THE FREEZE-DRYING OF *COWDRIA RUMINANTIUM*

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ABSTRACT

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Lyophilized tissues of mice and blood of sheep, infected with either the Kümm, the Welgevonden or the Ball 3 stock of *Cowdria ruminantium*, remain infective to mice and sheep after storage at 4 °C for 90 days. Freeze-dried tissues stored at −18 °C and −28 °C are still infective after 6 months and 2 years, respectively.

INTRODUCTION

Cowdria ruminantium is notorious for its rapid loss of infectivity and can only be effectively preserved at temperatures below − 70 °C (Logan, 1987). This is often a limiting characteristic of the heartwater agent, because maintenance of its infectivity plays a vital role not only in research on heartwater but also in present day vaccination procedures. Since the evaluation of the results of all experiments in which live organisms are used depends on the presence or absence of clinical disease, absolute certainty of the infectivity of the stabilate used in these cases is essential.

The current infection and treatment method of vaccination is totally dependent on the preservation of infectivity of the vaccine (Van der Merwe, 1987). Furthermore, for immunity to develop, a reaction in response to an infective inoculum is essential (Du Plessis & Malan, 1987b). The extreme lability of the heartwater agent is considered to be one of the major shortcomings of the present vaccine (Oberem & Bezuidenhout, 1987). There is little doubt that the successful freeze-drying of the heartwater vaccine would greatly facilitate its use and lead to vaccination on a much larger scale.

Since infectivity is a key factor in heartwater immunization, extensive use was made of the Kümm (Du Plessis, 1982) and Welgevonden (Du Plessis, 1985a) stocks of *C. ruminantium*. Their pathogenicity to mice enabled us to determine not only the effect of the freeze-drying process on the infectivity of the product, but also on its preservation after storage for varying periods of time and at different temperatures.

MATERIALS AND METHODS

Infected tissues freeze-dried

In 10 experiments tissues infected with either the Kümm, the Welgevonden or the Ball 3 stocks were lyophilized (Table 1). The spleens, lungs and hearts of mice infected with either of the former 2 stocks were homogenized in buffered lactose peptone (BLP: 181 g $Na_2HPO_4.12H_2O$, 26,4 g KH_2PO_4, 30 ℓ distilled water, 2 % Difco peptone, 10 % lactose) on a 20 % mass/volume basis.

Sheep blood, infected with either the Ball 3 or the Welgevonden stocks and prepared in the manner described for the heartwater vaccine currently issued (Oberem & Bezuidenhout, 1987), was also freeze-dried.

Stabilizing additives

In the case of Exp. 2 (Table 1), 10 % sucrose and 10 % peptone were added to BLP as additional stabilizers. In 3 further experiments, 1 volume of Scowgard stabilizer (10,4 % casein hydrolysate, 7 % saccharose, 4 % gelatin) was added to 3 volumes of homogenized tissues in BLP.

Freeze-drying cycle

In Exps. 1–7 and 10, volumes of 1,5 to 5 mℓ in standard 8 mℓ glass bottles, sealed with rubber stoppers of the slit type, were freeze-dried in an Edwards freeze-drying machine with a 20 ℓ capacity. The stoppers on the bottles were inserted in the correct position for freeze-drying under laminar flow.

The condenser temperature was −40 °C and the shelves were pre-cooled to −30 °C. The samples placed on the shelves to freeze, reached −30 °C in 3 h. The butterfly valve was opened to a vacuum of 6 kPa. The temperature was set to −10 °C and, depending on the volume, the samples were dried for 10–42 h. The temperature was then set to +10 °C and the samples dried for another 4–7 h. With the aid of a pneumatic stoppering system, the bottles were sealed off under vacuum. Care was taken not to exceed a temperature of +10 °C. Metal caps were fastened onto the bottles with a capping machine.

