A METHOD FOR DETERMINING THE *COWDRIA RUMINANTIUM* INFECTION RATE OF *AMBLYOMMA HEBRAEUM:* EFFECTS IN MICE INJECTED WITH TICK HOMOGENATES

J. L. DU PLESSIS, Veterinary Research Institute, Onderstepoort 0110

ABSTRACT

DU PLESSIS, J. L., 1985. A method for determining the *Cowdria ruminantium* infection rate of *Amblyomma hebraeum:* Effects in mice injected with tick homogenates. *Onderstepoort Journal of Veterinary Research*, 52, 55–61 (1985).

Amblyomma hebraeum ticks, collected in the field and individually homogenized, were injected into mice. Thirteen out of 240 ticks were shown to be infected with the heartwater agent. Antibodies against *Cowdria ruminantium* were detected in the sera of the mice by means of the indirect fluorescent antibody test. Giemsa-stained smears, prepared from the haemocytes of the ticks, revealed morphologically different forms of the heartwater agent.

A strain of *C. ruminantium*, designated the Welgevonden strain, was isolated in mice from one of the infected ticks and passaged in mice for 8 generations. When inoculated intravenously, it was highly infective to mice, sheep and cattle. The murinotropism of the Welgevonden strain is compared with that of other strains previously described.

INTRODUCTION

The 2 most important factors directly related to the immune status of the cattle on a particular farm that determine the role played by the tick vector (*Amblyomma* spp.) in the epidemiology of heartwater are the tick burden and the *Cowdria ruminantium* infection rate of the ticks. The tick load can be ascertained by means of tick counts, and the immune status of the cattle with the aid of the indirect fluorescent antibody (IFA) test (Du Plessis, 1982b). There is as yet no dependable method for assaying the percentage ticks infected by *C. ruminantium*.

The high degree of specificity and sensitivity of the IFA test makes an attempt to identify infected ticks by injecting them into mice and subjecting the sera of the mice to the IFA test a worthwhile investigation.

But strains of *C. ruminantium* appear to vary considerably in their infectivity to mice (Mackenzie & Van Rooyen, 1981; Uilenberg, 1983). Strains such as the Nigerian D225 are entirely non-infective and non-pathogenic, whereas the Ball 3 strain, used for the production of a vaccine at the Veterinary Research Institute, Onderstepoort, is non-pathogenic, but may be passaged in mice once or at the most 2 or 3 times in succession by the intraperitoneal route (K. E. Weiss, personal communication, 1971). The Malagasy K2 strain (Ramisse, cited by Uilenberg, 1983) does not elicit clinical signs in mice, but can be serially passaged indefinitely if the intravenous (i.v.) route is used. Further along the spectrum, the Kwanyanga strain (Mackenzie & Van Rooyen, 1981) is highly pathogenic to mice but only when inoculated intravenously (i.v.) and not intraperitoneally (i.p.), whereas the Kumm strain (Du Plessis & Kumm, 1971; Du Plessis, 1982a), also referred to as the Du Plessis strain (McHardy & Mackenzie, 1984; Uilenberg, 1983), is highly pathogenic to mice irrespective of whether the i.v. or the i.p. route is used.

MATERIALS AND METHODS

Experiment 1

Partially engorged adult *A. hebraeum* ticks of both sexes were collected from cattle throughout the main heartwater endemic areas of the Republic of South Africa as part of a study to determine the role of the tick vector in the epidemiology of the disease.

Five male and 5 female ticks per farm were examined within 3–6 days after collection or after being stored in liquid nitrogen. A preliminary experiment had shown that there was no loss of infectivity to mice of adult *A. hebraeum* ticks, infected as larvae with the Kumm strain

of *C. ruminantium* and stored in liquid nitrogen in an intact state for 2 months (unpublished observation, 1983).

Conventional, outbred, Swiss white mice, 4–6 weeks of age, were used. All procedures were carried out at 4 °C.

Individual ticks were homogenized in 1 mℓ of phosphate buffered saline (PBS) by means of a homogenizer equipped with a glass cylinder and a teflon piston. The homogenates were centrifuged at 250 g for 5 minutes and 0,3 and 0,1 mℓ of the supernatant fluid were inoculated i.p. into 2 mice per tick. Four to 5 weeks later, serum samples were collected from all surviving mice. If both mice that were inoculated with the homogenate from the same tick, survived, their sera were pooled.

