Onderstepoort J. vet. Res., 54, 497–506 (1987)

THE EFFICACY OF ALTERNATIVE ROUTES FOR THE INFECTION OR VACCINATION OF ANIMALS WITH *COWDRIA RUMINANTIUM*

J. D. BEZUIDENHOUT[1], J. A. OLIVIER[1], B. GRUSS[2] and J. V. BADENHORST[1]

ABSTRACT

BEZUIDENHOUT, J. D., OLIVIER, J. A., GRUSS, B. & BADENHORST, J. V., 1987. The efficacy of alternative routes for the infection or vaccination of animals with *Cowdria ruminantium*. *Onderstepoort Journal of Veterinary Research*, 54, 497–506 (1987).

The efficacy of subcutaneous, intramuscular and intraperitoneal inoculation of heartwater infective blood or nymph suspensions was tested in 5 experiments involving a total of 199 sheep. The success rate of subcutaneous injections varied greatly (0–100 %) in the different groups. However, it was found that certain additives to the inoculum, such as dimethyl sulphoxide or uninfective brain tissue, increased the efficacy of the subcutaneous route. Indications are that the site of inoculation and especially the dose volume are important factors in the success rate of such inoculations. Intramuscular injection with infective nymph suspensions containing bradikynin or hyaluronidase produced heartwater reactions in 11 out of 14 sheep. In 2 experiments, involving 25 cattle, it was found that, although very few animals showed definite reactions after the subcutaneous or intramuscular injection of nymph suspension and additives, the majority were afterwards immune to challenge. This phenomenon, which was also present in some sheep, needs further investigation.

INTRODUCTION

Artificial infection or vaccination of animals with heartwater-infected material is usually effective only when it is carried out intravenously. Most farmers, though, find it difficult to give intravenous inoculations, and this is probably the main reason for the limited use of the present heartwater vaccine. An easier route for vaccination should promote the use of the vaccine, which should in turn result in a higher degree of immunity amongst the livestock populations presently at risk. Furthermore, a different route of inoculation may prevent the shock reactions that sometimes occur after either single or repeated intravenous injections of blood (Fick & Schuss, 1952; Barnard, 1953) or tick suspensions (Bezuidenhout & Oberem, 1985; J. D. Bezuidenhout, unpublished data, 1986).

With these problems in mind it is not surprising that a number of previous workers have attempted to inject or vaccinate animals with *Cowdria ruminantium* infective material using alternative routes of inoculation. Their results, plus those of the present study, are summarized in Table 7.

Ticks secrete certain salivary components during feeding, some of which assist them to attach, to develop a feeding lesion or to release themselves from the host (Moorhouse & Tatchell, 1966; Binnington, 1978; Binnington & Kemp, 1980).

Some substances that may be present in tick salivary glands, such as histamine, hyaluronidase, prostaglandines and bradikynin-like substances (Neitz & Vermeulen, 1987), affect the cell membrane. This results in increased permeability of the bloodvessels and thus plays a role in the distribution and uptake of tissue fluids (Howell, Neitz & Potgieter, 1975; Chinery & Ayitey-Smith, 1977). We therefore decided to add some of these and other similar substances, as well as carrier drugs such as dimethyl sulphoxide (DMSO), to the inoculum in an attempt to facilitate the subcutaneous and intramuscular uptake of organisms.

The subcutaneous injection of infective brain material will usually set up a heartwater reaction (Anonymous, 1952; Ilemobade, 1976; Uilenberg, 1983). This interesting finding was taken a step further by using non-infective brain material as an additive for subcutaneous inoculation.

Experiments presented in this study were conducted in both sheep and cattle, and the results of 1 experiment were often used when planning the next.

EXPERIMENTS WITH SHEEP

Five different experiments were conducted with Merino sheep 6–9 months old. They were obtained from an area in the Republic of South Africa which is free of heartwater and were kept under tick-free conditions during the course of the experiments.

Routes of vaccination

Subcutaneous injections were given in the side of the neck. The only exception was in Experiment 5 where sheep were inoculated on the inside of the back leg. Intramuscular injections were given in the semimembranous muscle *(Muscularis semimembranosus)*. When the dose volume exceeded 7 mℓ, it was divided into 2 halves, each of which was injected into a different site. Intraperitoneal inoculations were given according to the method of Hurter (1987).

Monitoring of heartwater and immune reactions

Rectal temperatures were recorded daily for at least 25 days after injection or intravenous (i.v.) challenge with 5 mℓ blood vaccine. Some reacting animals were treated with oxytetracycline at a dosage rate of 10 mg/kg, while others were left untreated. Autopsies were performed on all dead animals and a diagnosis of heartwater was confirmed by the demonstration of *C. ruminantium* organisms in Giemsa-stained brain smears.

Experiment 1

The object was to determine the effect of certain additives, at different concentrations, on the efficacy of the subcutaneous and intramuscular routes of injection.

Infective inoculum. Heartwater infective nymph suspensions prepared from *Amblyomma hebraeum* ticks as described by Bezuidenhout (1981) were used. This *C. ruminantium* infected material (Ball 3 isolate) was stored in liquid nitrogen (−196 °C). Shortly before use it was thawed under running tap water. The different substances were then added to 2 mℓ of nymph suspension and sheep were inoculated subcutaneously or intramuscularly (Table 1).

Additives

Bradikynin[1]*:* Bradikynin triacetate powder was added in quantities ranging from 0,05–2,0 mg per dose immediately before injection into animals.

