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COWDRIA RUMINANTIUM: STABILITY AND PRESERVATION OF THE ORGANISM

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ABSTRACT

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Blood collected in either sodium heparin or disodium edetate vacutainers from febrile goats infected with 4 isolates of *Cowdria ruminantium* and cryopreserved with 10 % dimethyl sulphoxide at −70 °C and −196 °C was an effective stabilate to initiate heartwater infections in goats. A homogenized pool of whole *Amblyomma variegatum* ticks in Snyder's buffer, maintained at −196 °C, was used to infect a goat with *C. ruminantium*. Liver and spleen collected from Swiss mice infected with the Kwanyanga isolate of *C. ruminantium* were homogenized in Snyder's buffer, maintained at −196 °C and were used to initiate infections in mice. Fresh blood collected from febrile goats and maintained at 4 °C for as long as 72 h was infectious to mice. Neutrophils separated from blood of *C. ruminantium* infected goats and maintained in modified RPMI medium at 37 °C for 68 h were infectious for a goat. Similarly neutrophils from a 2nd infected goat maintained for 96 h at 37 °C were infectious for mice.

INTRODUCTION

The short period of viability of *Cowdria ruminantium* in blood, mammalian organ homogenates, and tick homogenates has seriously handicapped heartwater research for a number of years. Before the advent of cryopreserved stabilates of blood, organ or tick suspensions of *C. ruminantium*, the preservation of the organism was limited to passage through susceptible animals or maintainance in *Amblyomma hebraeum* ticks.

Alexander (1931) reviewed his attempts and those of others to preserve the infectivity of *C. ruminantium* in blood at room temperature and 4 °C. They were able to maintain *C. ruminantium* in defibrinated blood at room temperature for as long as 38 h. Alexander stated that *C. ruminantium* generally does not survive for more than 24 h at room temperature, and in some cases the viability is lost within 12 h. Henning (1956) described an exceptional case in which *C. ruminantium* remained infectious in blood held at room temperature for 4 days. Alexander (1931) was unable to stabilize or preserve *C. ruminantium* in blood at room or refrigerated temperatures by the addition of any of the following: glycerine, calcium-oxalate-phenol-glycerine (OCG), saccharose, glucose, liquid paraffin, formalin, isotonic sodium citrate, isotonic locke-gelatine-dextrose or isotonic dextrose. Attempts to maintain the infectivity of *C. ruminantium* by desiccation also failed. Alexander (1931) comments that storage at low temperatures appeared to have a favourable influence on the viability of *C. ruminantium* but that no data were yet available on the effects of freezing. Haig (1952) later notes that Alexander had stored *C. ruminantium* for a period of up to 2 years in a dry ice cabinet. Weiss, Haig & Alexander (1952) report the details of the successful preservation of *C. ruminantium* in 10 % phosphate buffered spleen homogenates at −74 °C. Haig (1955) reported that *C. ruminantium* infected blood frozen on dry ice at −45 °C was used in the transport of heartwater vaccine to distant sites. Collection and handling of the blood was not described. Thomas & Mansvelt (1957) later reported using sheep blood drawn in an equal volume of citrated buffered lactose peptone (BLP) that was rapidly frozen and stored on dry ice. These early heartwater researchers had developed the first method for cryopreserving *C. ruminantium* in organ homogenates and blood at −45 °C and −74 °C. Neitz (1968), working at the same institute, reported that *C. ruminantium* in blood and spleen homogenates maintained at −76 °C for 2 years was infectious to animals.

Karrar (1960) was unable to preserve infected citrated or defibrinated blood at −32 °C. Rahim & Shommein (1978) also failed to preserve *C. ruminantium* in goat blood collected in disodium edetate as an anticoagulant and frozen at −70 °C.

