Onderstepoort J. vet. Res., 53, 153–159 (1986)

HEARTWATER: AN *IN VITRO* STUDY OF THE ULTRASTRUCTURE OF *COWDRIA RUMINANTIUM*

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ABSTRACT

PROZESKY, L., BEZUIDENHOUT, J. D. & PATERSON, CAMILLA L., 1986. Heartwater: An *in vitro* study of the ultrastructure of *Cowdria ruminantium*. *Onderstepoort Journal of Veterinary Research*, 53, 153–159 (1986).

Notwithstanding morphological differences, the ultrastructure of *Cowdria ruminantium* cultured *in vitro* concurred to a large extent with that in previous *in vivo* studies. Two distinct forms of the organism, elementary and reticulate bodies, and a 3rd group of intermediate organisms were identified. Organisms within a particular vacuole were generally a specific form, but in cells containing many colonies different forms were present in the same colony. Most organisms were enveloped by 2 membranes and a few were surrounded by a 'capsule'. *C. ruminantium* multiplies mainly by binary fission, but it appears that multiplication can also take place by means of budding. The taxonomy of *C. ruminantium* is briefly discussed.

INTRODUCTION

Pienaar (1970) described the morphology of *Cowdria ruminantium* in endothelial cells in the choroid plexus of sheep infected with the Ball 3 strain. The Ball 3 strain was morphologically indistinguishable from the Welgevonden strain, which is infective for mice (Prozesky & Du Plessis, 1985).

Heartwater organisms were grouped into small, intermediate, large and very large (giant) forms by Pienaar (1970). He pointed out that within a colony the organisms had the same form and internal structure although an extreme variation in size was often seen in colonies containing mostly giant forms. The small organisms in these colonies despite their size, structurally resembled the very large forms.

The purpose of this *in vitro* study was to describe the morphology of *C. ruminantium* and to compare it with that in previous *in vivo* studies. The taxonomy of *C. ruminantium* is briefly discussed.

MATERIALS AND METHODS

Cell cultures

Tick derived stabilate, prepared from *Amblyomma hebraeum* nymphae infected with *C. ruminantium* (Bezuidenhout, 1981), was cultivated in plastic culture flasks containing irradiated endothelial cells in Eagle's medium (Bezuidenhout, Paterson & Barnard, 1985). Cell cultures inoculated with tick derived stabilate from non-infected *A. hebraeum* nymphae served as controls. Cells were collected for transmission electron microscopy at various days after inoculation (Table 1).

Electron microscopy

Eagle's medium was removed from the culture flasks, leaving only a thin layer covering the cells. Approximately 3 mℓ of freshly prepared 2½ % sodium cacodylate-buffered glutaraldehyde (pH 7,2–7,3) at room temperature was added with a syringe and allowed to stand for 1 min. After the glutaraldehyde-medium mixture was poured off, the cells were covered with *c.* 10 mℓ of freshly prepared 2½ % sodium cacodylate-buffered glutaraldehyde, and allowed to stand for *c.* 4 h. The cells were scraped off with a Costar disposable scraper* and centrifuged for 10 min at 4 000 × g. The pellet obtained in this manner was rinsed in sodium cacodylate buffer and post-fixed in 2 % osmium tetroxide for 1 h, dehydrated in a graded ethanol series (50–100 %), passed through propylene oxide as the intermediate solvent, and embedded in Polaron 812**. Thin sections (70–90 nm) were cut and stained for 30 min in a saturated aqueous uranyl acetate solution and for 10 min in Reynold's lead citrate at room temperature (Kay, 1965). An image analyser was used to measure the maximum diameter of the organisms.

Inoculation of sheep

Sheep were periodically injected intravenously with *c.* 10 mℓ of tissue culture cells suspended in medium (Table 2). The animals were stabled and observed daily, and temperatures were recorded every morning. Febrile animals were treated on the 2nd or 3rd day of the febrile reaction with a long-acting oxytetracycline preparation*** at a dosage level of 20–40 mg/kg live mass (Table 2).

RESULTS

Electron microscopy

Tissue cultures inoculated with infected tick stabilate

Heartwater organisms were identified as early as 7 days post-inoculation (Table 1). Single or multiple intracytoplasmic membrane-bound colonies and many extracellular organisms were noted (Fig. 1 & 2). Segments of the vacuolar membrane surrounding the organisms were infrequently absent. In a few colonies, a fine fibrillar matrix and small electron-dense granules were visible between the organisms (Fig. 3).

Most organisms were enveloped by 2 membranes (Fig. 4). Two main forms of the organism were identified from the morphology of the internal structure, namely medium electron-dense and electron-dense, as well as an intermediate form with an electron density between these 2 forms.