Experiment 2

Because of the difficulty of interpreting the trace amounts of antibody detected in mice injected i.p. with the homogenates of some of the ticks suspected of infection in Exp. 1, the homogenates were injected both i.v. and i.p. in a 2nd experiment. Furthermore, following preliminary observations on haemocytes of infected ticks, it was hoped that the serological evidence would be supported by the demonstration of the heartwater agent in the haemocytes of infected ticks. Twenty male and 20 only slightly engorged female adult ticks were therefore collected from cattle on each of 2 farms from which several infected ticks had been obtained during the first collection. It had also been found that few ticks from the first collection on these farms had caused non-specific deaths in mice.

Three to 4 days after collection, the ticks were homogenized in 1,5 mℓ of PBS, as described, and 0,3 and 0,1 mℓ of the supernatant fluid were injected i.v. into 2 mice and i.p. into another 2 mice. Before the ticks were homogenized, smears were prepared from the haemocytes of each tick by nipping off the distal portion of one or more of its legs with a pair of scissors. The exuding haemolymph was spread over the surface of a 1 cm diameter circle drawn with a diamond pencil on a microscope slide as well as over one of the wells of a 15-well multitest slide* for fluorescent antibody staining. The smears on the microscope slide were air-dried, fixed in methyl alcohol, stained for 50 min in 5 % Giemsa at a pH of 7,4 and studied microscopically under oil immersion.

Since inclusions which were morphologically very similar to *C. ruminantium* were detectable in the haemocytes of a tick used in this experiment (Tick 4, Table 4), 0,2 mℓ of its homogenate stored in liquid nitrogen was

* Flow Laboratories

added to 4 mℓ of buffered lactose peptone (BLP) (Du Plessis & Kumm, 1971) and inoculated i.v. into a heart-water susceptible, 6-month-old Merino lamb to determine whether the tick was infected or not.

Monitoring of mice injected with tick homogenates

Mouse mortalities were recorded daily. Those that occurred 1–7 days after inoculation were considered to be non-specific. Mice found dead at a later stage were autopsied and their hearts and lungs fixed in 10 % formalin for the preparation of conventional H & E stained sections.

The mouse given 0,1 mℓ of the homogenate of the tick from which the Welgevonden strain was isolated *(vide infra)* was killed and its peritoneal cells collected for the preparation of Giemsa-stained smears as previously described (Du Plessis, 1982a). Peritoneal cells were also placed on a 15-well multitest slide for fluorescent antibody staining. Specimens of liver and spleen were collected and individually homogenized in BLP on a 10 % mass/volume basis. Two groups of 6 mice were inoculated i.p. with the spleen and liver homogenates at a dosage rate of 0,2 mℓ per mouse.

When the mice showed clinical signs, 3 of them were killed and their spleens, livers and peritoneal cells were collected to carry out the next passage by the same route. Mortalities and clinical signs were recorded and 8 passages performed in this manner. Only mice showing clinical signs were used for successive passages. Peritoneal cells were collected in sterile PBS and the pooled cells from the 3 mice divided into 2 aliquots. Smears for Giemsa and IFA staining were prepared from one of the aliquots and an equal volume of BLP was added to the other for the sub-inoculation of mice in the next generation, 0,3 mℓ being injected i.p. per mouse.

Preparation of Welgevonden strain stabilate

The livers, spleens, hearts and lungs of 8 mice inoculated with infected peritoneal cells at the 6th passage level were pooled and homogenized in BLP. Suitable quantities of the homogenate were stored in liquid nitrogen as an infective inoculum to compare the infectivity of the Welgevonden strain to different animal species. Four serial tenfold dilutions in BLP were prepared from the stabilate and injected into mice, sheep and cattle along the i.v. route at a dosage level of 0,2 mℓ per mouse and 5 mℓ per sheep and bovine. Adult Merino sheep and 15-month-old Afrikander-Simmentaler-cross heifers were used. Early morning temperatures of the sheep and heifers were recorded. All the sheep, except one that was left to die, were treated with a long-acting preparation of oxytetracycline* on the 3rd or 4th day of the febrile reaction. The heifers were not treated. The sheep and heifers were challenged 4 months later by injecting them i.v. with 5 mℓ of sheep blood infected with the Ball 3 strain of *C. ruminantium*.

The mortality rate of the mice was recorded and the infectivity titre was calculated according to the method of Reed & Muench (1938).

Indirect fluorescent antibody test

To detect antibodies to *C. ruminantium* in the sera of the mice injected with the tick homogenates, the IFA test was carried out as previously described (Du Plessis, 1982b), except that antigen was prepared on 15-well multitest slides and stored in liquid nitrogen. The sera were tested first at a dilution of 1:10. Tenfold serial dilutions were subsequently prepared from all positives and the end-point of the reaction was determined in a 2nd test.

