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IN VITRO CULTIVATION OF *COWDRIA RUMINANTIUM*

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ABSTRACT

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Cowdria ruminantium was cultivated in a calf endothelial cell line after the cells had been irradiated at 45 & 90 GY. Another experiment in which the inoculum and non-irradiated cells were centrifuged together also yielded positive results. In some irradiated cultures, colonies of organisms could be demonstrated microscopically up to 70 days after the cultures had been inoculated with infected tick stabilate. The infectivity of cultures, even after 4 passages and 88 days post-inoculation*, was demonstrated by their intravenous injection in sheep.

INTRODUCTION

The prospective advantages that would result from the successful *in vitro* cultivation of *Cowdria ruminantium*, the causative agent of heartwater, have been recognized for many years. Antigen obtained in this manner could be used for immunization, serology, attenuation, screening of chemotherapeutics and other studies.

In the past, numerous attempts have been made to grow the organisms *in vitro*, but they have all been characterized by either failure or limited success. The persistence of *C. ruminantium* for up to 13 days in primary kidney cell cultures from reacting goats is the longest period reported for the presence of the organism *in vitro* (Jongejan, Van Winkelhoff & Uilenberg, 1979). There is one report (Haig, 1955) of chicken embryos being infective 9 days after inoculation with infective material, but all attempts at serial passage failed. It has also been reported that primary cell cultures from *Amblyomma hebraeum* ticks were infective 9 days after inoculation with infective blood (Andreasen, 1974). Recently, Sahu, Dardiri & Wool (1983) claimed to have detected some colonies of *C. ruminantium* in leucocyte cultures established from blood of infected goats, sheep and cattle. Unfortunately, the infectivity of these cultures was not tested.

Niewold (unpublished data, 1981, cited by Uilenberg, 1983) found that intestinal material of backless *A. hebraeum* ticks, cultured according to the method of Bell (1980), were infective for a goat after 4 days of incubation.

Other attempts to cultivate the organism, which, however, yielded negative results, are reviewed by Uilenberg (1983). In our laboratory primary cell cultures from *A hebraeum* tissues were difficult to establish and maintain (Bezuidenhout, unpublished results, 1979). Such sensitive and slow-growing cultures, in our opinion, would have limited application for the *in vitro* cultivation of *C. ruminantium*.

We decided to apply some of the methods described for the cultivation of rickettsiae and other similar organisms, such as *Chlamydia*, namely, irradiation of cultures (Gordon, Dressler, Quan, McQuilkin & Thomas, 1972) and centrifugation of inoculum and cells onto each other (Weiss & Dressler, 1960).

This paper describes the results of our experiments.

MATERIAL AND METHODS

Cell culture

A calf endothelial cell culture, known as the E5 cell line, was used. The culture, currently in its 70th passage, was specifically established for the cultivation of *Cowdria* at the Veterinary Research Institute, Onderstepoort (B. J. Erasmus, unpublished results, 1969). Eagle's medium, containing 10 % locally prepared bovine serum, sodium benzylpenicillin (200 i.u./mℓ), streptomycinsulphate (200 μg/mℓ) and Fungizone (Amphotericin B) (2,5 μg/mℓ), was used throughout the experiments.

Cell cultures were propagated at 37 °C in a variety of containers, such as plastic culture flasks (FF)*, tissue culture chambers (TCC)** and 50 mℓ wide-neck glass bottles (GB). The latter contained glass cover slides and were sealed with rubber stoppers. The TCC were incubated in a CO_2 incubator at 37 °C and 5 % CO_2. Medium of all cultures was changed once a week. Using standard techniques, stock and inoculated cultures were divided or passaged at various intervals (Tables 1, 2 & 3).

Inoculum

Tick-derived stabilate, prepared from *Amblyomma hebraeum* nymphae as previously described (Bezuidenhout, 1981) and currently issued as a vaccine against heartwater (Bezuidenhout & Oberem, 1985), was used as an inoculum. This vaccine (Batch 200) contained 1/8 of a nymph infected with the Ball 3 strain of *C. ruminantium* per 2 mℓ dose. Forty mℓ of vaccine was removed from the liquid nitrogen freezer (−196 °C), thawed under tap water and centrifuged in a tube at low speed (2 000 rpm) for 5 minutes at 4 °C. The supernatant was collected and centrifuged at 30 000 × g to pellet the organisms (Oberem, Bezuidenhout, Viljoen, Neitz & Vermeulen, 1984). The resultant pellet was resuspended in medium and the cultures were inoculated at a rate of 2 doses of vaccine per 25 cm^2 flask surface.

Irradiation of cells

Eight 25 cm^2 plastic FF, each with a monolayer of E5 cells (4 days old), were irradiated, using a Cesium source. Four flasks were irradiated at 45 GY and 4 at 90 GY, and their medium was changed daily for the following 2 days. All the cultures were inoculated on the 3rd day after irradiation. After the medium was poured off, a thin layer of inoculum (2 mℓ) was added carefully to the cells, which were then incubated for 1 hour at 37 °C before more medium was added, and then incubated further. Cultures were divided and passaged as illustrated in Tables 1, 2 & 3. Most passages were done on normal non-irradiated cells. However, from 70 days after the initial inoculation of the cultures, passages were again done on irradiated cultures.