Medium electron-dense organisms ranged in size from 0,3–6 μm in diameter (average 0,7–1,2 μm) and were mainly coccoid in shape, although the very large forms (giant organisms) were pleomorphic (Fig. 5 & 6). Often the membrane (inner membrane) surrounding the internal structure was segmentally visible, and occasionally the organisms were surrounded by a single membrane. A few organisms were enveloped by a clearly demarcated, fine floccular layer *c.* 19 nm in diameter (Fig. 7). The internal structure of the medium electron-dense organisms consisted of a fine fibrillar material and multifocal aggregates of medium electrondense ground substance in which small electron-dense granules were embedded

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Received 26 March 1986—Editor

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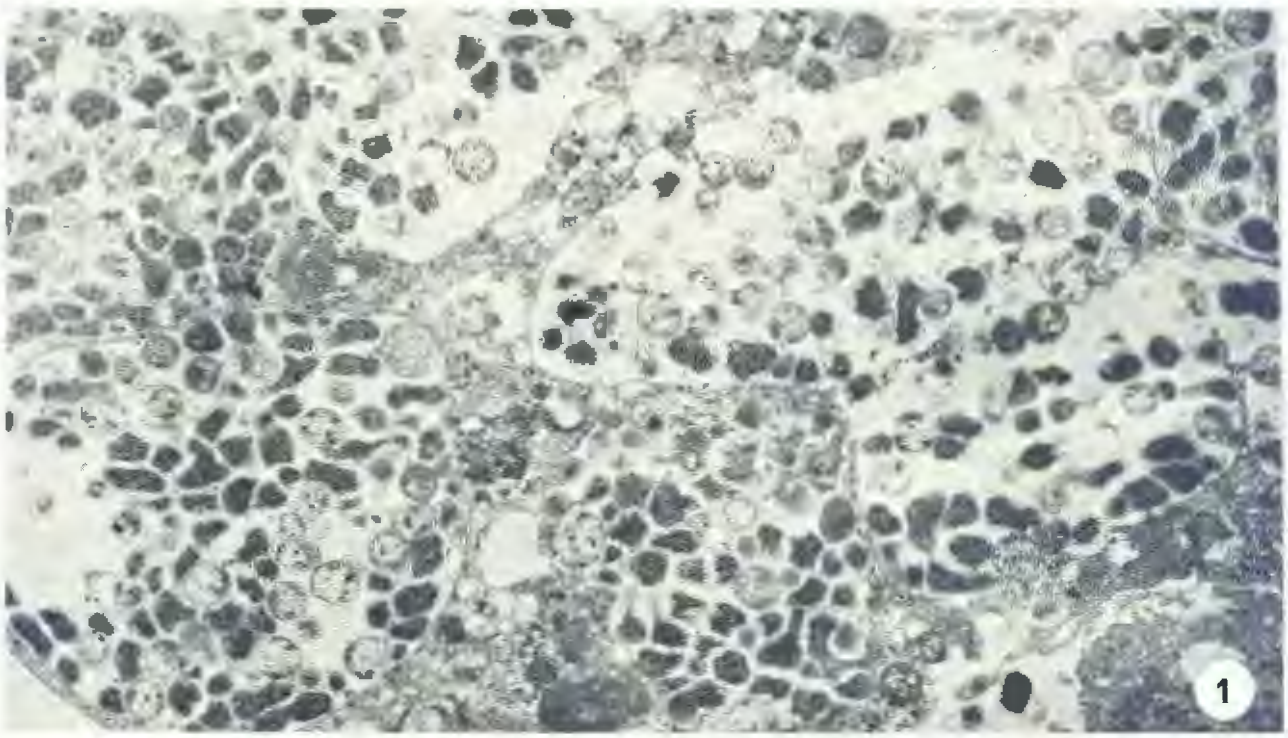
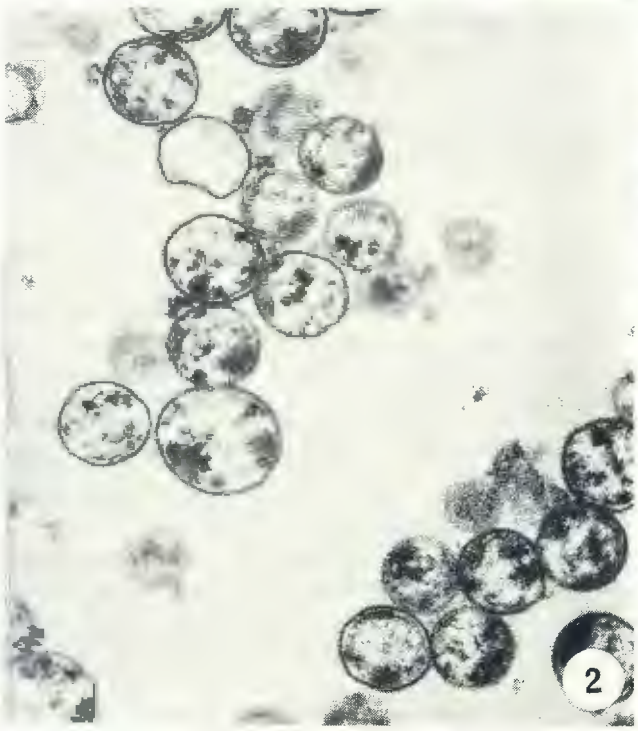