* Liquamycin/LA, Pfizer

The pooled serum from several uninfected mice at a dilution of 1:10 and positive serum at a dilution of 1:100, prepared from mice infected and challenged 4 weeks later with the Kumm strain, were included in each test as negative and positive controls.

Optimum reactivity of the anti-mouse conjugate* was ensured by titrating its reactivity against the control positive serum at regular intervals.

Fluorescent antibody staining of haemocytes and peritoneal cells

The indirect fluorescent antibody technique was used to determine the identity of the *C. ruminantium*-like inclusions in the haemocytes and peritoneal cells. The cells were fixed by immersion of the slides in cold methyl alcohol. A drop of the IFA test positive control mouse serum at a dilution of 1:100 was placed on each well containing the cells. Incubation, washing and staining of the cells with anti-mouse conjugate was carried out in the conventional manner.

RESULTS

Experiment 1

It can be seen from Table 1 that, whereas one or both mice died 1–7 days after inoculation with the homogenates of only 7 male ticks, those injected with the homogenates of 39 females died during this interval. Half, to almost fully engorged, females were chiefly but not exclusively responsible for these non-specific per-acute deaths which invariably occurred during the first 2 days after inoculation and which were usually confined to the ticks collected on specific farms.

Apart from the tick from which the Welgevonden strain was isolated *(vide infra)*, the mice inoculated with 0,3 mℓ homogenate of 2 female ticks died 11–14 days later. Because of the presence of post-mortem changes no attempt was made to isolate *C. ruminantium* from these mice, but typical colonies of the heartwater agent were demonstrable in histological sections of their lungs and myocardium. The mice injected with 0,1 mℓ of homogenate of these ticks did not show any clinical signs, but were serologically positive to titres of 1:100 and 1:1000. In Table 1, these 2 infected ticks are recorded under specific mouse mortalities and not under the serologically positive mice.

It can also be seen from Table 1 that the mice injected with one other male and one female tick failed to show clinical signs, but had high levels of serum antibodies. The serum from mice injected with 3 male and 2 female ticks reacted positively in the IFA test at a dilution of only 1:10.

Isolation of the Welgevonden strain. The mouse, injected with 0,3 mℓ of homogenate of a male tick collected on the farm Welgevonden in the district of Pretoria, died 14 days later. Typical colonies of the heartwater agent were demonstrable in histological sections of its lungs and myocardium. The mouse given 0,1 mℓ of homogenate showed clinical signs of depression, a ruffled haircoat and dyspnoea, and was killed *in extremis*.

The mortality rate of mice infected i.p. over the course of 8 serial passages with tissues from this mouse is shown in Table 2. It can be seen that passage of the agent in liver and spleen was readily achieved along the i.p. route for 4 generations of mice, but then changed abruptly at the 5th passage level, when only 1 out of 6 mice inoculated with spleen died. Subinoculation of peritoneal cells along the i.p. route, however, consistently resulted in almost 100 % mortality of the injected mice. No attempt was made to pursue subinoculations beyond the 8th passage level.

* Miles-Yeda Ltd

TABLE 1 The number of ticks injected i.p. that elicited fatal infection and serum antibodies in mice

Number of ticks inoculated		Ticks that caused mouse mortalities		Ticks to which mice became serologically +ive: Serum dilutions	
		Specific	Non-specific	1:10	1:100 and higher
Males	100	1*	7	3	1
Females......	100	2	39	2	1
Total	200	3	46	5	2

* Tick from which Welgevonden strain was isolated

TABLE 2 Pathogenicity of Welgevonden strain passaged in mice by the i.p. route

Passage level	Number of mice inoculated			Mortality/ morbidity	Incubation period in days
	Liver	Spleen	Peritoneal cells		
1	6			4	12
		6		2	15
2	6			2	16
		6		5	15
3	6			6	13
		6		6	14
			5 ,	5	14
4	6			6	15
		6		6	18
			5	4	13
5	6			0	
		6		1	15
			5	5	12
6			5	5	12
7			5	5	12
8			5	5	10

Mice, sheep and cattle infected i.v. with the Welgevonden strain. The infectivity of the Welgevonden strain injected i.v. into mice, sheep and cattle is shown in Table 3. It is evident that by this route the strain is highly infective to mice, since it attained a titre of $10^{5,4}$ which even surpasses some of the highest titres recorded with the Kumm strain infected i.p. (Du Plessis, 1982a).