Ramisse & Uilenberg (1970) first reported the use of 10 % dimethyl sulphoxide (DMSO) as a cryoprotectant for blood stabilates of *C. ruminantium*. Blood was collected in sodium citrate, versene or heparin and snap frozen. Blood maintained at −75 °C was equally as infectious as blood maintained at −180 °C. Slow freezing of blood with glycerol failed to preserve the organisms. Rapid freezing of blood with no cryoprotectant gave negative results, as did slow freezing of blood with 7,5 % DMSO and rapid freezing with 20 % DMSO. Ilemobade, Blotkamp & Synge (1975) preserved the infectivity of heparinized blood snap frozen at −85 °C or −196 °C without using a cryoprotectant.

Bezuidenhout (1981) successfully produced tick stabilates of *C. ruminantium* for use as a heartwater vaccine. Engorged infected nymphae were homogenized in BLP and 5 % DMSO, snap frozen and maintained on dry ice or in liquid nitrogen.

Brain homogenates made in phosphate buffered saline (PBS) and frozen with and without DMSO have been shown to be infectious to animals (Ilemobade & Blotkamp, 1978; Uilenberg, 1983).

A study in which 13 solutions were compared as to their ability to maintain *C. ruminantium* over a 3-h period at room temperature (21 °C) was conducted using outbred Swiss mice. Snyder 1 buffer was significantly better at maintaining the viability of *C. ruminantium* at the later time periods than were the other solutions studied (Birnie, Endris & Logan, 1986). This trial was performed with 1 isolate of *C. ruminantium* at room temperature and the effectiveness of Snyder 1 buffer with other isolates or cryopreserved stabilates was not evaluated.

The widespread distribution of *A. variegatum* in the Caribbean and the recent knowledge of the presence of heartwater on 3 Caribbean islands represents a potential disease threat to the United States livestock industry. In the past the exotic disease status of heartwater has prevented research on *C. ruminantium* within the continental United States (US). This work was initiated to determine how best to preserve *C. ruminantium* in samples. The collection of blood in vacutainer tubes by US veterinarians is very common and, in the event of an outbreak of heartwater, veterinarians would most likely resort to this method of collecting blood.

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The objectives of this study were to adapt reliable and convenient methods to cryopreserve *C. ruminantium;* to determine how long blood or diluted blood could be refrigerated before infectivity was lost, and to determine if neutrophil cultures could be used to initiate infections in animals.

MATERIALS AND METHODS

Animals

Adult goats of mixed breeds ranging in age from 1–5 years were housed in animal rooms at 22 °C and given free choice of pelleted alfalfa (lucerne) feed, water and salt-mineral blocks. Female albino Swiss mice (42–49 days old) were obtained from a closed outbred colony maintained at the Plum Island Animal Disease Center and housed in cages with free access to water and mouse chow.

Cowdria ruminantium *source*

Amblyomma hebraeum nymphae infected as larvae with the Kwanyanga isolate were provided by P. K. I. MacKenzie[1]. *A. hebraeum* (Spesbona isolate) nymphae, infected as larvae with the Kümm isolate, were provided by J. L. du Plessis[2]. The Mali 1 isolate was made from *A. variegatum* adults collected from cattle in Mali that were provided by T. G. Galvin and M. Toure[3]. The Gardel isolate from Guadeloupe was received as a frozen blood stabilate from N. Barré and E. Camus[4].

Method of infection

C. ruminantium infections were initiated in goats by allowing adult *A. hebraeum* infected with either the Kwanyanga isolate or Kümm isolate to feed on goats or with frozen blood stabilates as previously described (Logan, Endris, Birnie & Mebus, 1985). Three mℓ of pooled samples of tick stabilates made from homogenized adult *A. variegatum* ticks frozen in Snyder 1 with 10 % DMSO was used to initiate a *C. ruminantium* (Mali 1) infection in a goat.

Four isolates of *C. ruminantium* were passaged at intervals in goats. These infections were initiated in goats by intravenous (i.v.) injections of either frozen blood stabilates or fresh blood. One goat was injected i.v. with 5 mℓ of a neutrophil culture separated from the blood of a goat infected with the Mali 1 isolate of *C. ruminantium*. Brain smears were made from goats that died and were examined as described by Purchase (1945).