Centrifugation

Inoculum was prepared as described above except that the final pellet was not resuspended. Five-day-old E5 cells were trypsinized with Activated Trypsin Versene (ATV) and centrifuged at 1 000 rpm for 10 minutes. The sedimented cells were resuspended in a small volume (2 mℓ) of a medium and carefully pipetted onto the pellet of inoculum in the centrifuge tube. This was then centrifuged at 4 500 × g for 20 minutes at 4 °C to bring about close contact between the cells and the inoculum. The pellet obtained was resuspended in 45 mℓ of medium and transferred to one 25 cm^2 FF and 8 × 50 mℓ GB.

* Since this article went to press, the infectivity of a 113-day-old culture has been demonstrated by sheep inoculation

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* Nunc Laboratory Products

** Flow Laboratory Products

Control cultures

Three flasks of non-irradiated E5 cells were inoculated as described under the irradiation experiment.

Testing for the presence of C. ruminantium *in cultures*

Cultures were examined for the presence of *C. ruminantium* at various days after inoculation or passage (Tables 1, 2, 3, & 4). In the case of the FF, the medium was poured off and the bottom part of the flasks cut up for microscopical examination. In some instances, flasks were held at an angle and about 2/3 of the cell layer carefully trypsinized. After the trypsin was neutralized with medium containing 10 % bovine serum, the detached cells were carefully aspirated with a syringe and a long needle. Such material was used for animal inoculation and/or for passage. The remaining layer of cells on the top third of the flasks was cut out for microscopical examination. When 50 mℓ glass bottles were used coverslips were removed for microscopy. The slide at the base of the tissue culture chambers was used for microscopical examination. In some cases, where the content of the TCC were passaged further, cells were released with ATV, as was previously described.

Microscopic examination

Coverslips from the GB and pieces from the FF obtained, as described above, were carefully rinsed in medium, fixed in methanol and stained with Giemsa (5 % solution for 55 minutes). From time to time the cultures were also examined, using the direct fluorescent antibody technique (Oberem *et al.*, 1984) or the indirect fluorescent antibody technique described by Du Plessis (1981). Light microscopy, immunofluorescence as well as microphotography were used, using a Reichert Polyvar microscope. On 2 occasions, cells from irradiated cultures were examined under the transmission electron microscope. Cells from 26-day-old cultures (45 & 90 GY) as well as from Day 71 (45 GY) were processed for electron microscopy, according to previously described methods (Kay, 1965).

Animal inoculation

The infectivity of some cultures was tested at various stages during the course of the investigations by being injected into sheep (Tables 1, 2, 3 & 4). After decanting the media from such cultures, cells were released using ATV and the action of trypsin neutralized with medium containing 10 % bovine serum. Detached cells and media were then mixed with the corresponding supernatants and injected intravenously into susceptible Merino sheep. Rectal temperatures of the sheep were recorded daily for at least 21 days after inoculation. Reacting sheep were left to die or, in some cases, were sacrificed. Necropsies were performed on dead animals and Giemsa-stained brain smears were examined for the presence of colonies of *C. ruminantium* in endothelial cells (Purchase, 1945). Sheep that did not react or that survived were challenged with 1 dose of tick vaccine.

RESULTS

Irradiated cultures

The differences in morphology between irradiated and non-irradiated cells were not studied in any detail. However, the cells of the irradiated cultures had noticeably larger cytoplasms, were often multinucleated and also showed a much slower rate of division.

The morphology of colonies of *C. ruminantium* in infected cultures was surprisingly similar to that seen in brain smears of heartwater sheep (Jackson, 1931; Pienaar, 1970) and infected ticks (Cowdry, 1925; Bezuidenhout, 1984). Various sizes of intracytoplasmic colonies of organisms were observed in preparations stained with Giemsa and for direct immunofluorescence (Fig. 1 & 2). The majority of colonies consisted of densely packed, small organisms, but other forms were also seen from time to time. Many cells contained more than one and in some cases as many as 15 colonies of *C. ruminantium*. Initially, in unpassaged cultures, only a few cells were parasitized and they showed a rather patchy distribution. However, after the 1st passage the number of infected cells increased noticeably (Fig. 3).

The direct fluorescent antibody test was an excellent diagnostic aid as far as determining the presence of colonies in the cultures is concerned. However, individual organisms could not be distinguished with immunofluorescence.

Forty-five GY irradiated cultures

Tables 1a & 1b summarize the results of the microscopic and animal inoculation studies obtained from cell cultures initially irradiated at 45 GY.

Sheep 6602, injected with the total content of a FF inoculated 11 days previously with tick vaccine, contracted heartwater after an incubation period of 11 days and it subsequently died. Sheep 6448 also died of heartwater after it had received a 26-day-old culture. However, in this case the incubation period was only 5 days, an indication that there had been a remarkable increase in the number of organisms during the additional 15 days allowed for the incubation of the latter culture.

The sheep (3082) that received the contents of a 50 mℓ glass bottle on Day 56 also developed heartwater and died. In this instance, the incubation period was only 4 days. The organisms in this culture had already been passaged twice, i.e., on Days 26 and 46. Five days after Sheep 3082 was infected with the culture described above, 250 mℓ of its blood was collected and injected intravenously into another sheep which contracted heartwater and died. This finding is a further indication that the early temperature reaction shown by sheep 3082 was, in fact, a heartwater reaction.

