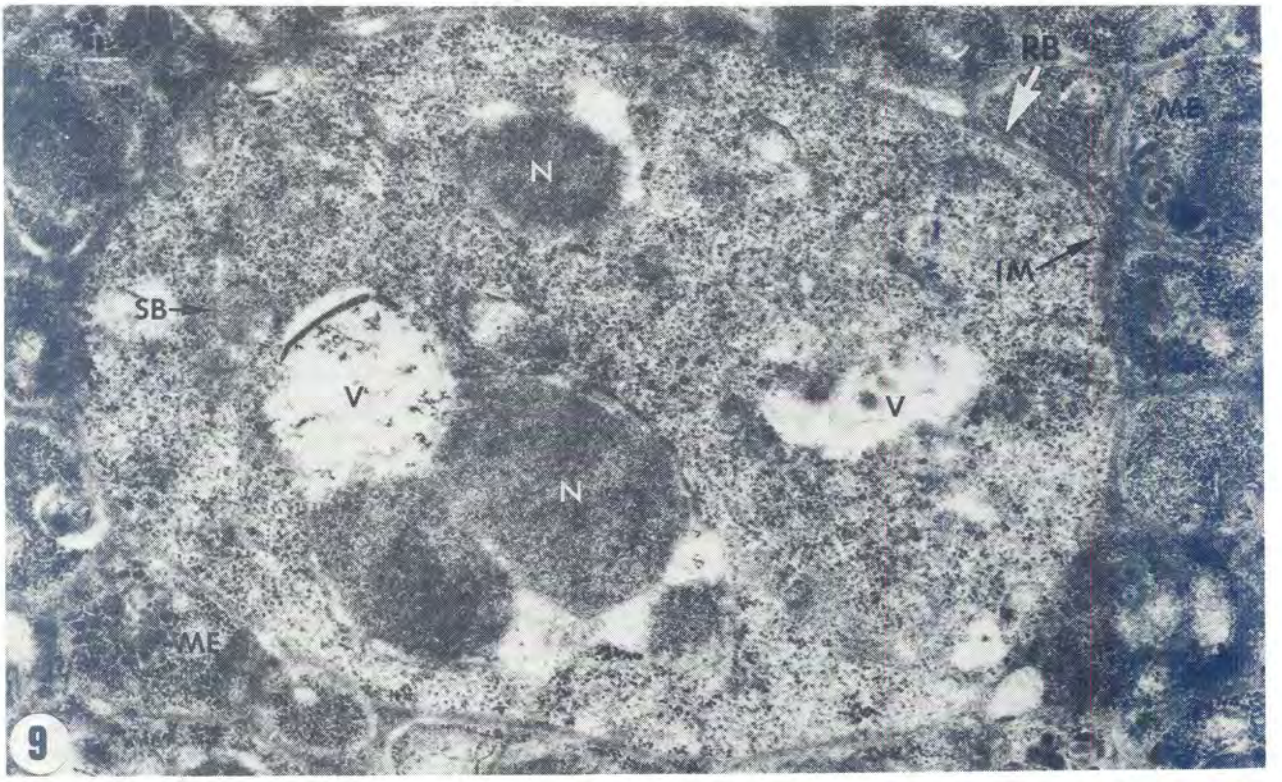


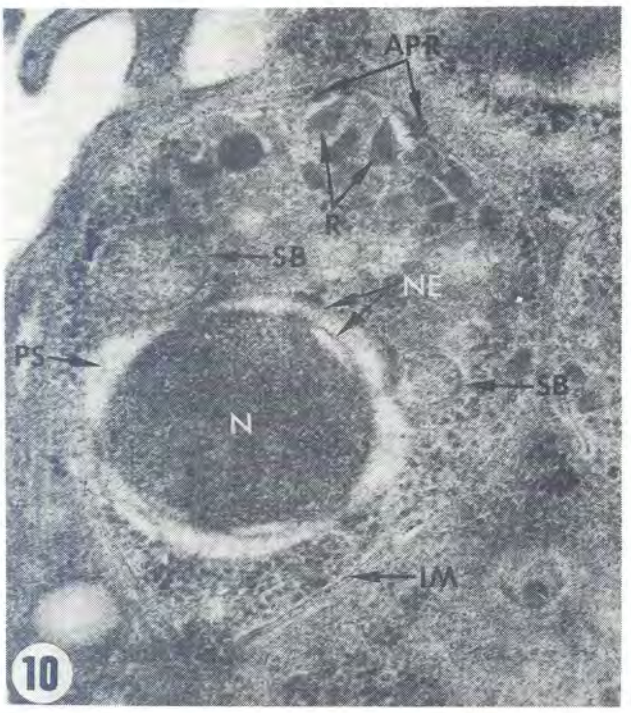
FIG. 7–11 Electron micrographs of a schizont of *B. bovis* in the salivary gland of a *B. microplus* larva