It can also be seen from Table 3 that the strain is highly infective to sheep and cattle. A sheep inoculated i.v. with the infective inoculum diluted to 10^{-4} developed a fatal reaction. A brain squash smear prepared from this animal showed numerous typical colonies of *C. ruminantium* in the cytoplasm of capillary endothelial cells. The 3 sheep that recovered failed to show any febrile reaction when they were challenged with Ball 3 blood, thus proving the immunogenicity and identity of the strain.

One of 2 heifers infected with a 10^{-2} dilution of the inoculum died, whereas the 2nd one and 3 others injected with higher dilutions developed severe febrile reactions, but recovered without treatment. They showed some or all of the following clinical signs: anorexia, depression, grinding of the teeth, inco-ordination, nystagmus and hypersensitivity. A brain smear prepared from the animal that died was positive for heartwater. All 4 heifers that recovered were solidly immune to challenge with Ball 3 blood.

Experiment 2

Antibodies were detected in the serum of 8 out of 40 ticks collected on 2 farms (Table 4). It is evident that in the case of Ticks 1 and 4 significantly higher levels of antibodies were recorded in the sera of mice injected i.v. than in mice inoculated i.p. Two other homogenates, those of Ticks 3 and 8, elicited low levels of antibody by the i.v. and no response by the i.p. route. In the case of

the other 4 ticks, only trace amounts of antibody were recorded, irrespective of the route of inoculation. The intravenous route therefore appears to have only some advantage over the intraperitoneal route.

Great value was attached to the observations made on Tick 4. None of the mice, not even the one injected i.v. with 0,3 mℓ of homogenate, showed any clinical signs, and yet high antibody titres were recorded in their sera. The lamb injected with the same homogenate, however, developed acute, fatal, heartwater. The febrile reaction commenced 9 days after inoculation, attained a maximum temperature of 42 °C on the following 2 days and was followed by death another 2 days later. At autopsy a mild oedema of the lungs and mild splenomegaly were the only lesions observed. The brain smear, however, revealed numerous medium to large typical colonies of *C. ruminantium.*

Appearance of C. ruminantium *in Giemsa-stained peritoneal macrophages and tick haemocytes*

Giemsa-stained smears prepared from the peritoneal cells of the mouse infected with the tick homogenate from which the Welgevonden strain had been isolated as well as those from cells of mice killed over the course of 8 passages, revealed inclusions which were indistinguishable from those previously described in mice infected with the Kumm strain (Du Plessis, 1975).

Various morphologically district forms of the parasite were seen in the peritoneal macrophages. No particular effort was made to study the sequential development of the inclusions, but strongly basophilic, homogeneous, round bodies of high density (Fig. 1, 2 & 4, arrows) appeared to be the earliest developmental stage seen in the cytoplasm of peritoneal macrophages. These bodies apparently assume a vaguely granular appearance prior to developing into the next stage, in which the inclusions consist of circumscribed agglomerations of granules interspersed by what appear to be as yet undivided fragments of the dense body (Fig. 3 & 4). A 3rd form of parasitic inclusion were well-circumscribed, intracytoplasmic colonies, consisting of clearly distinguishable individual organisms (Fig. 5).

Giemsa-stained smears prepared from the haemocytes of the 8 ticks that elicited an antibody response in the mice injected with them, revealed inclusions in haemocytes (Table 4) which closely resembled those described in the mouse peritoneal macrophages. Here too, several morphologically distinct forms of what appear to be the heartwater agent were demonstrable in the haemocytes. Dense bodies (Fig. 6, 7 & 8) were those seen most frequently, and they could be distinguished from well-circumscribed morular colonies (Fig. 9 & 10) containing granular but as yet undivided fragments and individual organisms. These closely resembled the organisms seen in colonies of *C. ruminantium* in brain capillary endothelial cells in size, shape and staining affinity. In what appears to be a 3rd form of intracellular development, the morular colonies disintegrate to give rise to clumps of organisms (Fig. 11) or to yet smaller clumps and single particles distributed throughout the cytoplasm of the haemocyte (Fig. 12).