Thirteen days later, i.e. 69 days post-inoculation, the cells from 2 GB similar to the one injected into Sheep 3082 were mixed and injected into Sheep 5960 and 5968. The coverslips from these GB were stained with Giemsa for microscopic examination, but colonies could not be demonstrated with any certainty. Neither of the 2 sheep injected contracted heartwater, and both were found to be susceptible when challenged. The other GB, seeded on Day 46, was incubated until Day 70, when the coverslip was removed and stained with Giemsa for microscopy. The cells and supernatant were processed for electron microscopy. The FF seeded on day 46 post-inoculation was incubated for another 25 days, i.e., until Day 71, when 2/3 of the cells and all the supernatant were injected into Sheep 5973. However, Sheep 5973 did not contract heartwater and proved to be susceptible when challenged. On Days 74 and 84 cultures were examined microscopically and passaged, as indicated in Table 1a. The cultures were suspicious microscopically, but well-defined colonies could not be distinguished. On Day 88, however, the contents of a large FF were injected into Sheep 6204 which developed symptoms of heartwater, and subsequently died. Necropsy findings were positive for heartwater.

Ninety GY irradiated cultures

The results obtained from these cultures are summarized in Tables 2a & 2b.

Both sheep injected respectively with cultures 11 and 26 days old, died of heartwater. The incubation period in the first sheep (6577) was 11 days and in the second (6364) 6 days, an indication of a marked increase in the number of organisms during the additional 15 days during which the second culture was incubated.

TABLE 1(a) *Cowdria ruminantium* in 45 GY irradiated bovine endothelial cells. Experimental outlay and results of microscopic and animal inoculation studies

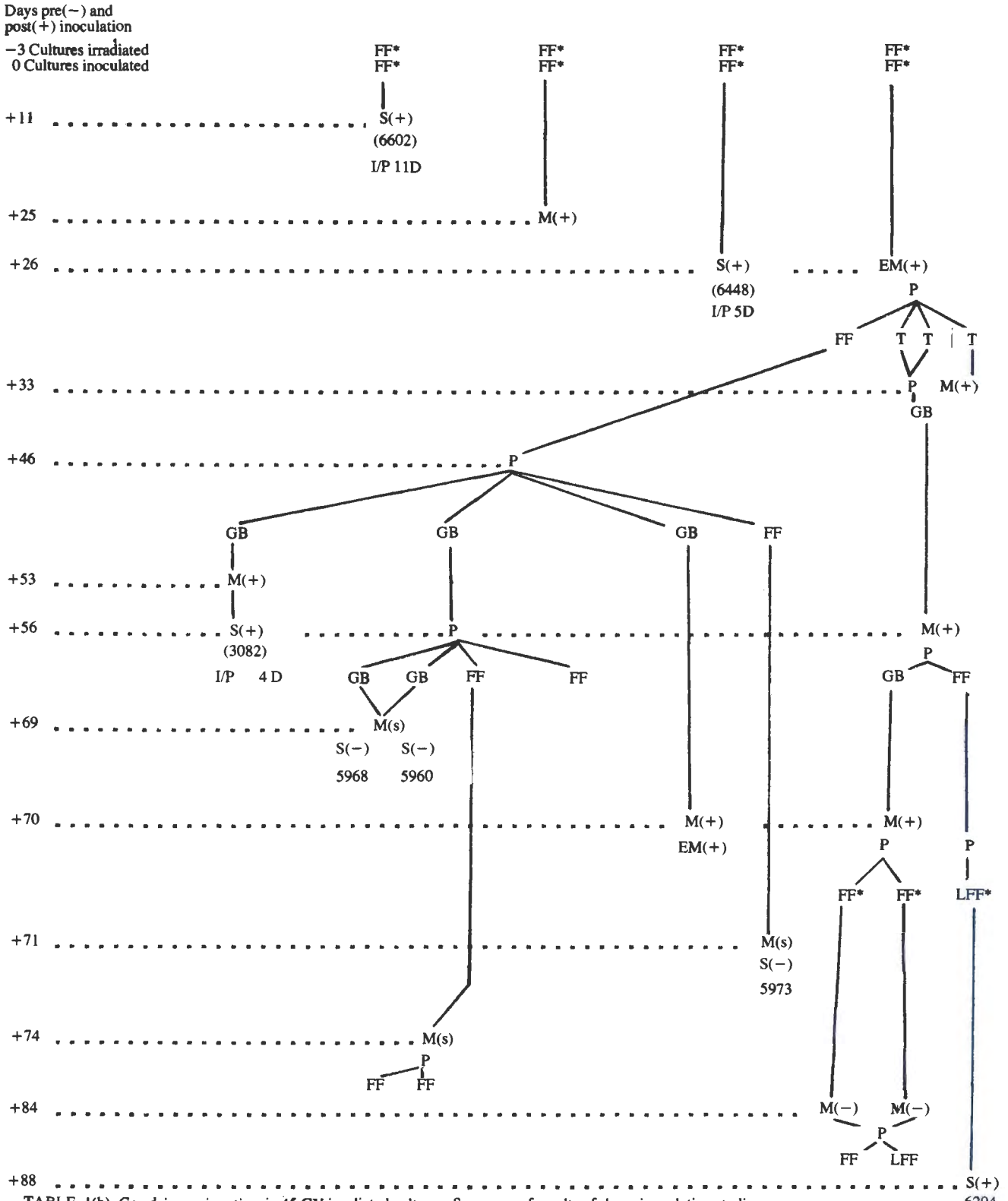


TABLE 1(b) *Cowdria ruminantium* in 45 GY irradiated cultures. Summary of results of sheep inoculation studies