In Exps. 8 and 9, an Edwards freeze-drying machine with a condenser capacity of 24 kg was used. The condenser temperature was −55 °C and the shelves were pre-cooled to −39 °C. The samples reached −30 °C in 3 h. With the chamber vacuum at 6 kPa and the temperature at 0 °C, the samples were dried for 15 h. The temperature was then set to +10 °C and the samples dried for another 12 h.

Storage

Suitable numbers of bottles were stored at room temperature (20–23 °C), + 4, −18 and −28 °C. A small number was also stored in the gas phase of liquid nitrogen.

Assaying of infectivity

Samples taken prior to freeze-drying, immediately thereafter and at varying periods after storage, were injected intravenously into mice and sheep. The lyophilized samples were reconstituted with a volume of sterile distilled water equal to the original volume of the sample. In the case of mice, the infectivity was titrated by preparing 10-fold serial dilutions of the reconstituted sample in BLP and injecting 0,2 mℓ per mouse. The mortalities of the mice were recorded and the titre of infectivity calculated according to the method of Reed & Muench (1938). The specificity of the mouse mortalities was confirmed as previously described (Du Plessis & Malan, 1988).

Immunization of cattle

To ascertain the immunogenicity of the lyophilized sheep blood, 20 Bonsmara calves 5–18 days old and 20 Bonsmara oxen 13–14 months old were inoc-

TABLE 1 Details of 10 freeze-drying experiments

Exp. No.	Tissue	Volume (mℓ)	*Cowdria* stock	Stabilate	Duration of freeze-drying cycle (h)
1	Mouse organs	1,5	Kümm	BLP+ Scowgard	17
2	Mouse organs	1,5	Kümm	BLP+ 10 % sucrose + 10 % peptone	17
3	Mouse organs	1,5	Welgevonden	BLP+ Scowgard	17
4	Mouse organs	1,5	Welgevonden	BLP+ Scowgard	17
5	Sheep blood	3	Welgevonden	BLP	53
6	Sheep blood	1,5	Welgevonden	BLP	28
7	Sheep blood	4	Welgevonden	BLP	53
8	Sheep blood	5	Welgevonden	BLP	30
9	Sheep blood	5	Ball 3	BLP	30
10	Sheep blood	5	Ball 3	BLP	53

TABLE 2 Infectivity to mice of freeze-dried Kümm and Welgevonden infected tissues before and after storage at various temperatures

Exp. No.	Storage temperature	Prior to FD*	Directly after FD	Days after freeze-drying									
				2	3	5	21	60	90	180	210	365	730
1	Room	3,8	2,8		2,4	0							
2		4,8	3	1,2		0							
3		3,4	3,2			0							
8						0							
1	4 °C	3,8	2,8					2	1,5				
2		4,8	3				3	2,6					
3		3,4	3,2				1,8						
4		4,4	3,3				2,8	1,6					
5		2,5	1,8					1,4	1	0,8			
8		3,8	2,5										
3	−18 °C	3,4	3,2							2,4			
4		4,4	3,3					2,3					
5		2,5	1,8						1,5	0,8			
6		2,4	1,6					1,6					
5	− 28 °C	2,5	1,8					1,8	1,8	1,7			1,4
7		2,8	1,8					1,8					1,4
2	Liquid nitrogen	4,8	3					3					
3		3,4	3,2				2,5					1,8	
4		4,4	3,3						3,3		3,3		
5		2,5	1,8						1,8				1,8

* FD – freeze-drying

ulated i.v. with 3 mℓ of reconstituted sheep blood, freeze-dried in Exp. 5 and stored at −28 °C for 3 months. Early morning rectal temperatures were recorded and the oxen treated with oxytetracycline[1] at a dosage level of 10 mg/kg body mass on either the 2nd, 3rd or 4th day of the febrile reaction. Six animals were treated 16 days after infection before a rise in body temperature and 4 were left untreated. None of the calves were treated. Six months later the vaccinated animals and suitable controls were challenged with a deep-frozen stabilate of sheep blood infected with the Welgevonden stock at a dose of 5 mℓ. No treatment was given when the animals were challenged. Fourfold serial dilutions of sera collected from the oxen a month after vaccination were subjected to the indirect fluorescent antibody (IFA) test (Du Plessis & Malan, 1987a).