The detection of *C. ruminantium* inclusions in tick haemocytes is difficult and time-consuming because, firstly, only a small percentage of cells are infected. (On an average 200–600 haemocytes per tick were examined, 1–2 % of which were found to be parasitized.) Secondly, the haemocytes of many ticks examined were invaded by commensal organisms of many different shapes and sizes. The great majority of these organisms are extracellular, but those occurring intracellularly can only with difficulty be differentiated from the final stage of the

A METHOD FOR DETERMINING THE *COWDRIA RUMINANTIUM* INFECTION RATE OF *AMBLYOMMA HEBRAEUM*

TABLE 3 Infectivity of the Welgevonden strain to mice, sheep and cattle by the i.v. route

		Reactions to infection: Inoculum dilution					Treatment/outcome	Reactions to challenge
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}		
Mice		10/10	10/10	10/10	10/10	8/10[(1)]		
Sheep	1	7/8/41,6[(2)]					Treated, recovered	None
	2		8/7/41,3				Treated, recovered	None
	3			9/8/41,9			Treated, recovered	None
	4				12/4/42		Not treated/died	—
Heifers....	1		12/7/41,2				Not treated/died	—
	2		14/7/40,9				Not treated/recovered	None
	3			12/7/40,7			Not treated/recovered	None
	4			13/9/40,8			Not treated/recovered	None
	5				16/5/40,6		Not treated/recovered	None

[(1)] 8 out of 10 mice inoculated died
Titre of infectivity in mice $10^{5,4}$
[(2)] The febrile reaction commenced on Day 7, lasted 8 days and attained a maximum of 41,6 °C

TABLE 4 Correlation between the serological response to tick homogenates in mice and *C. ruminantium* in the haemocytes of the ticks

Tick No.		Mouse mortality/morbidity		IFA titres of mice		Parasitic form in haemocytes		
Male	Female	i.v.	i.p.	i.v.	i.p.	Dense bodies	Morular colonies	Disintegrated colonies
1		—	—	1:1000	1:10	—	—	+
2		—	—	1:10	1:10	+	+	+
3		—	—	1:10	—	+	—	—
	4	—	—	1:1000	1:100	+	+	+
	5	—	—	1:10	1:10	+	—	—
	6	—	—	1:10	1:10	+	—	—
	7	—	—	1:10	1:10	+	—	—
	8	—	—	1:10	—	+	—	—

heartwater agent in which the morular colonies become disintegrated. The commensals in various shades of blue tend to be bacillary in shape and not coccoid or coccobacillary like *C. ruminantium,* which stains a typical deep mauve. Dense bodies and morular colonies can much more readily be differentiated from commensal organisms.

Several haemocytes from Tick 4, which caused fatal heartwater in a lamb, contained good examples of the 3 forms of *C. ruminantium* inclusions just described. It can also be seen from Table 4 that the cells of 1 other tick (Tick 2) harboured all 3 forms of the parasite, whereas dense bodies unaccompanied by the other forms were detected in those of 5 others. Only low levels of antibody were detectable in the sera of the mice injected with these 6 ticks.

Immuno-fluorescence of infected peritoneal macrophages and tick haemocytes

Fluorescent antibody staining of both mice peritoneal macrophages and tick haemocytes revealed inclusions showing specific fluorescence, and both in numbers and morphology closely resembled those seen in Giemsa-stained smears. Dense bodies were seen as intensely fluorescent homogeneous structures. Inclusions consisting of poorly-circumscribed fluorescent clumps of varying size and in varying degrees of confluence were also seen. What appeared to be fully developed colonies were seen as a fluorescent mosaic consisting of densely packed, clearly distinguishable individual organisms in the form of minute ringlets, the fluorescence appearing to be concentrated on the envelopes of the particles.

DISCUSSION

The findings in this study suggest that the detection of antibodies in the serum of mice injected with tick homogenates, in conjunction with the demonstration of parasitic inclusions in the haemocytes of the ticks, can be used as a method to determine the *C. ruminantium* infection rate of ticks. The correlation between the serological response of the mice and the observation of inclusions in the tick haemocytes was good, since not one tick with parasitized haemocytes failed to elicit an antibody response and vice versa. While appreciable levels of antibody were recorded in the serum of mice injected with some of the tick homogenates, others induced the production of only trace amounts of antibody, even if they were injected i.v. Particularly in the case of the latter, the demonstration of parasitic inclusions in the tick haemocytes thus provides valuable supportive evidence.

Taking into account the tick from which the Welgevonden strain was isolated and counting only the ticks that induced moderate to strong serological reactions in mice, 2,5 % of the ticks in Exp. 1 were infected. If the low antibody levels recorded are also taken into consideration, 5 % of the ticks were infected. In Exp. 2 the infection rate was 20 %, but the selection of the ticks was biased.