Sheep No.	Days in culture	Inoculation with tissue culture			Challenged with tick stabilate	
		Reaction	I/P days	P.M.	Reaction	Immune status
6602	11	RD	11	HW+	N/A	N/A
6448	25	RD	5	HW+	N/A	N/A
3082	56	RD	4	HW+	N/A	N/A
5968	69	NR	N/A	N/A	RTD	Sc
5960	69	NR	N/A	N/A	RD	Sc
5973	71	SR	4	N/A	RTR	Sc
6204	88	RD	5	HW+	N/A	N/A

TABLE 2(a) *Cowdria ruminantium* in 90 GY irradiated bovine endothelial cells. Experimental outlay and results of microscopic and animal inoculation studies

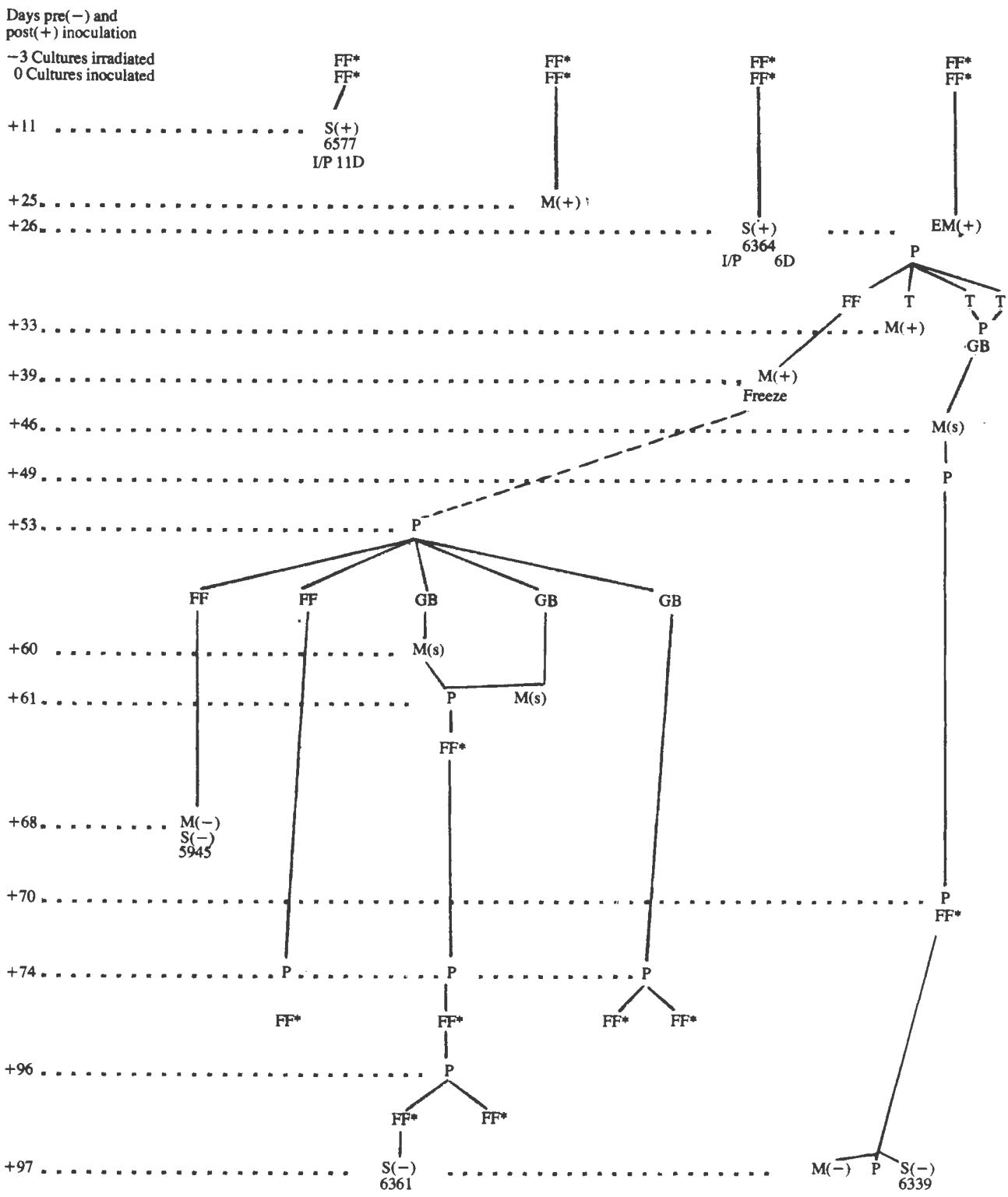


TABLE 2(b) *Cowdria ruminantium* in 90 GY irradiated cultures. Summary of results of sheep inoculation studies