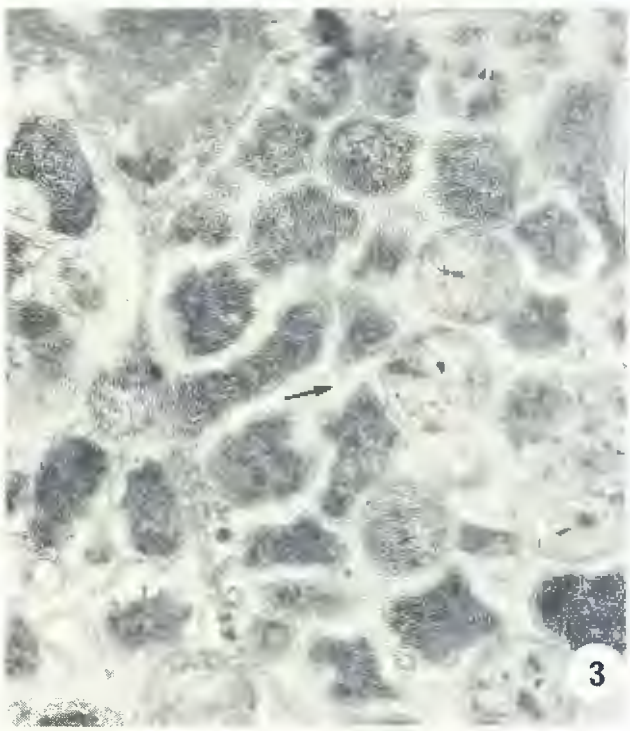
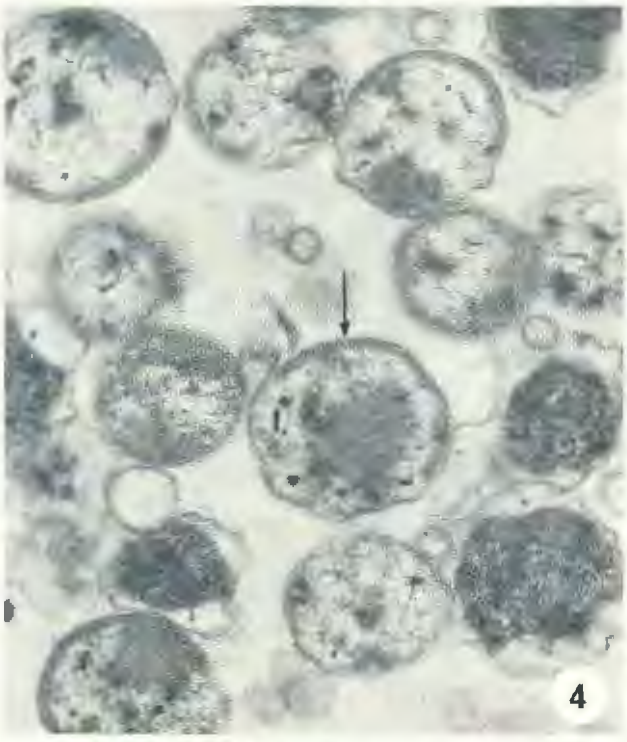
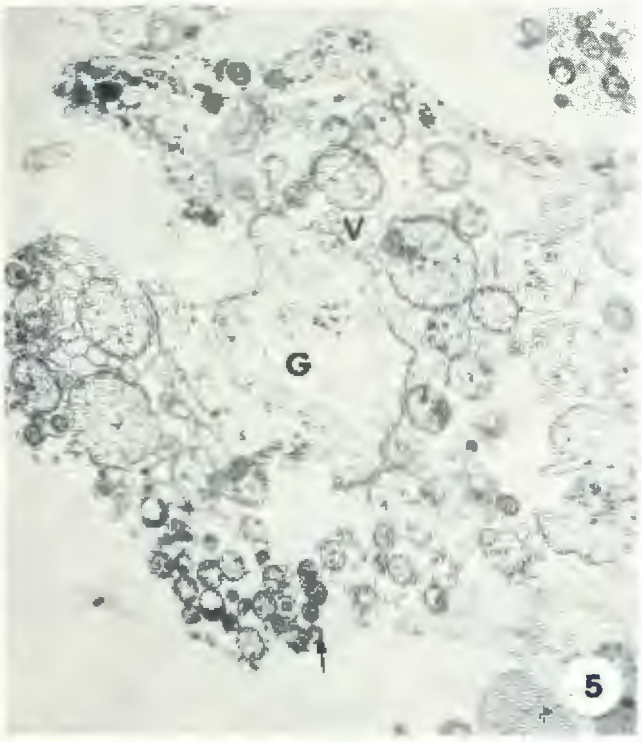