[1] Veterinary Research Institute, Onderstepoort, 0110

[2] Hoechst, Animal Health Division, P.O. Box 1758, Port Elizabeth 6000

Received 10 June 1987—Editor

[1] Sigma, P.O. Box 4508, St Louis, MO 63178, USA

TABLE 1 Heartwater and immune reactions in sheep after subcutaneous (s.c.) or intramuscular (i.m.) administration of infected *A. hebraeum* suspensions with certain additives

Additive and concentration (mg)	Route	Concentration of ticks in inoculum	Sheep No.	Heartwater reaction	Incubation period (days)	Interval (days)	Immunity test	
							Heartwater reaction	Immune status
Bradikynin								
0,05	i.m.	0,5	7963	Rr	9	36	NR	I
0,05	i.m.	0,5	8020	SRr	13	36	SRr	I
0,5	i.m.	0,5	7983	Rr	11	36	NR	I
0,1	i.m.	0,5	8052	Rr	11	36	NR	I
0,5	i.m.	1	7316	Nr	.	36	NR	I
0,5	i.m.	1	7322	RD	12		.	.
1,0	i.m.	1	7251	RD	13		.	.
2,0	i.m.	1	7162	NR	.	36	NR	I
0,5	s.c.	1	7248	NR	.	36	RTr	S
0,5	s.c.	1	5694	NR	.	36	RTr	S
1,0	s.c.	1	5787	NR	.	36	RTr	S
2,0	s.c.	1	7254	NR	.	36	RTr	S
Histamine								
0,1	s.c.	1	5672	NR	.	36	RTr	S
0,1	i.m.	1	6497	NR	.	36	RTr	S
0,01	s.c.	1	6489	NR	.	36	RTr	S
0,01	i.m.	1	6530	NR	.	36	RTr	S
Controls								
	s.c.	1	7287	NR	.	36	RTr	S
	i.m.	1	7277	NR	.	36	D	.
Hyaluronidase								
1,0	s.c.	2,5	4683	NR	.	36	RTr	S
10,0	s.c.	2,5	4690	NR	.	36	RTr	S
100,0	s.c.	2,5	4679	NR	.	36	RTr	S
100,0	i.m.	1	4763	NR	.	36	RTr	S
Hyalase								
(150 I.U.)	s.c.	0,1	6207/2	NR	.	150	RTr	S
(15 I.U.)	s.c.	0,1	4093/1	NR	.	42	RTr	S
(150 I.U.)	i.m.	0,1	6174/8	RTr	10	42	NR	I
(15 I.U.)	i.m.	0,1	3458/3	RTr	10	42	NR	I
Combinations								
Hist. (2,5 mg) + Hyal. (2,5 mg)	i.m.	0,5	7995	SRr	11	36	NR	I
Hyal. (1,0 mg) + Brad. (0,5 mg)	i.m.	0,5	7993	Rr	11	36	NR	I
Hyal. (1,0 mg) + Brad. (0,05 mg)	i.m.	0,5	7963	Rr	9	36	NR	I
Uninfected brain								
1,8	s.c.	0,5	7625	RD	14	36	.	.
1,8	s.c.	0,5	8090	Rr	12	36	NR	I
1,8	i.m.	0,5	8098	Rr	19	36	SRr	I
1,8	i.m.	0,5	7957	NR	.	36	Rr	S
Controls								
	i.m.	0,5	8092	Sr	11	36	NR	I
	i.v.	0,5	7992	RD	11	.	.	.

Rr = reacted, recovered
NR = no reaction
SRr = suspicious reaction, recovered
RD = reacted, died
RTr = reacted, treated, recovered
SR = suspicious reaction
D = died of another cause
I = immune
S = susceptible
I.U. = international units

Hyaluronidase: Two different formulations, namely hyaluronate glyconohydrolase[2] powder (250 USP units/mg) and hyalase[3], were used. Different quantities (mg) were mass-measured and added to the nymph suspensions.

Histamine[2]: Histamine dihydrochloride in quantities of 0,01 and 0,1 mg was added to some nymph suspensions.

Combinations of additives: Three sheep were injected with nymph suspensions which contained different combinations of the above-mentioned additives (Table 1).

Brain tissue: The brain of a healthy sheep, which had never been in contact with heartwater, was obtained from the abattoir at the Institute. Part of this brain was mass-measured and homogenized in phosphate buffered saline (PBS). Every 5 mℓ of the emulsion contained 1,8 g of brain material and this volume was added to 2 mℓ of infective tick suspension prior to inoculation into sheep.

Results (Table 1)

Bradikynin

Of the 8 sheep that were injected intramuscularly with nymph suspension to which bradikynin had been added (0,05–2 mg) 6 reacted, of which 2 died. None of the

[2] E. Merck, D-6100 Darmstadt, West Germany

[3] Fisons Pharmaceuticals (Pty) Ltd, Chloorkop 1624, R.S.A.

TABLE 2 Heartwater reactions and immune status of sheep after the injection of different volumes of infective blood containing 5 % DMSO and using different routes of inoculation

Route	Volume of blood (mℓ)	Sheep No.	Reaction	Incubation period (day)	Immune status
Subcutaneous	5	5081/8	RD	18	*
	5	5041/6	RD	13	*
	5	5038/5	RD	13	*
	5	5165/6	SR	20	S
	5#	5113/7	NR	*	d
	5#	5123/3	RD	12	*
	5#	5093/0	RD	13	*
	5#	5170/7	RD	13	*
	15	5101/5	RD	15	*
	15	5118/6	Rr	18	I
	15	5163/5	NR	*	S
	15	5036/9	SR	15	S
Intraperitoneal	5	5110/3	NR	*	S
	5	5146/7	NR	*	S
	5	5029/8	NR	*	S
	5	5150/5	SR	20	S
	5#	5125/88	RD	5	*
	5#	5102/3	NR	*	S
	5#	5073/9	NR	*	S
	5#	5083/4	NR	*	S
	15	5014/9	NR	*	S
	15	5034/4	NR	*	S
	15	5023/1	NR	*	S
	15	5011/9	NR	*	S
Intramuscular	15	5031/0	RD	12	*
	15	5126/6	Rr	12	I
	15	5044/0	SR	19	I
	15	5077/0	NR	*	I
Intravenous	5	5121/7	RD	10	*
	5	5166/8	RD	11	*
	5#	5079/6	RD	12	*
	5#	5105/6	RD	9	*

RD = reacted, died
NR = no reaction
d = died of another cause
* = not applicable
# = brain tissue (1 gm) added
Rr = reacted, recovered
SR = suspicious reaction
I = immune
S = susceptible

sheep were treated and after challenge the 6 survivors, even the 2 that did not react initially, were found to be immune.