Sheep No.	Days in culture	Inoculation with tissue culture			Challenged with tick stabilate	
		Reaction	I/P days	P.M.	Reaction	Immune status
6577	11	RD	11	HW+	N/A	N/A
6364	26	RD	6	HW+	N/A	N/A
5945	68	NR	N/A	N/A	RD	Sc
6361	97	NR	N/A	N/A	RTD	Sc
6339	97	NR	N/A	N/A	RTR	Sc

TABLE 3(a) *Cowdria ruminantium* in bovine endothelial cells after centrifugation of cells and inoculum onto each other. Experimental outlay and results of microscopic and animal inoculation studies

Days post-inoculation

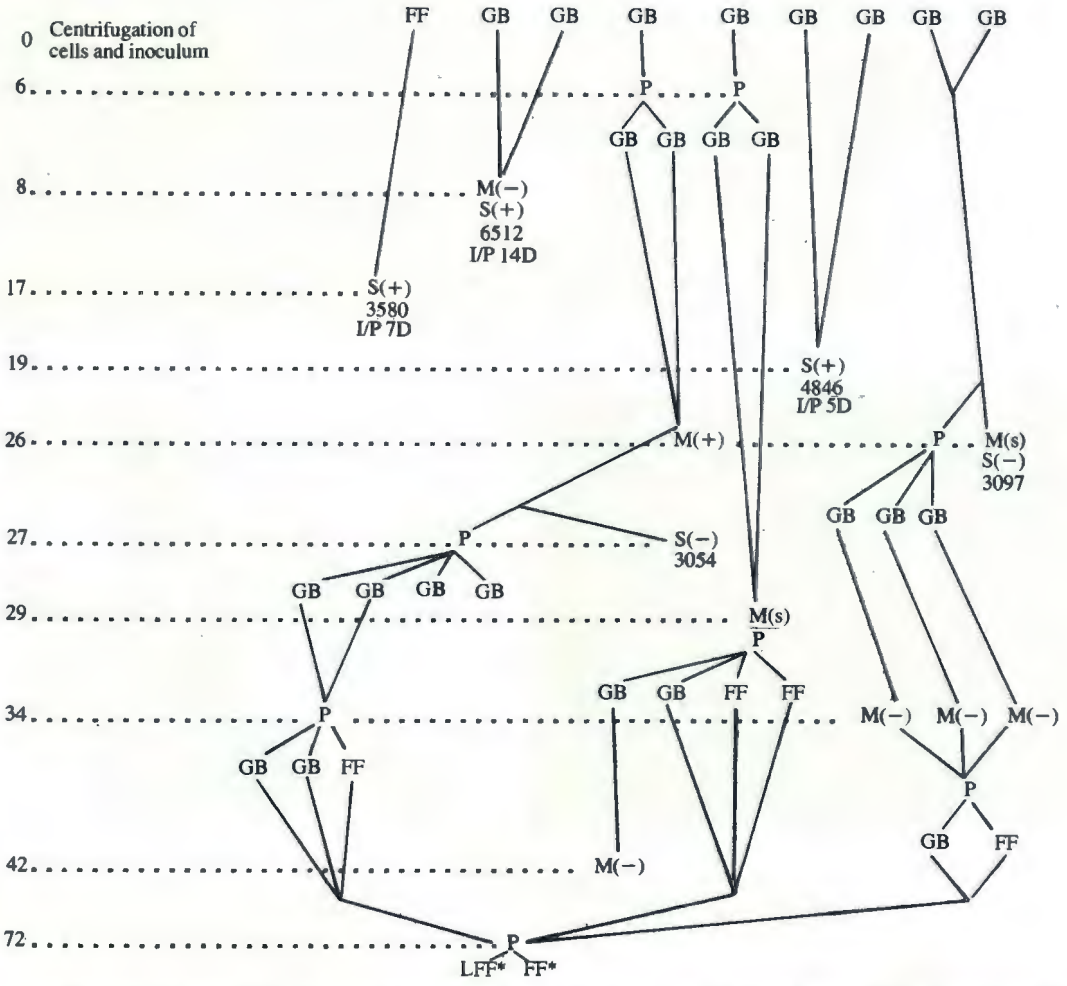


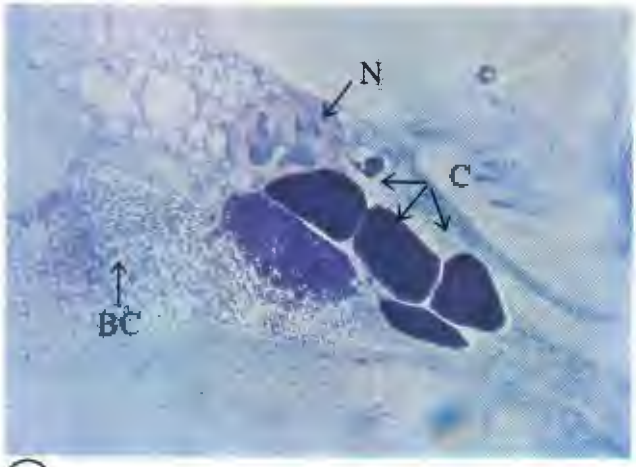
TABLE 3(b) *Cowdria ruminantium* in culture after centrifugation of cells and inoculum onto each other. Summary of results of sheep inoculation studies