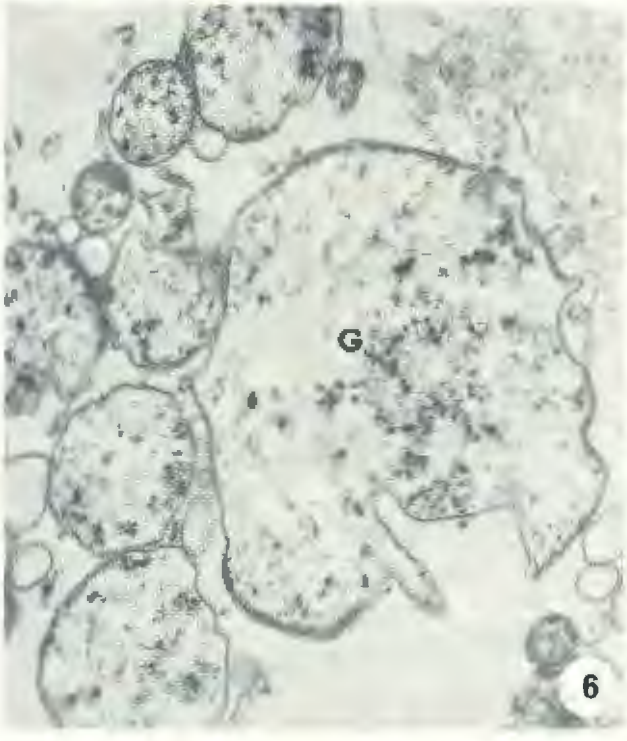
FIG. 1 Multiple colonies of heartwater organisms in an endothelial cell: × 10 300
FIG. 2 Extracellular organisms: × 22 000
FIG. 3 Fine fibrils are visible between the organisms (arrow): × 25 000

(Fig. 8 & 9). Colonies of medium electron-dense organisms frequently contained organisms with a faintly staining internal structure (Fig. 10), and large numbers of vesicular structures were visible between the organisms (Fig. 5).

Electron-dense organisms ranged in size from 0,7–1,7 μm in diameter and were mainly cocco-bacillary to coccoid in shape, although a few were pleomorphic (Fig. 11–13). While most organisms were enveloped by one membrane, a few were surrounded by a double membrane. A conspicuous feature of the majority electron-dense organisms was the rippled appearance of the surrounding membrane (Fig. 12). The internal structure consisted of a ground substance of intermediate electron density in which small electron-dense granules were embedded (Fig. 14). In a fair number of organisms, vesicular structures 0,1–0,2 μm in diameter were evident in the internal structure and, infrequently, between the organisms (Fig. 15).







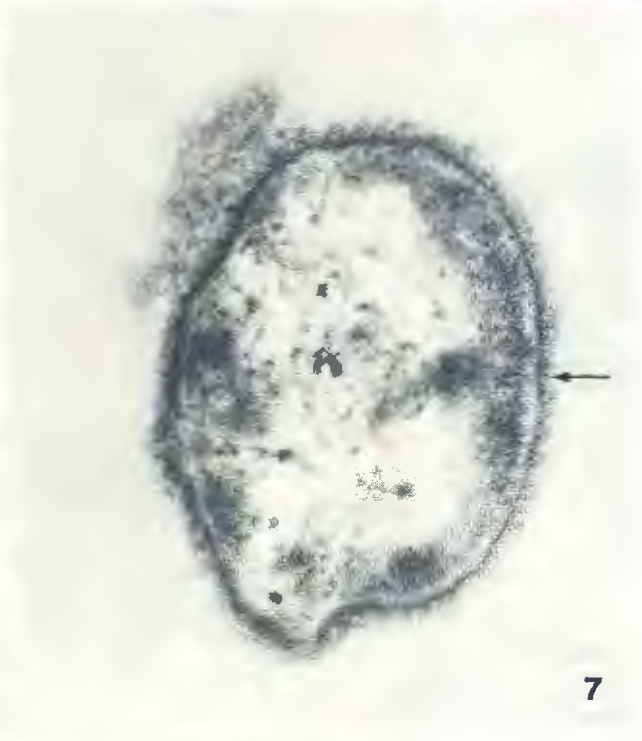


FIG. 4 A few organisms are enveloped by a double membrane (arrow); intermediate organism (I): × 38 600

FIG. 5 & 6 A colony with giant and minute organisms (arrow). Multiple vesicular structures are scattered between the organisms; giant organism (G); vesicular structures (V): × 5 700, × 14 400