Infections in mice were initiated using fresh or frozen mouse liver spleen homogenates, blood, or diluted blood by the i.v. injection of 0,1 mℓ of the infectious material or by the intraperitoneal administration (i.p.) of 0,2 mℓ.

Collection of blood

The goats were observed daily and rectal temperatures were recorded. At the height of the febrile response (>40,0 °C), blood was collected from the jugular vein of infected goats in both disodium edetate and sodium heparin vacutainers[5] and in syringes with either sodium heparin (10 iu/mℓ blood) or ACD solution (Logan, Whyard, Quintero & Mebus, 1987).

Preservation of blood

The blood was immediately transferred to a biological hood and processed. Blood from vacutainers was pooled and dimethyl sulphoxide (DMSO)[6] was added as 10 % of the final volume. The blood was kept on ice and was dispensed into either 1,8 or 4,5 mℓ cryotubes[7]. Blood stabilates were either frozen and maintained at −70 °C or snap frozen in liquid nitrogen and maintained at −195 °C. The blood stabilates were periodically thawed rapidly in a water bath (37 °C) and 4–13 mℓ was injected intravenously into susceptible animals.

Preservation of mouse liver and spleen

Mouse liver and spleen were sterilely collected from moribund mice according to the technique of MacKenzie & Van Rooyen (1981). The organs from an individual mouse were homogenised in 10 mℓ of Snyder 1 buffer (pH 7,2) (Jackson & Smadel, 1951). The supernate was used for serial passage in mice. For the preparation of frozen stabilates the suspension was centrifuged for 3 min at 200 × g, dispensed in 1,8 mℓ cryotubes, snap frozen in liquid nitrogen and maintained at −195 °C until needed.

Neutrophil cultures

Neutrophils were separated from infected goat blood collected in ACD solution using a modified protocol of Carlson & Kaneko (1973). The blood was divided into 35 mℓ aliquots in 50 mℓ polypropylene plastic centrifuge tubes and centrifuged at 700 × g for 15 min. The plasma, buffy coat and upper surface layer of the red cells were discarded. The red cells in the remaining fraction were lysed by the addition of 20 mℓ of cold distilled H_2O. After gentle mixing for 30 s, 10 mℓ of cold 2,7 % NaCl was added, mixed and the volume brought to 50 mℓ with cold Hanks Balanced Salt Solution (HBSS) lacking calcium and magnesium pH 7,2. The cell suspension was centrifuged at 200 × g for 10 min, and the supernatant discarded. The hypotonic lysis was repeated once, the cells were washed in HBSS and resuspended in RPMI medium supplemented with 15 % conditioned L929 medium, 10 % fetal bovine serum, 20 mM HEPES buffer, pH 7,3, and 1 mM l-glutamine. The cell suspension was incubated at 37 °C in a humidified atmosphere of 3 % CO_2 −97 % air (Logan *et al.*, 1987). Aliquots were removed at intervals and injected into mice or goats.

Stability of refrigerated blood

Venous blood collected in heparin from infected goats was immediately transferred to a biological hood and placed in glass vials as follows: 5 vials with blood alone; 5 vials with 1 part blood and 1 part RPMI, and 5 vials with 1 part blood and 1 part Snyder 1. The vials were refrigerated at 4 °C until needed. In order to deliver the same number of organisms 0,1 mℓ of whole blood or 0,2 mℓ of diluted blood was inoculated into mice.

RESULTS

Preservation of C. ruminantium

Over a 3 year period, *C. ruminantium* infections in goats were routinely initiated with stabilates of blood collected in sodium heparin vacutainers with 10 % DMSO, snap frozen and kept at −195 °C. Blood stabilates prepared in this manner from 4 different isolates of *C. ruminantium* were infectious in each of the 25 goats with no previous exposure to *C. ruminantium* (Table 1).