FIG. 7 Sections through a schizont showing densely packed differentiating merozoites. Note portion of large residual body found in schizont. × 34 500

FIG. 8a Part of a schizont showing a double unit membrane separating the developing small merozoites from the host cell cytoplasm. × 39 900

FIG. 8b High magnification of membranes surrounding the schizont as seen in Fig. 8a. × 110 000





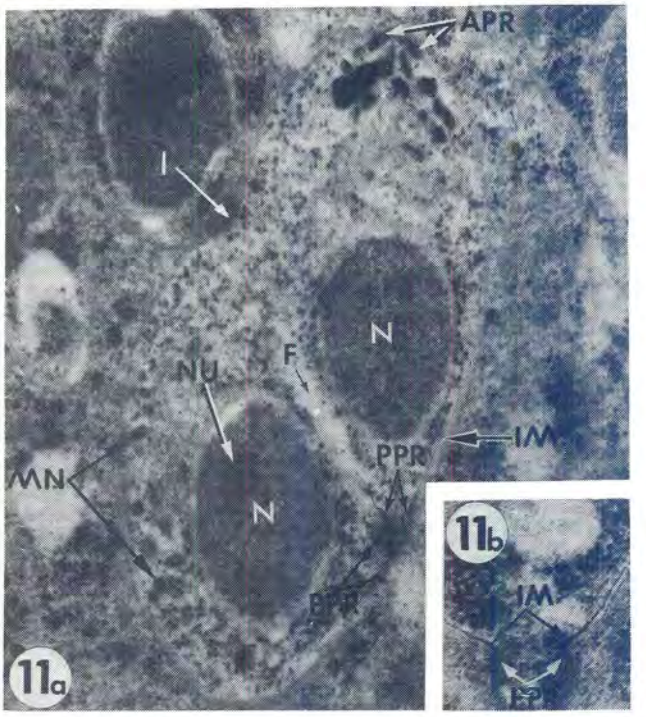


FIG. 9 A section through a residual body, surrounded by merozoites in a schizont. × 27 000
FIG. 10 Longitudinal section through a single small merozoite in a schizont. × 48 400
FIG. 11a Longitudinal section through a binucleate form. Note cytoplasmic invagination and fissure between the nuclei. × 26 000
FIG. 11b Posterior polar ring area of a small merozoite. × 52 000

Discussion

The light microscopic observations on the development of the small pyriform merozoites, which probably represent the infective forms of *B. bovis*, resemble those recorded for *B. argentina* by Riek (1966) and Mahoney & Mirre (1971). According to Riek (1966), the vermicules (large merozoites) enter a cell of the salivary gland and develop in the same way as described here for *B. bovis*, except for development of a spherical intermediate stage before the formation of the actual pyriform bodies. Riek gave no details of the structure of this intermediate stage but it may resemble that of the free-lying spherical bodies observed in this study which exhibited 2 and sometimes more red staining dots which were presumably nuclei. No such forms could be demonstrated in intact salivary gland cells, however.

It is apparently extremely difficult to demonstrate "infective forms" of *B. argentina*. Riek (1966) saw only small numbers 2–3 days after larval attachment, while Mahoney & Mirre (1971) observed "infective forms" only twice in larvae fed for 3 days. Mahoney & Mirre (1974) could not identify the infective forms of *B. argentina* with any certainty in their infective tick extracts prepared from larvae 3–5 days after attachment, and only a few organisms resembling the infective forms described by Riek (1966) were seen by them. Potgieter & Van Vuuren (1974) found the highest incidence of "infective forms" of *B. bovis* in smears made from larvae 3 days after infestation but the number of infected larvae was extremely low, an observation which also applies to this study.