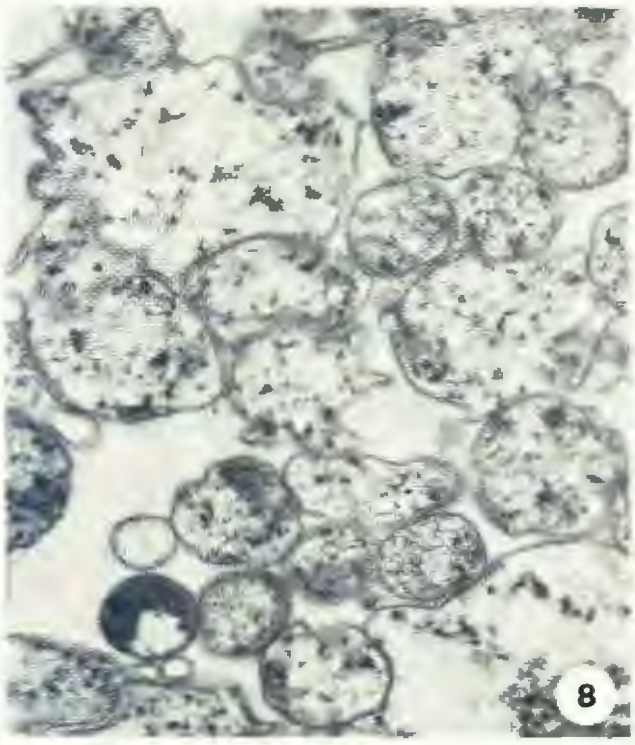
FIG. 7 An organism surrounded by a 'capsule' (arrow): × 151 000

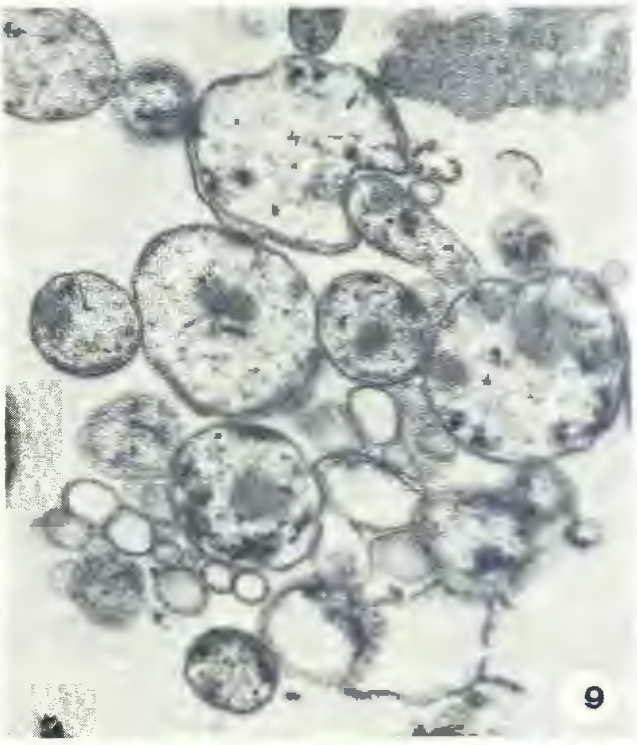
The internal structure and surrounding membranes of the intermediate organisms had features in common with both medium and electron-dense organisms (Fig. 4).

In cells with single *C. ruminantium* colonies, the organisms within a particular vacuole were usually of the same form, whereas in cells with multiple colonies different forms of organisms (mixed colonies) were present (Fig. 16–18).

In a few colonies, the organisms showed changes indicative of degeneration, such as vacuolation of the internal structure and the absence or poor delineation of the surrounding membranes. Numerous membranebound vacuoles were present between these orgaisms (Fig. 19). Cytopathogenic changes in the endothelial cells containing the degenerative colonies included swelling and pycnosis of the mitochondria.

The morphology of single medium electron-dense and electron-dense organisms suggested replication by means of binary fission. Indications were also found that medium electron-dense organisms can replicate by means of budding.





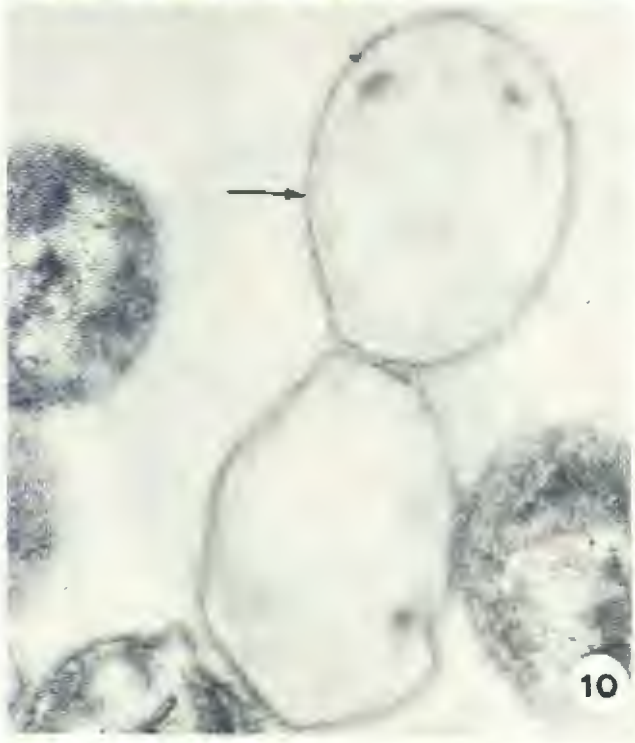


FIG. 8 Medium electron-dense organisms (reticulate bodies): × 18 000

FIG. 9 Intermediate organisms: × 23 000

FIG. 10 A faintly staining internal structure is visible in the 2 organisms enclosed by a 'capsule' (arrow): × 77 000

Tissue cultures inoculated with non-infected tick stabilate

No organisms were identified in the samples.