None of the 4 sheep that were injected subcutaneously reacted and after challenge all were found to be susceptible to heartwater.

Histamine

The vaccination of sheep with nymph suspensions to which histamine had been added was unsuccessful: none of the sheep reacted, regardless of whether they were injected intramuscularly or subcutaneously. All the sheep in this group were found to be susceptible when challenged.

Hyaluronidase

Two of the 3 sheep injected intramuscularly with nymph suspensions containing hyaluronidase reacted and both died. None of the 5 sheep injected subcutaneously reacted and all survivors were found to be susceptible when challenged (Table 1).

Combinations of additives

All 3 sheep injected intramuscularly with combinations of the above-mentioned additives reacted, recovered spontaneously and were immune when challenged.

Uninfective brain

Both sheep that were injected subcutaneously reacted, recovered spontaneously and were found to be immune after challenge. Only 1 of the 2 sheep that were injected intramuscularly reacted. After challenge this sheep (8098) was found to be immune while the other was susceptible.

Injection without any additives

The sheep injected intravenously reacted and died of heartwater. Of the 2 sheep injected intramuscularly 1 showed a suspicious reaction, and was immune when challenged, while the other (7277) died 5 days after challenge from another cause. The subcutaneous control did not react and was susceptible when it was challenged.

Experiment 2

The object was to compare the suitability of the subcutaneous (s.c.), intraperitoneal (i.p.) and to some extent the intramuscular (i.m.) route for the infection or vaccination of sheep with infective blood. At the same time the effect of brain tissue on the success rate of such injections was tested.

Infective inoculum

C. ruminantium infective blood (Ball 3 isolate) as issued by this Institute as a vaccine (Oberem & Bezuidenhout, 1987) was used. In some cases brain tissue was added to the inoculum. A brain, similar to that described above, was homogenized without any diluent. One gram of this brain pulp was added to every 5 mℓ of infective

TABLE 3 Results of Experiment 3 in which sheep were injected subcutaneously with different volumes of infected blood (with and without the addition of 5 % DMSO)

Volume of blood	DMSO (5 %) present	Sheep No.	Reaction	Incubation period (days)	Immune status after challenge
1,5 mℓ	Yes	5010/1	Rr	12	I
	Yes	5115/2	NR	*	S
	Yes	5007/5	NR	*	S
	Yes	5129/0	NR	*	S
	No	4844/1	RD	13	*
	No	5084/2	NR	*	S
	No	5165/0	NR	*	I
	No	5099/7	Rr	14	I
5 mℓ	Yes	5096/3	RD	16	*
	Yes	5043/2	NR	*	S
	Yes	5052/9	Rr	13	I
	Yes	5013/5	RD	12	*
	No	5008/8	RD	10	*
	No	5071/3	NR	*	S
	No	5124/1	NR	*	S
	No	5106/4	NR	*	S
10 mℓ	Yes	5116/0	RD	10	*
	Yes	5168/4	RD	19	*
	Yes	5129/2	Rr	13	I
	Yes	3936/2	RD	10	*
	No	5019/2	NR	*	S
	No	5112/9	NR	*	S
	No	5015/0	NR	*	d
	No	5100/7	NR	*	S
1,5 mℓ	Yes	5006/2	RD	7	*
i.v. control	No	5066/6	RD	10	*

Rr = reacted, recovered
NR = no reaction
RD = reacted, died
S = susceptible
I = immune
d = died due to another cause
* = not applicable

blood and mixed well (using a syringe and 14 G needle) before injection.

Thirty-two sheep were injected by different routes as set out in Table 2. The infected inoculum was injected in volumes of 5 mℓ (with or without brain material) or 15 mℓ.

Results

Seven of the 12 sheep injected subcutaneously with different volumes of blood containing either 5 % DMSO or blood-brain combinations reacted and died of heartwater. Of the remaining 5 sheep, 1 reacted but recovered spontaneously; 2 showed suspicious temperature reactions, and 2 did not react. When these 5 survivors were challenged, 3 were found to be susceptible to heartwater and died. One of those that had shown a suspicious temperature reaction previously reacted again but it survived without treatment. One sheep (5113/7) died 5 days after challenge from another cause.

Of the 12 sheep that were injected intraperitoneally with blood or blood-brain combinations, only 1 (5150/5) showed a slight temperature reaction on Day 20 while another 5125/8) died 5 days after injection from another cause. After challenge, 4 weeks later, all 11 of the survivors in this group were found to be susceptible; 6 died while 5 recovered without treatment.

Of the 4 sheep injected intramuscularly 2 reacted (of which 1 died); 1 showed a suspicious reaction on Day 19, and 1 did not react. All 3 survivors were immune when challenged.

All 4 intravenously injected controls reacted and died.

Experiment 3

The unexpected success obtained with the subcutaneous route in the previous experiment led to a comparison between the methods of Alexander (1931) and the current techniques used for the preparation of heartwater-infective blood. Apart from the differences in strains, volume of blood and diluents, DMSO, which is present in today's vaccine, was not used in Alexander's time. This led to the suggestion that DMSO could have been responsible for the successful subcutaneous infections. There had also been some difference in the rate of success according to whether 5 mℓ or 15 mℓ blood was used. It was decided that these 2 aspects should be investigated further.

Twenty-six sheep were used, divided into 6 groups of 4 sheep plus 2 individual control sheep. Thirteen sheep (including 1 control) received blood containing 5 % DMSO and the other 13 (including 1 control) blood without DMSO. The 2 control sheep were injected with 1,5 mℓ blood intravenously, while the other sheep were injected with either 1,5 mℓ, 5 mℓ or 10 mℓ blood subcutaneously.