An Angora goat was infected with the Kwanyanga isolate of *C. ruminantium* by 50 adult *A. hebraeum*. Blood was collected in EDTA vacutainers, 10 % DMSO was added and the stabilate was stored at −70 °C for 1071 days. It was tested in a female mixed-breed goat

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TABLE 1 Preservation of *Cowdria ruminantium*

Stabilate	Cryoprotectant	Storage days	Animal tested	Inoculum mℓ	Result
Goat blood	Heparin & 10 % DMSO	49–853[d]	25 goats	3–10[f]	All > 40,0 °C
Goat blood	EDTA & 10 % DMSO	1 071[e]	1 goat	13[f]	16 dpi = 41,9 °C, died 19 dpi, + brain + neutrophils
Goat blood	EDTA & 10 % DMSO	962[d]	1 goat	10[f]	13 dpi = 41 °C + neutrophils, tx
A. variegatum adults	Snyder 1 & 10 °C DMSO	62[d]	1 goat	3[f]	14 dpi = 41,6 °C, died 16 dpi, + brain + neutrophils
Mouse liver & spleen[a]	Snyder 1	214[d]	10 mice	0,1[f]	14 dpi all died
Mouse liver & spleen[a]	Snyder 1	273[d]	10 mice	0,1[f]	18 dpi all died
Mouse liver & spleen[b]	Snyder 1	60[d]	15 mice	0,1[f]	16 dpi all died
Mouse liver & spleen[c]	Snyder 1	97[d]	5 mice	0,2[g]	14 dpi 4 died 1 passaged

dpi = days post inoculation
tx = treated with oxytetracycline
[a] Kwanyanga isolate 49th passage in Swiss mice
[b] Kwanyanga isolate 54th passage in Swiss mice
[c] Kümm isolate 13th passage in Swiss mice
[d] stored at −196 °C
[e] stored at −70 °C
[f] Intravenous inoculation
[g] Intraperitoneal inoculation

TABLE 2 Stability of *Cowdria ruminantium* in whole blood[a] at 4 °C

Time h	Inoculum mℓ	Diluent[b]	Number of mice			
			Injected	Sick	Died	Survived
0	0,1	None	10	7	7	3
24	0,2	Snyder 1	10	9	7	3
24	0,2	RPMI	10	5	3	7
56	0,2	Snyder 1	10	2	2	8
56	0,2	RPMI	10	5	2	8
96	0,2	Snyder 1	10	0	0	10
96	0,2	RPMI	10	0	0	10

[a] Blood collected from a goat infected with *C. ruminantium* (Kwanyanga isolate), 13 dpi, 41,0 °C
[b] Diluent mixed 1:1 with blood

TABLE 3 Stability of *Cowdria ruminantium* in whole blood[a] at 4 °C

Time h	Inoculum mℓ	Diluent[b]	Number of mice			
			Injected	Sick	Died	Survived
0	0,1	None	10	10	7	3
0	0,2	Snyder 1	11	11	8	3
0	0,2	RPMI	10	10	8	2
24	0,1	None	5	2	0	5
24	0,2	Snyder 1	10	10	3	7
24	0,2	RPMI	10	6	0	10
48	0,1	None	10	3	2	8
48	0,2	Snyder 1	10	3	3	7
48	0,2	RPMI	10	6	3	7
72	0,1	None	10	3	2	8
72	0,2	Snyder 1	10	2	1	8[c]
72	0,2	RPMI	10	5	4	6

[a] Blood collected from a goat infected with *C. ruminantium* (Kwanyanga isolate), 12 dpi, 42,1 °C
[b] Diluent mixed 1:1 with blood
[c] One mouse died at 4 dpi, and was not counted as a heartwater death

TABLE 4 Stability of *Cowdria ruminantium* in neutrophils[a] at 37 °C

Time h	Inoculum mℓ	Diluent	Number of mice[b]			
			Injected	Sick	Died	Survived
0	0,1	RPMI	10	0	0	10
24	0,1	RPMI	5	0	0	5
56	0,1	RPMI	13	10	8	4
96	0,1	RPMI	10	10	10	0
168	0,1	RPMI	6	0	0	6

[a] Blood collected from a goat infected with *C. ruminantium* (Kwanyanga isolate) 13 dpi, 41,1 °C. Neutrophils separated and maintained in RPMI at 37 °C.
[b] Mice inoculated with neutrophil cultures at the same time periods as mice in Table 2

(Table 1). The goat developed a febrile response on 14 days post inoculation dpi and by 16 dpi the animal's temperature was 41,9 °C; the goat died 19 dpi. Both neutrophil cultures and brain smears were positive for *C. ruminantium* (Table 1).