The morphologically different forms of the parasitic inclusions in tick haemocytes closely resembled those observed in the peritoneal macrophages of mice infected with the Welgevonden strain of *C. ruminantium*. Their near identity was confirmed with immuno-fluorescence and positive brain smears in a sheep and a heifer. They were also indistinguishable from the inclusions described in lymph node reticulo-endothelial cells of sheep infected with the Ball 3 and Kumm strains (Du Plessis, 1970), as well as in peritoneal macrophages of mice infected with the Kumm strain (Du Plessis, 1975).

The demonstration of these inclusions in the haemocytes of a particular tick, the homogenate of which caused fatal heartwater in a lamb injected with it, is supportive evidence that they represent developmental forms of the heartwater agent. Specific immuno-fluorescence of the inclusions in the haemocytes of this tick and several others confirmed their identity with *C. ruminantium*.

The results of attempts to induce clinical *C. ruminantium* infection in sheep and mice by the i.v. inoculation of haemolymph collected from experimentally infected ticks have been inconsistent (Bezuidenhout, 1984). Spe-
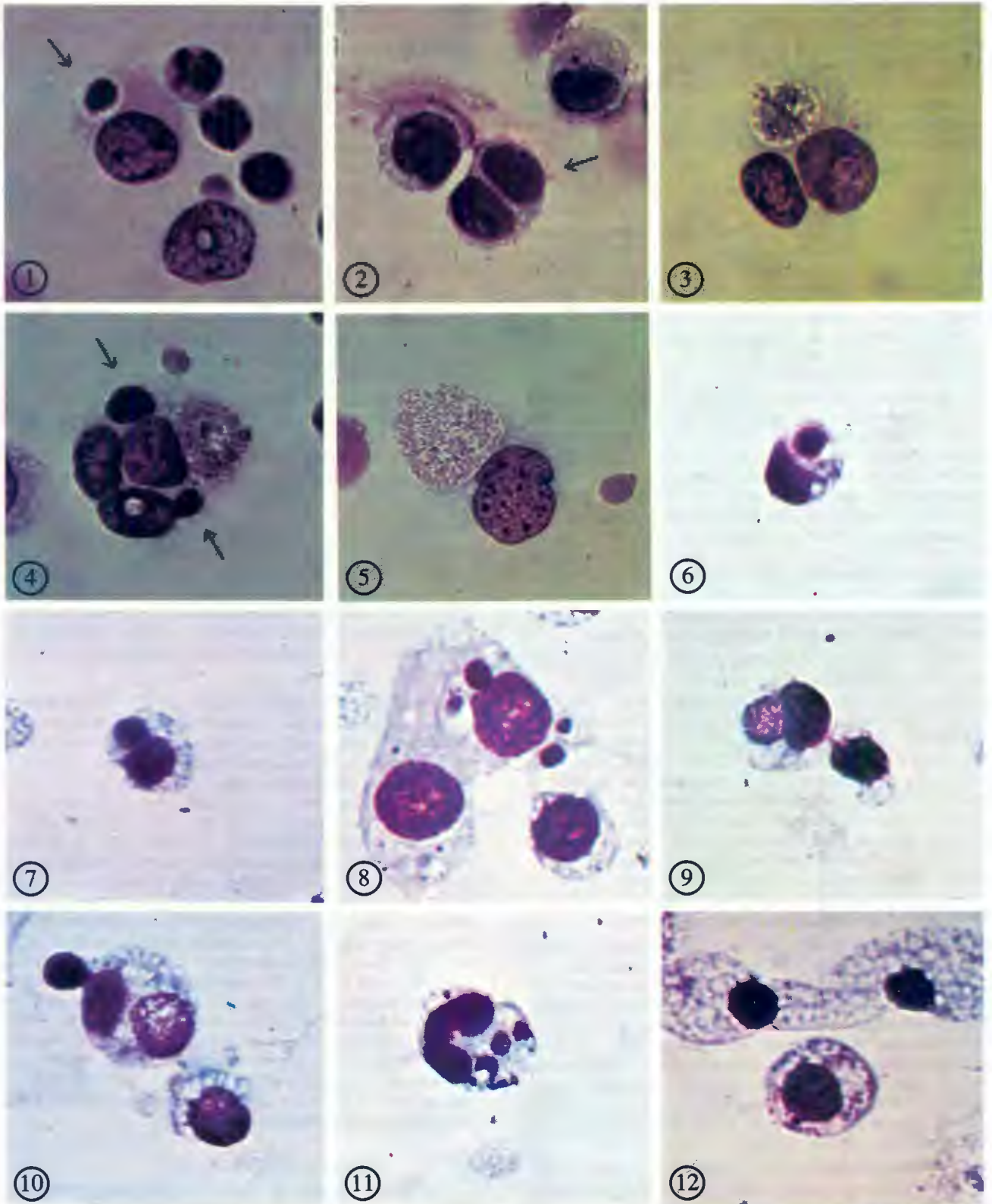
FIG. 1–12 *C. ruminantium* inclusions in mouse peritoneal macrophages (Fig. 1–5) and tick haemocytes (Fig. 6–12):
    × 1000, Giemsa