Results (Table 3)

Blood containing 5 % DMSO injected subcutaneously caused primary reactions in 8 of the 12 sheep, of which 5 died and the other 3 were immune when challenged. Blood without DMSO injected subcutaneously caused reactions in only 3 of the 12 sheep. The 1 sheep that did not show a primary reaction was found to be immune when challenged.

With regard to the dose volume, in the group that received blood containing 5 % DMSO 1 of the 4 sheep injected subcutaneously with 1,5 mℓ, 3 of the 4 injected with 5 mℓ, and all 4 of the sheep that received 10 mℓ of blood, reacted.

In the groups injected with blood without DMSO 2 of the 4 sheep injected with 1,5 mℓ, 1 of the 4 that received

TABLE 4 Results of an experiment in which the effect of DMSO, dose volume and concentration of organisms/dose on the efficacy of subcutaneous injections was determined

Concentration of organisms	Dose volume	DMSO in nymph suspension								Total							
		0 %		5 %		10 %		15 %									
	(mℓ)	R	I	R	I	R	I	R	I	R	I						
Single dose $^{1}/_{10}$ nymph/dose	2	$^{0}/_{2}$	$^{0}/_{2}$	$^{3}/_{4}$	$^{3}/_{3}$	$^{0}/_{4}$	$^{2}/_{4}$	$^{4}/_{4}$	$^{4}/_{4}$	$^{7}/_{14}$	$^{9}/_{13}$	R	$^{13}/_{28}$	(46,4)			
	10	$^{0}/_{2}$	$^{0}/_{2}$	$^{3}/_{4}$	$^{4}/_{4}$	$^{2}/_{4}$	$^{1}/_{3}$	$^{1}/_{4}$	$^{1}/_{4}$	$^{6}/_{4}$	$^{6}/_{13}$	I	$^{15}/_{26}$	(56,6)	R	$^{25}/_{56}$	(44,6)
Double dose $^{1}/_{5}$ nymph/dose	4	$^{0}/_{2}$	$^{0}/_{2}$	$^{1}/_{4}$	$^{3}/_{4}$	$^{2}/_{4}$	$^{3}/_{4}$	$^{2}/_{4}$	$^{4}/_{4}$	$^{5}/_{14}$	$^{10}/_{14}$	R	$^{12}/_{28}$	(42,8)	I	$^{31}/_{53}$	(58,4)
	10	$^{0}/_{2}$	$^{0}/_{2}$	$^{2}/_{4}$	$^{1}/_{3}$	$^{2}/_{4}$	$^{3}/_{4}$	$^{3}/_{4}$	$^{2}/_{4}$	$^{7}/_{14}$	$^{6}/_{13}$	I	$^{16}/_{27}$	(59,2)			
Group totals		$^{0}/_{8}$	$^{0}/_{8}$	$^{9}/_{16}$	$^{11}/_{14}$	$^{6}/_{16}$	$^{9}/_{15}$	$^{10}/_{16}$	$^{11}/_{16}$								
DMSO groups				R $^{25}/_{48}$ (52,0)			I $^{31}/_{45}$ (68,8)										
All groups				R $^{25}/_{56}$ (44,6)			I $^{31}/_{53}$ (58,4)										

R = number of animals/number in group, that reacted after inoculation
I = number of animals/number in group that were immune to challenge
( ) = percentage

5 mℓ and none of those that received 10 mℓ of infective blood subcutaneously contracted heartwater.

Both control sheep that were injected intravenously with infective blood, whether or not it contained DMSO, reacted and died.

Experiment 4

After the nymph vaccine became available commercially (Bezuidenhout & Oberem, 1985), it was decided to determine the effect of the DMSO concentration, dose volume and concentration of organisms per dose on the success rate of subcutaneous injections with nymph suspensions. Fifty-six sheep were used and injected subcutaneously with the nymph vaccine prepared according to the method of Bezuidenhout (1981).

Results (Table 4)

These are in many ways erratic, which makes it difficult to evaluate them. It is clear, though, that none of the 8 sheep which were injected with nymph suspension without the addition of DMSO reacted. After challenge all 8 were found to be fully susceptible to heartwater. In contrast 25 (52 %) out of 48 sheep reacted after they had been vaccinated with nymph suspensions that did contain DMSO.

Furthermore, when challenged 32 (69,5 %) of the 46 sheep that survived the initial vaccination were immune to heartwater.

With reference to the different concentrations of DMSO used, 80 % of the sheep that received 5 % DMSO, 60 % of those that received 10 % DMSO and 68,8 % of those that were injected with suspensions containing 15 % DMSO were immune when challenged.

When only the dose volume is taken into account, 82,6 % of the sheep which were injected with a low volume (2–4 mℓ) containing DMSO were immune as opposed to 54,5 % of those which received a high volume (10 mℓ).

With regard to the concentration of nymphae per dose (excluding the 0 % DMSO group) 62,5 % of the sheep injected with 1/10 of a nymph were immune against 64 % that received 1/5 of a nymph (double dose).

Experiment 5

The results of Experiment 4 indicated that subcutaneous vaccination with a single dose of 2 mℓ nymph vaccine containing 1/10 nymph per dose and 5 % DMSO might be effective. The efficacy of such a vaccination was therefore tested in 50 sheep. As the wool on these sheep was very long they were not injected in the neck area, but high up on the inside of the thigh. Unfortunately the results of this experiment were very disappointing. Only 3 sheep (6 %) showed a primary reaction and when challenged 5 (10 %) were found to be immune.

Experiments with Cattle

Two experiments were conducted with cattle in which the subcutaneous and intramuscular infection routes were tested. In the 1st experiment 9 Friesian calves, 1–2 months old, and in the 2nd experiment 16 Afrikander/Bonsmara crosses, 6–27 months old, were used.

Routes of injection

Subcutaneous injections were given into the side of the neck while intramuscular injections were given into the medial gluteus muscle *(Muscularis gluteus medius)*.