Sheep No.	Days in culture	Inoculation with tissue culture			Challenged with tick stabilate	
		Reaction	I/P days	P.M.	Reaction	Immune status
6512	8	RR	14	N/A	NR	I
3580	17	RD	7	HW+	N/A	N/A
4846	19	RD	5	HW+	N/A	N/A
3097	26	NR	N/A	N/A	RTR	Sc
3054	27	SR	16	N/A	RTR	Sc

TABLE 4 Control cultures. Summary of results of sheep inoculation studies

Sheep No.	Days in culture	Inoculation with tissue culture			Challenged with tick stabilate	
		Reaction	I/P days	P.M.	Reaction	Immune status
5849	8	NR	N/A	N/A	RR	Sc
5404	8	NR	N/A	N/A	RR	Sc
6101	8	NR	N/A	N/A	RD	Sc

EM Electron microscopy
FF Plastic culture flask
GB Glass bottle
HW+ Positive for heartwater
I Immune
I/P Incubation period
LFF Large plastic culture flask
M Microscopy
N/A Not applicable
NR No reaction
P Passage
P.M. Post-mortem
RD Reacted, died
RR Reacted, recovered
RTD Reacted, treated, died
RTR Reacted, treated, recovered
S Sheep
(s) Suspicious
Sc Susceptible
SR Slight reaction
T Tissue culture chamber
* Irradiated cells
+ Positive



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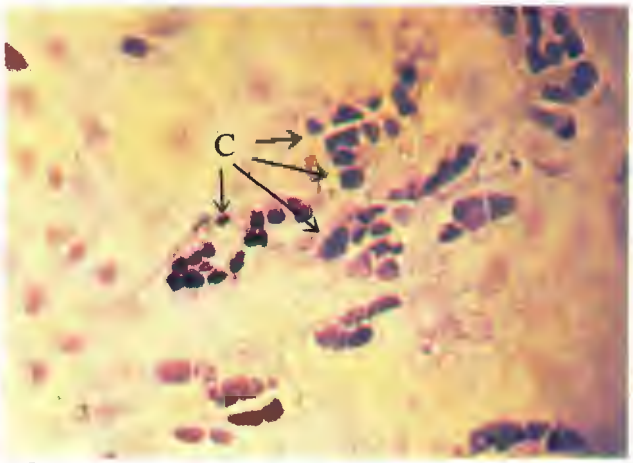

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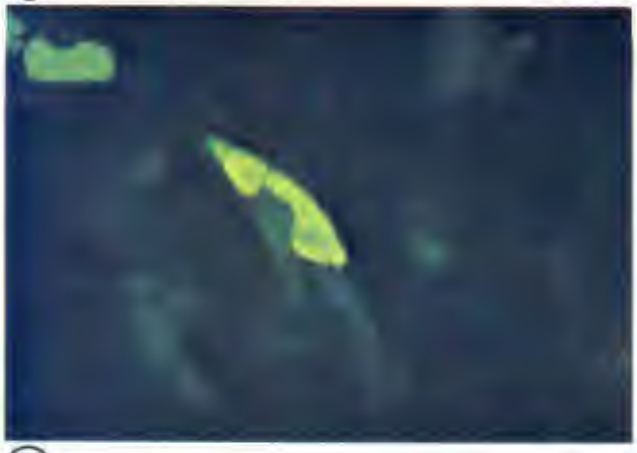

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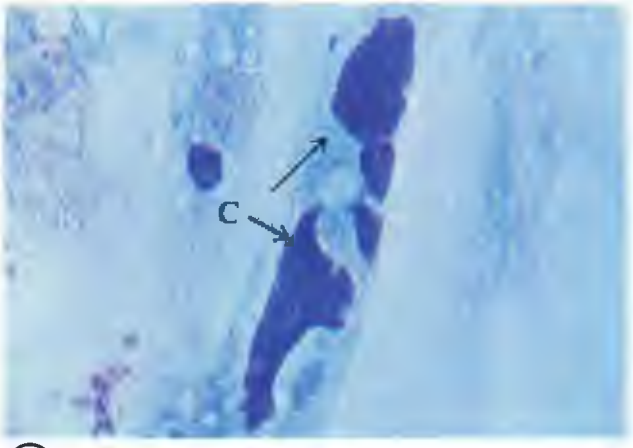

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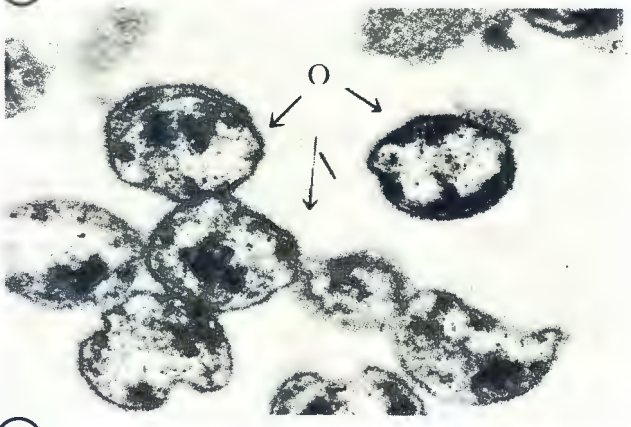