FIG. 1 & 2. Dense body stage (arrows) in 2 macrophages

FIG. 3. Colony of organisms in process of division

FIG. 4. Multi-nucleated macrophage with 2 dense bodies (arrows) and a colony in process of division

FIG. 5. Colony of fully developed particles

FIG. 6 & 7. Dense bodies in haemocytes

FIG. 8. Haemocyte with 3 dense bodies and another in process of division

FIG. 9 & 10. Morular colonies in process of division

FIG. 11. Clumps of particles in process of division

FIG. 12. Particles distributed throughout cytoplasm of haemocyte

cific antibodies, however, have been detected with the IFA test in the sera of some of these animals (J. D. Bezuidenhout, personal communication, 1984). The failure of the inoculum to cause clinical disease may have been due either to insufficient numbers of infective organisms or to the fact that the heartwater agent in the haemocytes was in a non-infective stage of development at the time of collection and inoculation.

Mackenzie & Van Rooyen (1981) and Uilenberg (1983) have stated that there appears to be a whole spectrum of pathogenicity to mice of strains of *C. ruminantium*. The present study confirmed this phenomenon and revealed the variation in murinotropism of the strains of the heartwater agent harboured by the 13 ticks that were found infected—5 ticks in Exp. 1 that elicited an appreciable antibody response in mice, and 8 ticks in Exp. 2.

At the one end of the spectrum, the Welgevonden strain proved to be highly pathogenic to mice, not only when they were infected by the i.v. route, but also during the early passages even when they were infected i.p. At the other end of the spectrum, the strains harboured by the 6 ticks in Exp. 2, the homogenates of which merely elicited the development of low levels of antibody in the mice injected with them, were only mildly murinotropic. Apart from antibodies detected with the IFA test in these mice, the demonstration of the parasite in haemocytes of the ticks proved that they were, in fact, infected. The other 6 occupy an intermediate position, their homogenates having elicited high antibody levels in the absence of clinical signs in the mice inoculated with them. Two of them, however, had caused a fatal infection in the mice injected at the higher dosage level.

Only 1 out of the 13 infected ticks in this study would have been identified if the mortality of mice injected with the ticks had been the only parameter. This emphasizes the necessity of employing other techniques to determine *C. ruminantium*-infection rates of *Amblyomma* ticks. Thus Barré & Camus (1983) found that not 1 out of 15 strains of *C. ruminantium* isolated in goats and sheep from *Amblyomma variegatum* ticks collected on the Caribbean island of Guadeloupe caused clinical signs or fatal infection in mice inoculated both i.v. and i.p. It may even be necessary to employ additional techniques as for example fluorescent antibody-stained, frozen sections of ticks (Bezuidenhout, 1984), to determine the infection rate more accurately.

The detection of only trace amounts of antibody in the mice injected with the mildly murinotropic strains is consistent with their low pathogenicity, since antibody levels detectable with the IFA test in domestic ruminants vary according to the severity of their reactions to artificial infection with the heartwater agent. High antibody titres are recorded in goats, sheep and susceptible cattle breeds that develop severe reactions, whereas more resistant cattle breeds that only develop mild to moderate febrile reactions have low levels of antibody (unpublished observation, 1983).

The Welgevonden strain isolated from an *A. hebraeum* male was highly pathogenic to mice if injected i.v. The sudden decrease in its infectivity to mice infected i.p. cannot be explained, but it is not unprecedented. The Kwanyanga strain (Mackenzie & Van Rooyen, 1981) also killed only a few of the mice injected i.p. and even appeared to lose its pathogenicity to mice infected i.v. after 15 or 19 sheep passages (P.K.I. Mackenzie, personal communication, 1983).