Monitoring of heartwater and immune reactions

Reactions after injection or challenge were monitored as described under the experiments with sheep.

Additives

Histamine (2,5 mg) plus *hyaluronidase* (2,5 mg) were added to suspensions containing concentrations ranging from 1,0–4,0 infective nymphae per dose. These were injected intramuscularly into 2 animals in each experiment (Tables 5 & 6).

Brain tissue, prepared as described under the 1st experiment with sheep, was mixed with nymph suspension and injected subcutaneously into 3 calves in the 1st experiment.

Oil-based adjuvant. One part of nymph suspension prepared in PBS which contained 1 % Tween 80 was emulsified with 2 parts oil (1 part Arlacel C plus 9 parts Buyol F). This mixture, which contained 4 nymphae per 5 mℓ dose, was injected subcutaneously into 4 animals and intramuscularly into another 4 animals in the 2nd experiment.

Unvaccinated control animals were kept in both groups in order to compare their immunity with that of the survivors in both experiments. Two animals in the 2nd experiment were also injected intramuscularly with nymph suspension which contained no additives.

Results

In the 1st experiment, in which 9 young Friesian calves were used (Table 5), only the 3 calves that were

TABLE 5 Heartwater and immune reactions in calves after intramuscular (i.m.) and subcutaneous (s.c.) administration of nymph suspensions with certain additives

Additive	Route	Concentration of nymph suspension	Cattle No.	Age (months)	Heartwater reaction	Interval (days)	Immunity test	
							Heartwater reaction	Immune status
Hist. + hyal.	i.m.	1	3398	1	Rr (II)	110	NR (IV)	I
Hist. + hyal.	i.m.	1	3397	1	Rr (II)	110	Rr (III)	I
Brain	s.c.	1	3354	1	NR	126	Rr (III)	I
Brain	s.c.	1	3304	1	NR	110	SRr (III)	I
Brain	s.c.	1	3403	1	NR	110	Rr (II)	S
None	s.c.	1	3400	1	NR	110	Rr (III)	I
None	s.c.	1	3399	1	RTr (II)	110	NR (IV)	I
None	.	.	3402	1	.	110	RTr (II)	S
None	.	.	3401	2	.	110	Rr (III)	I

Rr = reacted, recovered
NR = no reaction
SRr = suspicious reaction, recovered
RTr = reacted, treated, recovered
( ) = type of heartwater reaction according to Du Plessis & Bezuidenhout (1979)

TABLE 6 Heartwater and immune reactions in cattle after subcutaneous (s.c.) or intramuscular (i.m.) administration of infective *A. hebraeum* suspensions with certain additives

Additive	Route	Concentration tick/dose	Cattle No.	Age (months)	Heartwater reaction	Incubation period	Interval (days)	Immunity	
								Heartwater reaction	Immune status
Hist. + hyal.	i.m.	4	4337	12	NR (IV)		30	NR (IV)	I
Hist. + hyal.	i.m.	4	3925	12	SRr (III)	16	30	NR (IV)	I
Oil adj.	i.m.	4	3888	12	NR (IV)	.	30	NR (IV)	I
	i.m.	4	4080	12	NR (IV)	.	30	RTr (II)	S
	i.m.	4	4224	6	NR (IV)	.	30	SRr (III)	I
	i.m.	4	4371	10	NR (IV)	.	30	RD (I)	S
	s.c.	4	4091	10	NR (IV)	.	54	NR (IV)	I
	s.c.	4	4376	9	NR (IV)	.	54	SRr (III)	I
	s.c.	4	3484	27	NR (IV)	.	54	SRr (III)	I
	s.c.	4	4095	8	SRr (III)	10	54	NR (IV)	I
Controls									
None	i.m.	4	3926	12	SRr (III)	15	30	NR (IV)	I
None	i.m.	4	4082	12	SRr (III)	18	30	NR (IV)	I
	.	.	3481	12	.	.	.	RTr (I)	S
	.	.	3832	12	.	.	.	Rr (II)	S
	.	.	3833	12	.	.	.	RTr (I)	S
	.	.	3889	12	.	.	.	RTr (II)	S

NR = no reaction
SRr = suspicious reaction, recovered
RTr = reacted, treated, recovered
RD = reacted, died
I = immune
S = susceptible
Rr = reacted, recovered
( ) = type of heartwater reaction according to Du Plessis & Bezuidenhout (1979)
Hist. = Histamine
Hyal. = Hyaluronidase

injected intramuscularly with nymph suspensions showed a primary reaction. None of the 3 calves that were injected subcutaneously with nymph suspension and uninfected brain material or the calf that received only nymph suspension subcutaneously showed primary reactions.

When they were challenged 3,5 months later, though, it was found that 7 animals were immune. Only 1 of the 2 unvaccinated controls, and 1 calf that was injected subcutaneously with nymph suspension mixed with brain material, showed high temperature reactions. The control animal was then treated.

In the 2nd experiment, in which 16 older cattle were used (Table 6), only 4 animals (of which 3 had been injected intramuscularly) showed slight temperature reactions after 10–18 days. Two of these animals had

been injected intramuscularly with nymph suspension without any additive. Another had received nymph suspension with histamine and hyaluronidase and the 4th had been injected subcutaneously with nymph suspension in an oil adjuvant.

When they were challenged 30–54 days later 10 animals were immune, but the 4 unvaccinated controls, as well as one that was injected subcutaneously and another that received an intramuscular injection with oil adjuvant, were susceptible.

DISCUSSION

As far as is known, heartwater is the only animal disease where vaccination is practised and where intravenous inoculation is a prerequisite for successful vaccination. Other live blood vaccines, such as those against babesiosis or anaplasmosis, are effective when administered subcutaneously.