Inoculation of sheep

Five of the 7 inoculated sheep reacted with a febrile response and were treated (Table 2).

DISCUSSION

Notwithstanding morphological differences (*vide infra*) the ultrastructure of *C. ruminantium* cultured *in vitro* was generally similar to that described in *in vivo* studies of the choroid plexus of sheep (Pienaar, 1970) and the lungs of mice (Prozesky & Du Plessis, 1985). Pienaar (1970) described 3 distinct forms of organisms in sheep, namely, small (0,49 μm), large (1,04 μm) and very large or giant forms (up to 2,7 μm in diameter) as well as an intermediate group between the small and large organisms. According to Pienaar (1970), as a rule only organisms of the same form were found within a particular vacuole, although an extreme variation in size was often seen in colonies containing mostly giant forms.

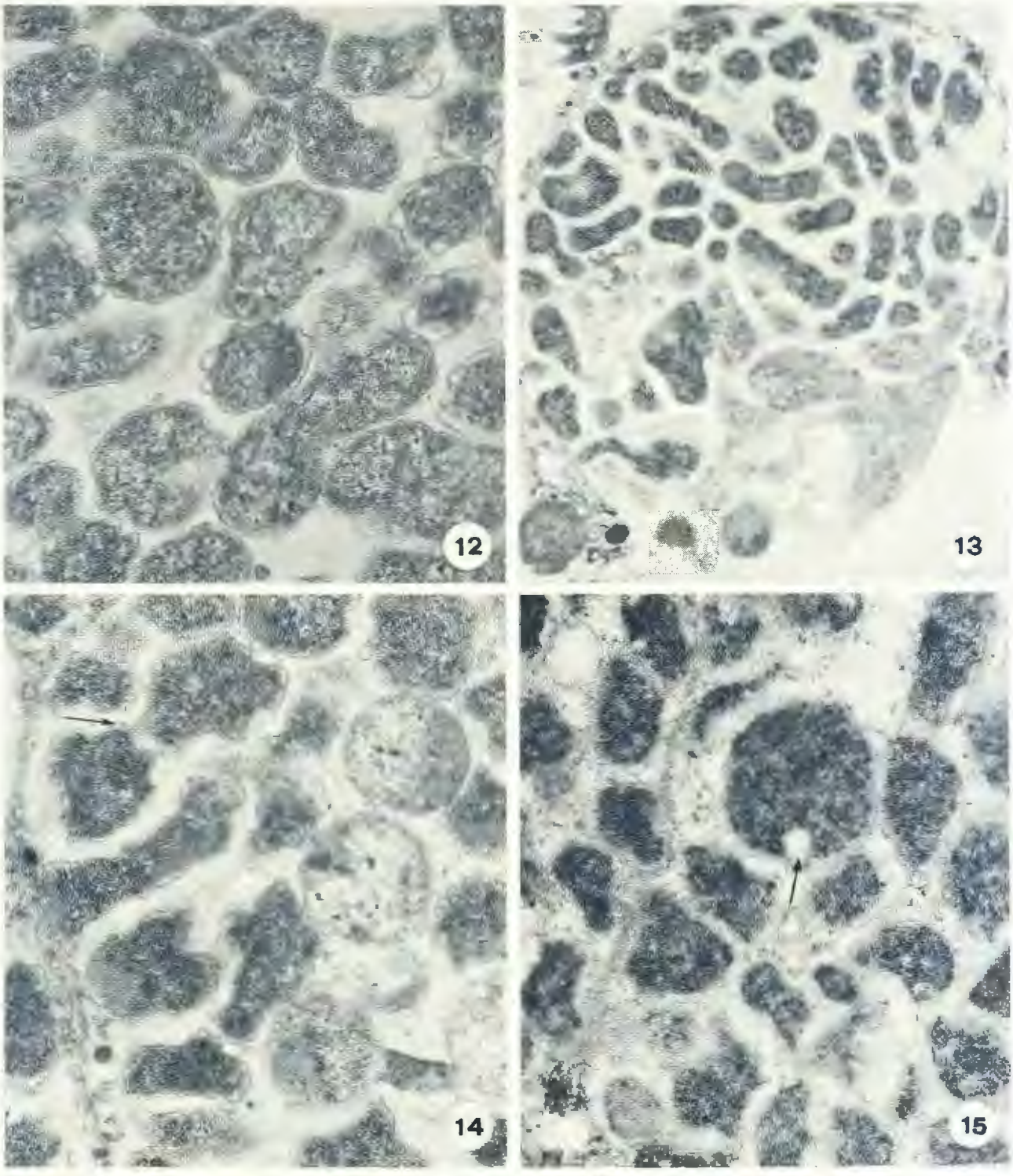


FIG. 11–13 Electron-dense organisms (elementary bodies): × 8 000, × 30 000, × 15 000
FIG. 14 The membranes enveloping the electron-dense organisms (elementary bodies) has a rippled appearance (arrow): × 48 000
FIG. 15 A vacuolated electron-dense organism (elementary body) (arrow): × 30 000