Although only 8 serial passages of the Welgevonden strain was carried out in mice, it was already clear that continued passaging would be possible, provided that peritoneal cells are injected by the i.p. and other tissues by the i.v. route. This observation is consistent with the finding that the Kumm strain can be readily passaged by the i.p. route with peritoneal cells, even if they are harvested from donor mice as early as 1 day after being infected. Passaging with organ suspensions from donor mice, though, could only be effected 5 days and more after infection of the donor mice (Du Plessis, 1982a). The explanation advanced here was that peritoneal cells, in contrast to emulsified organs, remain intact and viable during subinoculation and can therefore support the uninterrupted growth of the parasite even though it may be in a non-infective state of development when the cells are collected. This, however, does not explain the loss of infectivity of the Kwanyanga and Welgevonden strains injected i.p.

The Welgevonden strain has several characteristics in common with the Kwanyanga strain. Apart from both being highly infective to mice by the i.v. route, both strains are also pathogenic to sheep and cattle. According to limited cross-immunity tests, though, their immunogenic relationship to the Ball 3 strain is somewhat different. Whereas there appears to be complete cross-immunity between the Welgevonden and the Ball 3 strains in sheep, that between the Kwanyanga and the Ball 3 strains is not fully protective (Mackenzie & Van Rooyen, 1981).

The Kumm strain remains the most pathogenic strain passaged i.p. in mice to date, and differs from the Welgevonden strain not only in that it is consistently infective to mice injected i.p., but also in its very mild pathogenicity to cattle (Du Plessis, 1982a). The Welgevonden strain therefore occupies a position in the spectrum of murinotropism much closer to the Kwanyanga than to the Kumm strain.

In assessing the murinotropism of *C. ruminantium* strains, variation in the susceptibility to the heartwater agent of the strains of mice injected with material possibly infected with the agent should be borne in mind. McHardy & Mackenzie (1984) found that BALB/C inbred mice were more susceptible to the Kwanyanga strain of *C. ruminantium* than the CD-1 outbred and 4 other inbred strains (CBA/CA, C3H/HE, C57 BL/6 and DBA_2), but that there was no difference in the susceptibility of these 6 strains of mice to the Kumm strain. In a recent experiment the infectivity titre, calculated as described above, of a given infective inoculum to AKR inbred mice was $10^{3,3}$, whereas that recorded in BALB/C and C57 BL/6 inbred mice was $10^{2,5}$. In the outbred strain of mice used in the present study on ticks, however, an infectivity titre of $10^{3,2}$ was recorded with the same inoculum (unpublished observation, 1984). Although slight differences in the susceptibility of mouse strains may play a minor role in pathogenicity studies on *C. ruminantium*, such differences are unlikely in the present study, since the strain of mice used was no less susceptible than several others examined.

REFERENCES

BARRÉ, N. & CAMUS, E., 1983. Étude épidémiologique de la cowdriose (heartwater) aux Antilles. Rapport annuel, 1983, I.E.M.V.T., Mission Antilles-Guyane.

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, 213–215.

DU PLESSIS, J. L., 1970. Pathogenesis of heartwater: I. *Cowdria ruminantium* in the lymph nodes of domestic ruminants. *Onderstepoort Journal of Veterinary Research*, 37, 89–96.

DU PLESSIS, J. L. & KUMM, N. A. L., 1971. The passage of *Cowdria ruminantium* in mice. *Journal of the South African Veterinary Medical Association*, 42, 217–221.

DU PLESSIS, J. L., 1975. Histopathological studies on the pathogenesis of heartwater as manifested in mice infected with a strain of *Cowdria ruminantium*. M. Med. vet. thesis, University of Pretoria.

DU PLESSIS, J. L., 1982a. Mice infected with a *Cowdria ruminantium*-like agent as a model in the study of heartwater. D.V.Sc. thesis, University of Pretoria.

DU PLESSIS, J. L., 1982b. The effect of intensive tick control on the immunity of cattle to heartwater. *Proceedings of Symposium on Ectoparasites of Cattle*, Pretoria, 1982, 18–27.

MACKENZIE, P. K. I. & VAN ROOYEN, R. E., 1981. The isolation and culture of *Cowdria ruminantium* in albino mice. *Proceedings of International Congress on Tick Biology and Control*, Grahamstown, 1981, 33–39.

McHARDY, N. & MACKENZIE, P. K. I., 1984. Virulence of two strains of *Cowdria ruminantium* in mice and their use to predict drug activity against heartwater. *Tropical Animal Health and Production*, 16, 56–62.

REED, L. J. & MUENCH, H., 1938. A simple method of estimating fifty per cent endpoints. *American Journal of Hygiene*, 27, 493–497.

UILENBERG, G., 1983. Heartwater (*Cowdria ruminantium* infection): Current status. *Advances in Veterinary Science and Comparative Medicine*, 27, 427–480.