The single large schizont seen in the salivary gland of a larval tick which had fed for approximately 72 h provided valuable information on the ultra-structure of differentiating merozoites which develop into the small pyriform merozoites of the parasite. As a result of the displacement of host cell cytoplasm by the growing schizont, it is impossible to determine the exact identity of the host cell in the granule-secreting alveoli.

The fact that the schizont was surrounded by 2 unit membranes is difficult to interpret. Since no parasitophorous vacuole was observed, the most likely explanation is that the 2 unit membranes represent the limiting membranes of the parasitized cell and an adjoining epithelial cell. *B. ovis* schizonts in the salivary glands of *R. bursa* are also not found in parasitophorous vacuoles, the merozoites being in immediate contact with the host cell cytoplasm (Friedhoff *et al.*, 1972; Mehlhorn & Scholtyseck, 1974). In contradistinction to the case in *B. ovis*, no residual bodies consisting of cytoplasm of the host cell could be distinguished between the densely-packed differentiating merozoites of *B. bovis*.

The large multinuclear bodies in the schizont of *B. bovis* remain unidentified. No clear evidence could be obtained that the merozoites are produced from them, and therefore they apparently do not correspond to the ordinary residual bodies of cytoplasm found in differentiating schizonts. The large vacuoles in their cytoplasm may indicate that these bodies are in a state of degeneration.

An interesting feature is the occurrence of binucleate parasities that are probably undergoing fission. Whether this represents ordinary binary fission or the terminal phase of cytoplasmic differentiation of cytomere-like intermediate stages of the parasite is unknown at this stage. If uninucleate cytomeres are produced as the 1st phase of differentiation in the schizont, and if this is followed by a 2nd phase of fission producing 2 small merozoites from each cytomere, it bears direct resemblance to what Scholtyseck (1973) has described for *Eimeria tenella* and *E. stiedae*. It is possible that these binucleate forms correspond to the free-lying spherical bodies referred to above.

The fine structure of the individual small merozoites is basically the same as that of *B. ovis* (Friedhoff *et al.*, 1972). The pellicle of the parasite was poorly defined, however, and only the inner membrane could be identified in most cases. This is probably due to the incomplete differentiation of the merozoites observed.

It is not known whether the spherical bodies observed in *B. bovis* merozoites are the same as the single, larger bodies seen in *B. ovis*, some of which contain vesicles. Similar spherical bodies, almost the size of the nuclei, observed in *B. bovis* were found in red blood cells in the brain capillaries of a bovine host (Potgieter, unpublished observations). Wright (1972) also found *B. argentina* infected erythrocytes in brain tissue where the parasite showed 2–3 spherical bodies which he identified as nuclei. At this stage insufficient information is available to enable the spherical bodies seen in *B. bovis* to be defined, and it must be borne in mind that possibly only immature merozoites were observed in the electron microscope study.

No micropores or fixed arrangement and number of microtubules were observed, such as were present in developing *B. bovis* merozoites in the gut of engorged female *B. microplus*, described by Potgieter *et al.* (1976).

Certain developmental and morphological similarities exist between *B. bovis* described above and *B. argentina* (Riek, 1966). Other common features, viz. their close serological relationship (Goldman & Rosenberg, 1974), their having a common vector and a similar incubation period in the bovine host (Potgieter, unpublished observations, 1973), support the suggestion by Riek (1968) that these parasites are synonymous.

List of Abreviations used in Electron micrographs

APR—Anterior polar ring
DM—Double membrane
F—Fissure
HC—Host cell cytoplasm
I—Invagination
IM—Inner membrane
ME—Merozoite
MN—Microneme
MT—Microtubule
N—Nucleus
NE—Nuclear envelope
NU—Nucleolus
PC—Parasite cytoplasm
PPR—Posterior polar ring
PS—Perinuclear space
R—Rhoptries
RB—Residual body
SB—Spherical body
UM—Unit membrane
V—Vacuole

References

COWDRY, E. V. & HAM, A. W., 1932. Studies on East Coast fever. I. The life cycle of the parasite in ticks. *Parasitology*, 24, 1–49.