A blood stabilate, collected in EDTA, 1 passage removed from the Angora goat previously described, was stored at −195 °C for 962 days and was tested in a pregnant female Nubian goat (Table 1). The goat delivered 2 kids 12 dpi and the following day the temperature

rose to 41,0 °C. Blood was collected and neutrophils separated and cultured. Colonies of *C. ruminantium* were detected in neutrophils. The goat was treated once with oxytetracycline on 14 dpi and recovered.

A female Saanen goat injected with 3 mℓ of pooled tick stabilate developed a temperature rise to 40,4 °C 11 dpi, which peaked at 41,6 °C on 14 dpi. The goat died 16 dpi. Numerous colonies of *C. ruminantium* were seen in brain smears (Table 1).

Following 49 passages of the Kwanyanga isolate in Swiss mice, the liver and spleen from a moribund mouse were collected 8 dpi, homogenized in Snyder 1, snap frozen in liquid nitrogen and held 214 days. All 10 of the mice injected with this stabilate were dead by 14 dpi. After 273 days of storage the same lot of stabilate was injected into 10 mice and by 18 dpi all 10 mice had died (Table 1).

Following 54 passages of the Kwanyanga isolate in Swiss mice, the liver and spleen from a moribund mouse were collected 14 dpi, processed, and snap frozen. Sixty days later, 15 mice were injected with this stabilate and by 12 dpi 14 mice had died; the remaining mouse died 16 dpi (Table 1).

Neutrophils separated and held in culture from a goat that had been infected with adult *A. hebraeum* (Kümm isolate) were used to initiate an infection in mice. Following 13 passages of this neutrophil-derived Kümm isolate in mice, the liver and spleen from a moribund mouse were collected 14 dpi, processed, and snap frozen. After 97 days of storage, 5 mice were injected and by 14 dpi 4 of the mice were dead. The remaining sick mouse was used to begin a new passage in mice (Table 1).

Stability of blood at 4 °C

The duration of infectivity of the Kwanyanga isolate of *C. ruminantium* in goat blood held at 4 °C was tested in mice (Table 2, 3). Whole blood and blood diluted with either Snyder 1 or RPMI was injected into mice at intervals as shown. In the 1st trial (Table 2) blood diluted in either Snyder 1 or RPMI held for up to 56 h was infectious in 2 out of 10 mice inoculated. In the 2nd trial (Table 3), at 72 h *C. ruminantium* killed 2 out of 10 mice inoculated with blood, 1 out of 10 mice inoculated with blood and Snyder 1, and 4 out of 10 mice inoculated with blood and RPMI.

Neutrophil culture

Twenty mℓ of a neutrophil culture derived from a goat infected with the Mali 1 isolate was harvested after 68 h in culture. Colonies of *C. ruminantium* in neutrophils were seen in Dif-Quik stained cytocentrifugation preparations of the culture supernate (Logan *et al.*, 1987). The cells were spun down and resuspended in 5 mℓ of culture medium and injected into a female cross-bred goat. This inoculum was equivalent to 2 × 107 cells. Thirteen dpi the goat had a temperature of 41,6 °C and the following day it died. Brain smears had a remarkable number of colonies in capillary endothelial cells. *C. ruminantium* colonies were also seen in neurophils isolated and cultured from this goat.

Blood drawn at the same time from the goat used as a source of blood for the trial recorded in Table 2 was used as a source for neutrophil cultures. The number of cells in culture was adjusted to 1×10^6 per mℓ. Aliquots of neutrophils were inoculated into mice at 0, 24, 56, 96, 168 h later. At 56 and 96 h colonies of *C. ruminantium* were evident in neutrophils in the culture media and they were highly infectious to mice (Table 4).