In the present study, the morphology of the internal structure was used to distinguish between different forms of organisms. Medium electron-dense and electron-dense as well as a range of organisms (intermediate forms) intermediate between these 2 main forms were identified. Organisms of the same form were generally present in a particular vacuole. However, in cells containing many colonies, different forms of organisms (mixed colonies) were found within a particular vacuole. Mixed colonies were not found in previous *in vivo* studies (Pienaar, 1970; Prozesky & Du Plessis, 1985). Factors responsible for the formation of mixed colonies are unknown, and a possible explanation is that it is a consequence of unfavourable intracellular conditions resulting from the high concentration of organisms within a particular cell.

When environmental conditions *in vitro* are not specially favourable for rickettsiae and chlamydiae, altered (abnormal) forms are found [Popov, Shatkin, Avakyan & Prozorousky, (1977), cited by Avakyan & Popov, 1984]. Altered rickettsial forms include filamentous, vacuolated and spheroplast-like forms, and organisms with

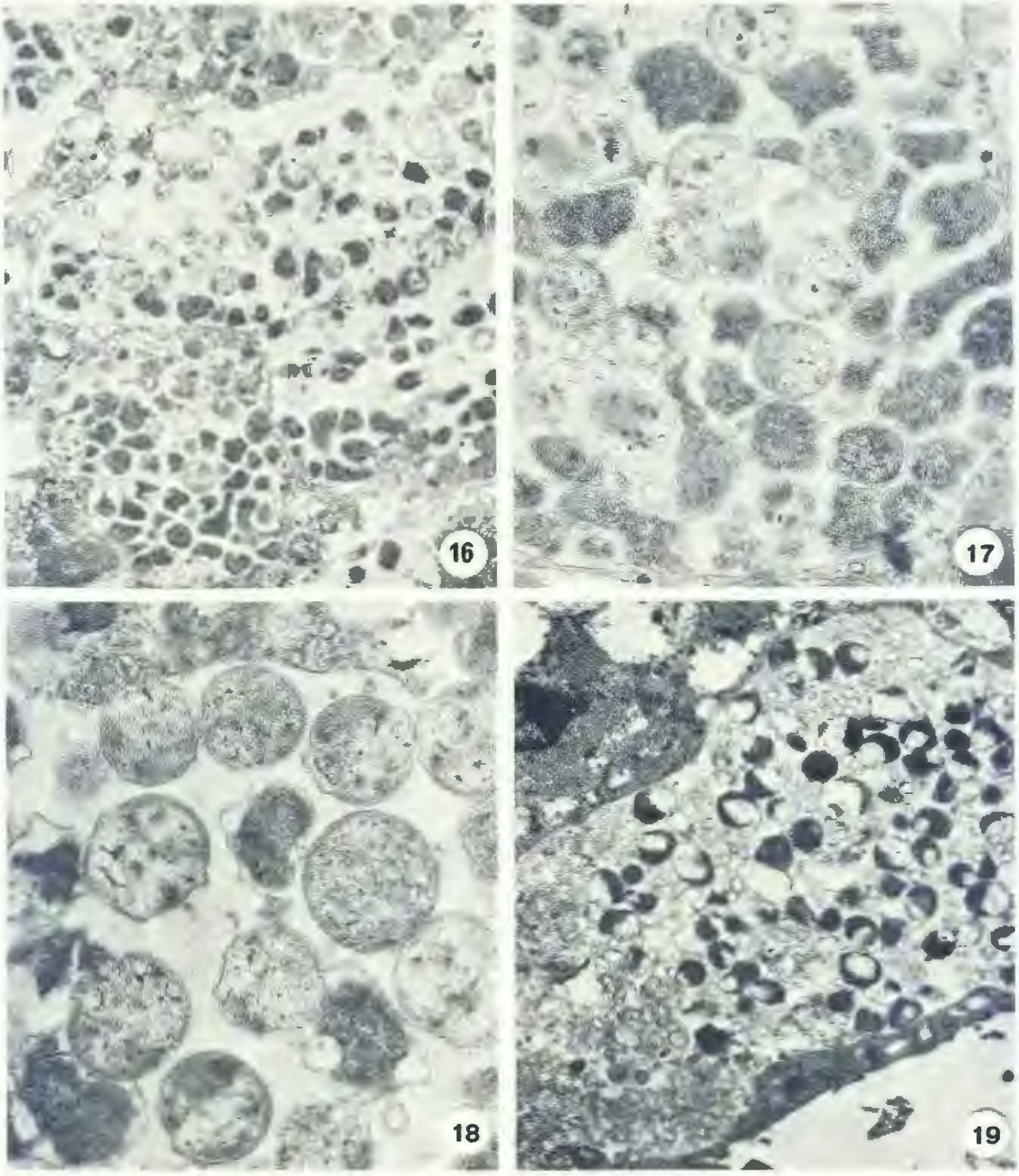


FIG. 16–18 Different types of organisms (mixed colonies) within membrane bound vacuoles: × 8 500; × 23 000; × 30 000