FRIEDHOFF, K., SCHOLTYSECK, E. & WEBER, G., 1972. Die Feinstruktur der differenzierten Merozoiten von *Babesia ovis* in den Speicheldrüsen weiblicher Zecken. *Zeitschrift für Parasitenkunde*, 38, 132–140.

GOLDMAN, M. & ROSENBERG, A. S., 1974. Immunofluorescence studies of the small *Babesia* species of cattle from different geographical areas. *Research in Veterinary Science*, 16, 351–354.

HOLBROOK, A. A., ANTHONY, D. W. & JOHNSON, A. J., 1968. Observations on the development of *Babesia caballi* (Nuttall) in the tropical horse tick *Dermacentor nitens* Neumann. *Journal of Protozoology*, 15, 391–396.

MAHONEY, D. F. & MIRRE, G. B., 1971. Bovine babesiasis: estimation of infection rates in the tick vector *Boophilus microplus* (Canestrini). *Annals of Tropical Medicine and Parasitology*, 65, 309–317.

MAHONEY, D. F. & MIRRE, G. B., 1974. *Babesia argentina:* The infection of splenectomized calves with extracts of larval ticks (*Boophilus microplus*). *Research in Veterinary Science*, 16, 112–114.

MARTIN, H. M., BARNETT, S. F. & VIDLER, B. O., 1964. Cyclic development and longevity of *Theileria parva* in the tick *Rhipicephalus appendiculatus*. *Experimental Parasitology*, 15, 527–555.

MEHLHORN, H. & SCHOLTYSECK, E., 1974. Die parasit-Wirtbezeihugen bei verschiedenen Gattungen der Sporozoen (*Eimeria, Toxoplasma, Sarcocystis, Frenkelia, Hepatozoon, Plasmodium* und *Babesia*) unter Anwendung spezieller Verfahren. *Microscopica Acta*, 75, 429–451.

POTGIETER, F. T. & VAN VUUREN, A. S., 1974. The transmission of *Babesia bovis* using frozen infective material obtained from *Boophilus microplus* larvae. *Onderstepoort Journal of Veterinary Research*, 41, 79–80.

POTGIETER, F. T., ELS, H. J. & VAN VUUREN, A. S., 1976. The fine structure of merozoites of *Babesia bovis* in the gut epithelium of *Boophilus microplus*. *Onderstepoort Journal of Veterinary Research*, 43, 1–10.

RIEK, R. F., 1964. The life cycle of *Babesia bigemina* (Smith and Kilborne, 1893) in the tick vector *Boophilus microplus* (Canestrini). *Australian Journal of Agricultural Research*, 15, 802–821.

RIEK, R. F., 1966. The life cycle of *Babesia argentina* (Lignières, 1903) (Sporozoa: Piroplasmidea) in the tick vector *Boophilus microplus* (Canestrini). *Australian Journal of Agricultural Research*, 17, 247–254.

RIEK, R. F., 1968. Babesiosis. *In* Weiman, D. & Ristic, M. (Ed.), 1968. Infectious Blood Diseases of Man and Animals, Vol. II, pp. 219–268. New York & London: Academic Press.

SHORTT, H. E., 1973. *Babesia canis:* The life cycle and laboratory maintenance of its arthropod and mammalian hosts. *International Journal for Parasitology*, 3, 119–148.

SCHOLTYSECK, E., 1973. Die Deutung von Endodyogenie und Schizogony bei Coccidien und anderen Sporozoen. *Zeitschrift für Parasitenkunde*, 42, 87–104.

WRIGHT, I. G., 1972. An electron microscopic study of intravascular agglutination in the cerebral cortex due to *Babesia argentina* infection. *International Journal for Parasitology*, 2, 209–215.

YOUNG, A. S. & PURNELL, R. E., 1973. Observations on *Babesia equi* in the salivary glands of *Rhipicephalus evertsi*. *Bulletin of Epizootic Diseases of Africa*, 21, 377–383.

Printed by and obtainable from The Government Printer, Private Bag X85, Pretoria, 0001