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FIG. 1 Colonies of *Cowdria ruminantium* in an endothelial cell in tissue culture irradiated at 45 GY. Intact colonies (C arrow) and a colony in the process of breaking up (BC arrow) are indicated. The nucleus of the endothelial cell is also indicated (N arrow). Giemsa stain: × 3000

FIG. 2 Numerous intracytoplasmic colonies of *Cowdria ruminantium* in endothelial cell tissue culture irradiated at 90 GY. Giemsa stain: × 1200

FIG. 3 Colonies of *Cowdria ruminantium* in 90 GY irradiated endothelial cells in tissue culture showing specific fluorescence after direct fluorescent antibody staining: ×1200

FIG. 4 Endothelial cells irradiated at 45 GY containing colonies of organisms. A large colony (C arrow) is indicated, corresponding closely with the colony showing specific fluorescence in Fig. 3. Giemsa stain: × 3000

FIG. 5 Electron micrograph of *Cowdria ruminantium* organisms (O arrow) obtained from 71-day-old endothelial cell tissue culture irradiated at 45 GY: × 100 000

Bar scale = 200 nm

The sheep (5945) that was injected on Day 68 with the supernatant and half of the cells of a FF culture, did not develop heartwater and was found to be susceptible to the disease when challenged. On Day 97, 2 sheep, 6361 and 6339, were injected with the supernatant and cells of cultures, as indicated in Table 2a. Neither of the 2 sheep developed heartwater. Colonies of *C. ruminantium* were clearly visible in Giemsa-stained preparations from cultures until Day 39. Thereafter, only inclusions of a suspicious nature were seen. The FF culture harvested on Day 39 showed the highest percentage of infected cells of all the cultures in this study. On the same day, the remaining cells and supernatant of this culture were frozen in liquid nitrogen and stored for a period of 14 days. All the cultures subsequently inoculated with this frozen material were negative for the presence of *C. ruminantium*, both microscopically and biologically.

Centrifugation

The results obtained from centrifuging cells onto the inoculum are summarized in Table 3.

Sheep 6512 was injected with the contents of 2 GB on the 8th day after centrifugation of the cells and inoculum onto each other. This sheep contracted heartwater after an incubation period of 14 days. A 17-day-old culture in a FF was injected into sheep 3580 which developed heartwater after an incubation period of 7 days. Sheep 4846 injected on Day 19 with the contents of 2 GB developed heartwater after a 5-day incubation period. However, neither Sheep 3097 injected on Day 26 nor Sheep 3054 injected on Day 27 contracted heartwater, and both were susceptible when challenged. Microscopy entailed Giemsa staining of coverslips removed from the GB at various intervals, as indicated in Table 3a. How-

ever, organisms could never be demonstrated microscopically with any certainty.

Control cultures

The results of animal inoculation with control cultures are summarized in Table 4. None of the sheep inoculated with control cultures developed symptoms of heartwater, and all proved to be susceptible to challenge with the tick stabilate vaccine. Microscopical examinations were also negative.

Electron microscopy

Electron microscopy was done on cultures irradiated with 45 GY on Days 26 and 70. The culture examined on Day 26 was positive, but the organisms did not exhibit the typical structure and morphology as seen in tick and brain material, whereas the organisms in the Day 70 culture showed up more of the typical characteristics (Fig. 5). However, more detailed studies of the ultrastructure of *Cowdria* in tissue culture will have to be undertaken. Such studies have been planned for the future.

Discussion

It has been possible in these experiments to grow and passage *Cowdria ruminantium* in tissue culture for a minimum of 88 days. It would be difficult to identify all the factors contributing to the successful cultivation of the organism for this length of time. Some of the possible factors are the following:

(1) The E5 endothelial cell line used, apart from being the target cell parasitized by *Cowdria* in the mammalian host, also possesses exceptional phagocytosing properties (B. J. Erasmus, personal communication, 1984). However, the limited experiments done so far indicate that, following standard inoculation methods, non-irradiated E5 cells are not capable of becoming infected. This differs from the findings of previous experiments in which it was found that non-irradiated E5 cells could support the persistence or growth of *C. ruminantium* for 14 days during which period the cells were passaged once (B. J. Erasmus, personal communication, 1984). The cultures were infective for sheep during this period, but the organisms were never demonstrated microscopically with any certainty.

(2) The inoculum used, namely, the tick suspension vaccine, had not previously been used as an inoculum, only infected blood or infected organs. The tick suspension is a more concentrated source of organisms and thus the chances of infecting the cells are greater.

(3) Irradiation and centrifugation are techniques never before attempted in the cultivation of *C. ruminantium*, and in this investigation they appear to have facilitated at least initial growth of the organisms. At this stage, there appears to be little difference between the growth of *Cowdria* that took place in 45 GY and 90 GY irradiated cultures. However, the 90 GY cultures produced fewer viable cells for inoculation purposes. It would also appear that, because centrifugation does not affect cell division in the same way as irradiation does, the easily identifiable colonies of organisms (seen in the irraditated cultures) do not have a chance to develop in these more rapidly dividing cells, and thus microscopic identification is difficult with the centrifugation technique.

Thus, although the reasons why other workers have failed are not clear, it would appear that the source of the inoculum, choice of cell line and techniques of infection are important factors.