FIG. 19 A colony of organisms with changes indicative of degeneration, such as vacuolation of the internal structures and the poor delineation of their surrounding membranes: × 11 600

crystalline structures. Abnormal chlamydial forms are represented by giant, spheroplast-like bodies up to 5 μm in diameter, minute forms (100–250 nm) and vesicular structures (30–80 nm) in the inclusion cavity. Giant, minute and vacuolated forms as well as vesicular structures amongs the organisms were identified in the present study (*vide infra*). Although giant, minute, and vacuolated forms were reported in sheep infected with heartwater, vesicular structures were not found in previous *in vivo* studies (Pienaar, 1970; Prozesky & Du Plessis, 1985).

Rickettsiae multiply in the host cell cytoplasm without an obligate cycle of development (Avakyan & Popov, 1984). On the other hand, chlamydiae are characterized by a developmental cycle, consisting of a mandatory alteration of vegetative and spore-like forms (reticulate and elementary bodies) (Storz & Spears, 1977). Pienaar (1970) emphasized the close resemblance between the reticulate bodies of chlamydiae and the large forms of *C. ruminantium*, and between the small forms of *C. ruminantium* and the elementary bodies of chlamydiae. The

TABLE 1 Electron microscopic examination of tissue cultures infected with *C. ruminantium*

Collection of samples (days post-inoculation)	Relative concentration of *C. ruminantium* in samples
1	Neg
4	Neg
7	+
27	+
29	+
38	++++
62	++++
70	++++

+: Increasing severity (arbitrary relative units)
Neg: Negative

TABLE 2 Intravenous inoculation of sheep with *C. ruminantium* infected tissue culture suspensions

Sheep No.	Age of tissue culture suspension (day post-inoculation)	Reaction
1	13	Febrile reaction, treated, recovered
2	20	No febrile reaction
3	22	Slight febrile reaction
4	25	Febrile reaction, treated, recovered
5	33	Febrile reaction, treated, recovered
6	49	Febrile reaction, treated, recovered
7	60	Febrile reaction, treated, recovered

different forms of *C. ruminantium* present in mixed colonies may represent such structurally different particles as part of a developmental cycle. As a result of the ability of *C. ruminantium* to form mixed colonies, it is appropriate to suggest that the terms elementary, intermediate and reticulate bodies be used to describe the different forms of the organism. However, the morphological criteria to define the specific forms of *C. ruminantium* are understandably limited, and the grouping of transitional organisms as either a specific form or intermediate organism can be problematic.

According to Pienaar (1970), heartwater organisms are surrounded by 2 membranes. This concurs with our findings, although a few elementary bodies were enveloped by one membrane. Reticulate and intermediate bodies were occasionally enveloped with an electron-dense layer surrounded by a well-demarcated fine fibrillar layer. Morhpologically, this layer corresponds to the so-called 'capsule' (slime layer) present in many bacteria and rickettsiae (Avakyan & Popov, 1984). Factors responsible for the formation of 'encapsulated' heartwater organisms are unknown. Structurally, these organisms are compatible with bacteria surrounded by a similar capsule and are often referred to as cell-wall deficient forms (Kordova, 1978).

Our study confirmed the findings of Pienaar (1970) that *C. ruminantium* multiplies mainly by binary fission. Furthermore, Pienaar (1970) infrequently observed organisms undergoing unequal division (multiple budding). In the present study, the morphology of a few reticulate bodies indicated replication by means of budding (a process in which small organisms are pinched off from larger organisms.) This may explain the presence of numerous vesicular structures in colonies with reticulate bodies. Budding was reported in chlamydiae by Higashi (1960), cited by Moulder (1962), and Avakyan & Popov (1984). Prozorousky, Beskina, Popov & Barkhatova (1979), cited by Avakyan & Popov (1984), considered budding of chlamydiae as altered (abnormal) forms.

Criteria to differentiate rickettsiae and chlamydiae include morphological parameters (normal anatomy and formation of altered forms), features of interaction with the host cell, such as the localization of the parasite in the cell and characteristics of the developmental cycle (Avakyan & Popov, 1984). Both *Rickettsiaceae* and *Chlamydiaceae* are classified in a special class of obligate intracellular scotabacteria *Intracytobiotes* (Shatkin & Popov, 1982). Furthermore, Avakyan & Popov (1984) suggested that the terms *chlamydia-likeness* or *rickettsia-likeness* should facilitate the differentiation of rickettsia- and chlamydia-like organisms. In view of the available data, the term *chlamydia-like* appears to be appropriate to describe the close association between chlamydiae and *C. runinantium*.

ACKNOWLEDGEMENTS

The authors wish to thank the technicians of the Section of Electron Microscopy for their assistance.

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