These studies have shown that the chances for successful subcutaneous or intramuscular infection with heartwater are perhaps not as remote as previously thought. It is also encouraging to learn that the efficacy of these routes can be increased by the use of certain additives. For example, the subcutaneous injection of 10 mℓ of infective blood which contained 5 % DMSO was found to be effective in 4 out of 4 sheep, whereas none of the 4 sheep that received 10 mℓ of blood without DMSO reacted. In Experiment 3, in which nymph suspensions were used, DMSO also played a major role in increasing the efficacy of the subcutaneous route. However, these results suggest that, besides DMSO, the dosage volume also had a definite influence on the success rate of subcutaneous inoculation. This has greatly complicated the interpretation of the results.

The results of experiments in which DMSO was used as an additive indicate that the subcutaneous infection of animals can sometimes be very effective (up to 100 %). In other instances it was completely ineffective. The reasons for these erratic results are not understood at present.

The incubation period of the disease varies considerably when the subcutaneous route is used (10–20 days), which is a further disadvantage. This variation is probably due to differences in the number of viable organisms that are taken up by individual animals. In the case of intravenous injection it is well known that the length of the incubation period is indirectly proportional to the number of viable organisms injected (Ilemobade, 1976). Unless the incubation period can be standardized, it will be very difficult to decide on a specific day for the block treatment of animals after vaccination.

The addition of uninfective brain tissue to the inoculum was successful in most sheep that were subcutaneously inoculated. Ilemobade (1976) had similar results but he used heartwater infective brain material. Our results indicate that it is not a specific stage of the organism in the brain but merely the brain tissue itself which makes the subcutaneous route effective. In Experiment 2, though, brain tissue was added to blood which contained 5 % DMSO, so it is impossible to say whether the brain tissue or the DMSO was responsible for the successful subcutaneous inoculation.

In mice, J. L. du Plessis (unpublished data, 1986) found the subcutaneous route highly effective when they were inoculated with the Kümm isolate (Du Plessis & Kümm, 1971) but completely ineffective when the Welgevonden isolate (Du Plessis, 1985) was used (Table 7). However, in sheep he succeeded only once in 6 sheep with the Welgevonden isolate and twice in 13 sheep when the Kümm isolate was used: the 3 sheep that reacted to the subcutaneous infection were immune to subsequent challenge but the other 16 animals proved to be fully susceptible to challenge.

The intramuscular route for the infection or vaccination of sheep is promising. Of the 16 sheep injected intramuscularly with nymph suspensions containing additives, 11 reacted. When challenged, 2 of the 5 animals that did not react initially were found to be immune. Unfortunately, though, only 2 control animals, of which one reacted, were included in this experiment, so it is impossible to determine conclusively whether the additives were responsible for the success or whether i.m. injection in itself has a high degree of success.

When sheep were injected intramuscularly with 15 mℓ of blood containing 5 % DMSO, 3 out of 4 animals reacted. In this case the 15 mℓ dose was halved and each half injected into a different site. At present it is not known what effect the dosage volume has on the success rate of intramuscular injections.

Intraperitoneal inoculation of infective blood with or without brain material was found to be completely ineffective, both in infecting animals with *C. ruminantium* or in stimulating their immunity to the disease. These results differ from those of Hurter (1987) who found that, despite the fact that no primary reactions were obtained after i.p. vaccination, 8 out of 14 sheep were immune to subsequent challenge. However, it does support his findings with goat kids, in which no reactions were seen and no immunity was stimulated after intraperitoneal vaccination.

When applied to mice, the intraperitoneal route seems to present a different picture. In mice the strain or isolate of *C. ruminantium* appears to be a very important factor in the success rate of intraperitoneal inoculations. The Kümm isolate is highly pathogenic when injected intraperitoneally (Du Plessis & Kümm, 1971), while other isolates, such as the Kwanyanga (MacKenzie & Van Rooyen, 1981) or Welgevonden isolates (Du Plessis, 1985), have a low rate of success compared to intravenous inoculation.

In the experiments with cattle, very few animals reacted after subcutaneous or intramuscular injection. The only exceptions were the 2 calves that were injected intramuscularly with nymph suspension to which a combination of histamine and hyaluronidase had been added, and 1 calf that received only nymph suspension subcutaneously. When challenged, though, the majority of animals were found to be immune. A comparison of the reactions of vaccinated and unvaccinated animals suggests that at least some immunity was stimulated by the injections.

However, because of the high percentage of non-reactors that are encountered during heartwater experiments in cattle, we feel that no final conclusions can be drawn from these results. The same phenomenon, i.e. the apparent development of immunity after only a slight temperature reaction or even no reaction, was also seen in some sheep that were injected subcutaneously or intramuscularly. This was most pronounced in Experiment 4, in which only 52 % of the sheep showed a definite primary reaction but 68,8 % were found to be immune when they were subsequently challenged. These findings are in sharp contrast to the general opinion that immunity to heartwater follows only after a definite primary reaction (Dixon, 1899; Alexander, 1931; Du Plessis & Malan, 1987). However, it is in agreement, at least to some extent, with others who found that an immunity may develop without a strong vaccination reaction (Spreull, 1922; Mare, 1972).

TABLE 7 Summary of results regarding the inoculation of Heartwater infected material by routes other than the intravenous route