Other observations made during the experiments were:

(1) There are indications that multiplication of the organisms took place in tissue culture, i.e. the shortening of the incubation period of the disease in sheep that were inoculated and the increase in the number of colonies during microscopic investigations. The fact that the contents of a 50 mℓ GB, 56 days post-inoculation and after 2 passages, produced heartwater in a sheep with an incubation period of only 4 days is seen as a remarkable finding, as this is the shortest incubation period ever reported for heartwater.

(2) Serial passage of the organisms in culture has been established, but it is impossible to determine whether the increase of parasitised cells after the 1st passage was due to infection of new non-irradiated cells or merely to a survival of the infected irradiated cells in the new cultures. However, the latter is unlikely after 2 or 3 passages, as parasitised cells would not be likely to remain viable for such a long period.

(3) The recently developed direct fluorescent antibody test for the demonstration of *Cowdria* in its vertebrate and invertebrate host (Oberem *et al.*, 1984) proved to be a useful technique in confirming the presence of organisms in culture.

The importance of this study is the fact that cell line, inoculum, media, antibiotics and techniques used are suitable for the *in vitro* cultivation of *C. ruminantium*. If these results are used as a starting point, it may be possible to find better and more efficient methods for the mass production of the organisms in cell cultures.

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References

ANDREASEN, M. P., 1974. Multiplication of *Cowdria ruminantium* in monolayer of tick cells. *Acta Pathologica et Microbiologica Scandinavica*, Section B, 82, 455–456.

BELL, L. J., 1980. Organ culture of *Rhipicephalus appendiculatus* with maturation of *Theileria parva* in tick salivary glands *in vitro*. *Acta Tropica*, 37, 319–329.

BEZUIDENHOUT, J. D., 1981. The development of a new heartwater vaccine, using *Amblyomma hebraeum* nymphae infected with *Cowdria ruminantium*. *Proceedings of the International Conference on Tick Biology and Control*, Grahamstown, Republic of South Africa, 41–45.

BEZUIDENHOUT, J. D., 1984. Demonstration of *Cowdria ruminantium* in *Amblyomma hebraeum* by fluorescent antibody techniques light and electron microscopy. *Onderstepoort Journal of Veterinary Research*, 51, 231–215.

BEZUIDENHOUT, J. D. & OBEREM, P. T., 1985. New vaccine against heartwater. *South African Journal of Science*, 81, 2–3.

COWDRY, E.V., 1925. Cytological studies on heartwater. II. *Rickettsia ruminantium* in the tissues of ticks capable of transmitting the disease. *11th and 12th Reports of the Director of Veterinary Education and Research, Union of South Africa*, 180–196.

DU PLESSIS, J. L., 1981. The application of the indirect fluorescent antibody test to the serology of heartwater. *Proceedings of the International Conference on Tick Biology and Control*, Grahamstown, South Africa, 47–52.

GORDON, F. G., DRESSLER, H. R., QUAN, A. L., McQUILKIN, W. T. & THOMAS, J. I., 1972. Effect of ionising irradiation on the susceptibility of McCoy cell cultures to *Chlamydia trachomatis*. *Applied Microbiology*, 23, 123–129.

HAIG, D. A., 1955. Tick-borne Rickettsioses in South Africa. *Advances in Veterinary Science*, 2, 307–325.

JACKSON, C., 1931. The microscopic diagnosis of heartwater. A preliminary note on the valuc of intima smears. *17th Report of the Director of Veterinary Services and Animal Industry, Union of South Africa*, 1, 161–173.

JONGEJAN, F., VAN WINKELHOFF, A. J. & UILENBERG, G., 1980. *Cowdria ruminantium* (Rickettsiales) in primary goat kidney cell cultures. *Research in Veterinary Science*, 29, 392–393.

KAY, D. H., 1965. Techniques for electron microscopy. Oxford: Blackwell Scientific Publications.

OBEREM, P. T., BEZUIDENHOUT, J. D., VILJOEN, G., NEITZ, A. W. H. & VERMEULEN, N. M. J., 1984. Attempts at the purification of *Cowdria ruminantium* and the development of serological techniques for heartwater. *Proceedings of the XIIIth World Congress on Diseases of Cattle*, Durban, 531–535.

PIENAAR, J. G., 1970. Electron microscopy of *Cowdria (Rickettsia) ruminantium* (Cowdry, 1926) in the endothelial cells of the vertebrate host. *Onderstepoort Journal of Veterinary Research*, 37, 67–78.

PURCHASE, H. S., 1945. A simple and rapid method for demonstrating *Rickettsia ruminantium* (Cowdry, 1925) in heartwater brains. *Veterinary Record*, 57 (3), 413–414.

SAHU, S. P., DARDIRI, A. H. & WOOL, S. H., 1983. Observation of *Rickettsia ruminantium* in leucocyte cell culture from heartwater-infected goats, sheep and cattle. *American Journal of Veterinary Research*, 44 (6), 1093–1097.

UILENBERG, G., 1983. Heartwater *(Cowdria ruminantium* infection): Where do we stand? *Advances in Veterinary Science and Comparative Medicine*, 27, 427–479.

WEISS, E. & DRESSLER, H. R., 1960. Centrifugation of rickettsiae and viruses onto cells and its effect on infection. *Proceedings of the Society for Experimental Biology and Medicine*, 103, 691–695.