Discussion

Past studies as well as those presented here demonstrate that *C. ruminantium* can be effectively preserved in a variety of organ suspensions at low temperatures (−70 °C to −196 °C) for indefinite periods of time. Collection of blood in either sodium heparin or disodium edetate vacutainers was an effective method of obtaining blood for stabilates. Snyder 1 was effective in preserving the infectivity of *C. ruminantium* in samples of mouse liver and spleen and adult *A. variegatum*. Stabilates can be retrieved, thawed, and immediately injected into animals. Sufficient numbers of infective *C. ruminantium* were preserved in these samples to initiate infections in animals with *C. ruminantium*.

Inoculations of 0,1 mℓ whole infected goat blood (Kwanyanga isolate) into mice caused 70 % mortality. Some mice became clinically ill with the Kwanyanga isolate, and later recovered. After serial passage with mouse liver and spleen homogenates, the mortality reached 100 % (Table 1).

In trials listed in Tables 2 and 4, blood was collected from the same goat infected with frozen blood stabilate. Seven out of 10 mice became ill when injected with whole blood at time 0 but none became ill when injected with the neutrophil fraction at time 0 or at 24 h. The infective dose of *C. ruminantium* in 1×10^5 neutrophils in a 0,1 mℓ inoculum was insufficient to make the mice clinically ill. These same cultures, however, killed 8 out of 10 mice at 56 h and 10 out of 10 at 96 h. Colonies of *C. ruminantium* were easily detected in neutrophils in culture media at these time periods. The monitoring of cultures of neutrophils from infected goats was a helpful means of diagnosing infections and provided a safer method than brain biopsy (Synge, 1978).

Storage of organisms at low temperatures arrests reproduction and stabilizes the biological characteristics of organisms. The word "stabilate" was proposed to define a population of an organism preserved in a viable condition (Lumsden & Hardy, 1965). Foggie, Lumsden & McNeillage (1966) refer to *Ehrlichia phagocytophila*-infected blood frozen with DMSO as a stabilate. The term "stabilate" appropriately describes cryopreserved specimens containing *C. ruminantium*.

The fragility of *C. ruminantium* differs from that of other closely related members of the Ehrlichia tribe. *Ehrlichia (Cytocetes) ondiri,* the etiologic agent of bovine petechial fever, remains infective in blood for as long as 9 days when maintained at 4 °C, or 2 days at 37 °C. *E. ondiri* was not successfully stored at either −20 °C or lyophilized (Haig & Danskin, 1962).

Adapting the method for preserving trypanosomes, Foggie *et al.* (1966) successfully produced the first stabilates of *Ehrlichia (Cytocetes) phagocytophila,* the etiologic agent causing tick-borne fever. Sheep and guinea pig blood infected with *E. phagocytophila* was cryopreserved with both glycerol and dimethyl sulphoxide at −79 °C for 13 months. Blood held at room temperature remained infective for up to 13 days at 4 °C. At room temperature, glycerol had a detrimental effect on infected sheep blood while DMSO did not. Both DMSO and glycerol-treated blood remained infectious following a controlled gradual freezing to −50 °C in 1 h.

Blood smears have a very high percentage of infected neutrophils in both *E. phagocytophila* and *E. ondiri* (Foggie, 1951; Haig & Danskin, 1962) while the number of *C. ruminantium* colonies seen in circulating neutrophils is low (Logan *et al.*, 1987). This may explain the much longer stability at room temperature of these 2 *Ehrlichia* spp. than with *C. ruminantium*.

The adaptation of *C. ruminantium* to cell culture

(Bezuidenhout, Paterson & Barnard, 1985) should enable researchers to initiate definitive studies on the best means of preserving *C. ruminantium*. Effective storage and preservation are important for maintaining isolates of *C. ruminantium* for research and for the eventual development and distribution of heartwater vaccines.

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