Route	Animal	Infective material	Results	References
Subcutaneous	Sheep	Blood	Negative in 3 sheep	Dixon, 1898([10]
			1 out of 11 reacted	Alexander, 1931
			Small doses of blood at monthly intervals apparently stimulated immunity	Spreull, 1922
			Negative in 1 sheep	Uilenberg, 1971
			3 out of 12 reacted	Present study
		Blood + DMSO	8 out of 12 reacted	Present study
		Blood + DMSO + uninfective brain	3 out of 4 reacted	Present study
		Tick suspensions	Negative in 11 sheep	Present study
		+ additives	Negative in 11 sheep	Present study
		+ DMSO (5–15 %)	25 out of 48 reacted	Present study
		+ DMSO 5 %	3 out of 50 reacted	Present study
		+ uninfective brain	2 out of 2 reacted	Present study
		Mouse tissue homogenates (Welgevonden isolate)		
		+ mouse brain	1 out of 2 reacted	J. L. du Plessis, unpublished results, 1986
		+ thioglycolate	Negative in 4 sheep	J. L. du Plessis, unpublished results, 1986
		(Kümm isolate)		
		+ sheep brain	1 out of 6 reacted	J. L. du Plessis, unpublished results, 1986
		+ mouse brain	Negative in 5 sheep	J. L. du Plessis, unpublished results, 1986
		+ Lactobacillus[2]	1 out of 2 reacted	J. L. du Plessis, unpublished results, 1986
	Goats	Hydropericardial fluid and bile	Negative in 4 sheep	Dixon, 1898
		Blood	2 out of 2 reacted, but usually very rare	Spreull, 1904
			23 out of 100 died	Dixon, 1899
			4 out of 6 reacted after the 3rd inoculation	Robertson, *In:* Dixon, 1910
		Tick suspension	1 out of 2 reacted	A. J. van Winkelhoff, unpublished data in Uilenberg, 1983
		+ uninfective brain	1 out of 2 reacted	A. J. van Winkelhoff, unpublished data in Uilenberg, 1983
		Infective brain	1 out of 2 reacted	Anonymous, 1952
			6 out of 6 reacted	Ilemobade, 1976
				Ilemobade & Blotkamp, 1978
			Reacted in most cases	A. J. van Winkelhoff, A. Starink & G. Uilenberg, unpublished data. *In:* Uilenberg, 1983
		Lung Macrophages	Negative in 3 goats	Ilemobade & Blotkamp, 1978
		Spleen pulp	3 out of 3 reacted	Dixon, 1898
Subcutaneous	Cattle	Blood	Negative in 1 case	Uilenberg, 1971
			Negative in 20 cases[3]	Uilenberg, 1971
		Nymph suspensions	1 out of 2 reacted	Present study
		+ uninfective brain	None out of 3 reacted	Present study
		+ oil adjuvant	1 out of 3 reacted	Present study
	Mice		Reciprocal of infectivity titre	J. L. du Plessis, unpublished results, 1986

	s.c.	i.p.	i.v.
Mouse tissue homogenate (Kümm isolate)	1,7	3,25	*
+ sheep brain	3,5	3,8	
	3,8	*	*
+ thioglycolate	3,6	4,25	*
+ Lactobacillus	2,8	4,25	*
Mouse tissue homogenate (Welgevonden isolate)			
+ sheep brain	0	*	3,0

Intramuscular	Sheep	Blood + 5 % DMSO	3 out of 4 reacted	Present study
		Nymph suspension	1 out of 2 reacted	Present study
		+ additives	11 out of 14 reacted	Present study
	Cattle	Nymph suspension	2 out of 2 reacted	Present study
		+ additives	3 out of 4 reacted	Present study
		+ oil adjuvant	None out of 4 reacted but 2 were immune when compared to unvaccinated controls	Present study
Intraperitoneal	Sheep	Blood	Rarely positive	Alexander, 1931
			No primary reaction but 8 out of 14 were immune afterwards	Hurter, 1987
			No primary reaction and no immunity in 10 sheep	Present study
		Blood + uninfective brain	No reaction in 5 sheep and no immunity afterwards	Present study
	Goats	Blood	No primary reaction and no immunity afterwards in 7 kids	Hurter, 1987
		Brain	1 case of fatal heartwater	Balozet, 1936

TABLE 7 Summary of results regarding the inoculation of heartwater infected material by routes other than the intravenous route

Route	Animal	Infective material	Results	References
	Mice	Blood; mouse tissue homogenates	Very effective for some isolates e.g. Kümm, but less effective for others e.g. Kwanyanga, Nonile and Welgevonden	MacKenzie & McHardy, 1987
Intrathoracic	Goat	Blood	Negative	Dixon, 1898
Intracerebral	Sheep	Brain	Positive?	Balozet, 1936
Intratracheal	Sheep	Blood	Negative	Alexander, 1931
Intradermal	Sheep	Blood	Negative	Alexander, 1931
		Tick suspension	Negative in 2 cases	J. D. Bezuidenhout, unpublished data, 1979
Intranasal	Sheep	Duodenal fluid	Negative	Dixon, 1898
Per os	Sheep	Blood	Negative	Alexander, 1931

(1) Many more animals were inoculated subcutaneously with blood. However, reactions after vaccination were not monitored and the results are difficult to interpret

(2) Discovered as an incidental contaminant in inoculum

(3) Animals originated from a tick infested area and some animals therefore could have been immune

* Not done

FUTURE STUDIES

Unless a major breakthrough with an alternative vaccine against heartwater can be achieved the urgency to find another effective route of inoculation will remain. It is therefore important to assess the successes achieved so far in this regard, however limited they are, and to investigate other less expensive and more stable additives. It will also be important to obtain a solid knowledge of the factors that are involved in the distribution and absorption of materials inoculated subcutaneously or intramuscularly.

Not only is the number of infective organisms that are deposited important, but also the dosage volume and the concentration of the additive. Possible toxic effects of the additive on *C. ruminantium* should also be investigated, and both tissue cultures and mice may prove to be valuable systems for this purpose.

For obvious reasons, the subcutaneous route is the route of choice and it should therefore receive the most attention. Uninfected brain material and DMSO were found to be promising additives and these should be explored further. It is important to determine the substance(s) or factor(s) in brain material that are responsible for successful subcutaneous inoculation. It may be possible to simulate such factors or obtain similar substances commercially. The use of brain material itself has many disadvantages, such as the possible spread of other diseases or the possibility of demyelination after repeated inoculation.

The site of injection in subcutaneous vaccination may be important and should be standardized.

Although it is the 2nd choice the advantages of the intramuscular route should not be overlooked. The fact that it is now possible, with tick suspensions and tissue cultures, to prepare highly infective doses in a small volume makes this a suitable route for small lambs and kids. Bradikynin seems to be a very effective additive for intramuscular inoculation but it is both very expensive and unstable. Hyaluronidase may be a more practical additive for this route. If it proves to be stable when diluted and in its frozen form it may be added to the vaccine or, in freeze dried form, supplied separately with the vaccine, to be mixed in just before use.

ACKNOWLEDGEMENTS

We gratefully acknowledge the assistance of Dr Jane B. Walker and Mrs Susan Brett in the preparation of this manuscript. Thanks are also due to Dr J. L. du Plessis for permission to quote from unpublished work.

REFERENCES

ALEXANDER, R. A., 1931. Heartwater. The present state of our knowledge of the disease. *17th Report of the Director of Veterinary Services and Animal Industry, Union of South Africa*, 89–150.

ANONYMOUS, 1952. Annual Report of the Veterinary Department, Kenya for 1950. pp. 16–18. Nairobi: Government Printer.

BARNARD, W. G., 1953. Heartwater immunization under field conditions in Swaziland. *Bulletin of Epizootic Diseases of Africa*, 1, 300–313.

BALOZET, L., 1936. Contribution a l'étude experimentale de la heartwater. *Archives de l'Institut Pasteur de Tunis*, 251–267.

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, Rhodes University, Grahamstown*, 41–45.

BEZUIDENHOUT, J. D. & OBEREM, P. T., 1985. New vaccine against Heartwater. *Suid-Afrikaanse Tydskrif vir Wetenskap*, 81, 2–3.

BINNINGTON, K. C., 1978. Sequential changes in salivary gland structure during attachment and feeding of the cattle tick *Boophilus microplus*. *International Journal for Parasitology*, 8, 97–115.

BINNINGTON, K. C. & KEMP, D. H., 1980. Role of tick salivary glands in feeding and disease transmission. *Advances in Parasitology*, 18, 316–341.

CHINERY, W. A. & AYITEY-SMITH, E., 1977. Histamine blocking agent in the salivary gland homogenate of the tick *Rhipicephalus sanguineus sanguineus*. *Nature, London*, 265, 366–367.

DIXON, R. W., 1898. Heartwater experiments. *Agricultural Journal of the Cape of Good Hope*, 12, 754–760.

DIXON, R. W., 1899. Heartwater experiments. *Agricultural Journal of the Cape of Good Hope*, 14, 205–207.

DIXON, R. W., 1910. Heartwater in sheep and goats. *Agricultural Journal of the Cape of Good Hope*, 36, 554–559.

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

DU PLESSIS, J. L. & BEZUIDENHOUT, J. D., 1979. Investigation on the natural and aquired resistance of cattle to artificial infection with *Cowdria ruminantium*. *Journal of the South African Veterinary Association*, 50, 334–338.

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.

DU PLESSIS, J. L. & MALAN, LETITIA, 1987. The block method of vaccination against heartwater. *Onderstepoort Journal of Veterinary Research*, 54, 493–495.

FICK, J. F. & SCHUSS, J., 1952. Heartwater immunization under field conditions in Swaziland. *Journal of the South African Veterinary Medical Association*, 23, 9–14.

HOWELL, C. J., NEITZ, A. W. H. & POTGIETER, D. J. J., 1975. Some toxic, physical and chemical properties of the oral secretion of the sand tampan, *Ornithodoros savignyi* Audouin (1827). *Onderstepoort Journal of Veterinary Research*, 42, 99–102.

HURTER, L. R., 1987. A preliminary investigation into the efficacy of intraperitoneal vaccination of sheep and goat kids against heartwater. *Onderstepoort Journal of Veterinary Research*, 54, 507–508.

ILEMOBADE, A. A., 1976. Study on heartwater and the causative agent *Cowdria ruminantium* (Cowdry, 1925) in Nigeria. Thesis Doctor in Physiology, Ahmadu Bello University, Zaria, pp. 299.

ILEMOBADE, A. A. & BLOTKAMP, C., 1978. Heartwater in Nigeria. II. The isolation of *Cowdria ruminantium* from live and dead animals and the importance of routes of inoculation. *Tropical Animal Health and Production*, 10, 170.

MARE, C. J., 1972. The effect of prolonged oral administration of oxytetracycline on the course of heartwater infection in sheep. *Tropical Animal Health and Production*, 4, 69–73.

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

MACKENZIE, P. K. I. & MCHARDY, N., 1987. *Cowdria ruminantium* infection in the mouse. A review. *Onderstepoort Journal of Veterinary Research*, 54, 267–269.

MOORHOUSE, D. E. & TATCHELL, R. J., 1966. The feeding processes of the cattle tick *Boophilus microplus* (Canestrini): a study in host parasite relations. Part I, Attachment to the host. *Parasitology*, 56, 623–632.

NEITZ, A. W. H. & VERMEULEN, N. M. J., 1987. Biochemical studies of the salivary glands and haemolymph of *Amblyomma hebraeum*. *Onderstepoort Journal of Veterinary Research*, 54, 443–450.

OBEREM, P. T. & BEZUIDENHOUT, J. D., 1987. The production of heartwater vaccine. *Onderstepoort Journal of Veterinary Research*, 54, 485–488.

SPREULL, J., 1904. Heartwater inoculation experiments. *Agricultural Journal of the Cape of Good Hope*, 24, 433–442.

SPREULL, J., 1922. Heartwater. (In afrikaans) *Journal, Department of Agriculture, Union of South Africa.*, 4, 236–245.

UILENBERG, G., 1971. Etudes sur la cowdriose à Madagascar. *Revue d'Élevage et de Médecine Vétérinare des Pays Tropicaux*, 33, 21–22.

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

VAN DER MERWE, LENTE, 1987. The infection and treatment method of vaccination against heartwater. *Onderstepoort Journal of Veterinary Research*, 54, 489–491.

Printed by the Government Printer, Private Bag X85, Pretoria, 0001