RESULTS

Mice infectivity test

The loss of infectivity during the process of lyophilization, as determined in mice, varied from as little as 0,2 log (Exp. 3, Table 2) to as much as 1,8 log (Exp. 2), with an average of 0,97 log. This variation did not seem to be related to either the stock of C.

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[1] Terramycin LA, Pfizer

ruminantium, the stabilizer or the duration of the freeze-drying cycle.

Infectivity declined rapidly after storage at room temperature. It was maintained for 3 days, but was totally lost after 5 days.

Samples stored at 4 °C showed a decrease in infectivity after 21 days, but were still infective after 90 days. At −18 °C and −28 °C the decline in infectivity in relation to the storage period was much more gradual and only commenced after 90 and 180 days, respectively. It was noteworthy that freeze-dried samples of sheep blood infected with the Welgevonden stock and stored at −28 °C for 2 years (Exp. 5 & 7, Table 2) were almost as infective as immediately after lyophilization. Except in the case of Exp. 3, there appeared to be no loss of infectivity after storage in liquid nitrogen for 1–2 years.

There did not appear to be any correlation between the gradual loss of infectivity after storage and the stabilizers used on one hand and the duration of the freeze-drying cycle on the other. The decline in infectivity appeared to be related rather to the temperature at which the samples were stored.

Infectivity tests: sheep

All 5 animals (Sheep 2–6, Table 3), inoculated with reconstituted freeze-dried sheep blood infected

TABLE 3 Infectivity to sheep of freeze-dried sheep blood infected with the Ball 3 and Welgevonden stocks

Exp. No.	Sheep No.	Inoculum sheep blood	Reaction
9	1	5 mℓ, diluted 1/10, prior to FD	10/6/41,6[1], died
9	2	5 mℓ, undiluted, directly after FD	11/7/41,8, died
9	3	5 mℓ, diluted 1/10, directly after FD	13/7/41,1, died
9	4	2,5 mℓ, undiluted, stored 90 days at 4 °C	18/9/42, died
9	5	2,5 mℓ, undiluted, stored 90 days at −28 °C	11/8/41,2, died
9	6	2,5 mℓ, undiluted, stored 220 days at −28 °C	8/7/41,8, died
10	7	5 mℓ, diluted 1/10, prior to FD	10/7/41,5, died
10	8	5 mℓ, undiluted, directly after FD	NR[2]
10	9	2,5 mℓ undiluted, directly after FD	NR
5	10	3 mℓ, undiluted, stored 60 days at −28 °C	11/5/41,3, died
5	11	3 mℓ, diluted 1/5, stored 60 days at −28 °C	13/12/41,9, died
5	12	3 mℓ, diluted 1/5, stored 60 days at −28 °C	18/5/41
5	13	3 mℓ, diluted 1/10, stored 60 days at −28 °C	11/6/42, died
5	14	3 mℓ, diluted 1/10, stored 60 days at −28 °C	17/8/40
5	15	3 mℓ, diluted 1/20, stored 60 days at −28 °C	NR
5	16	3 mℓ, diluted 1/20, stored 60 days at −28 °C	NR

[1] 10/6/41,6   = the febrile reaction of S 1 commenced 10 days p.i., lasted for 6 days and attained a maximum temperature of 41,6 °C

[2] NR   = no reaction

TABLE 4 Immunization of oxen with freeze-dried sheep blood infected with Welgevonden stock and subsequently challenged

Ox No.	Febrile reaction to immunization			Treatment	Reciprocal of IFA test titre 1 month after immuni- zation	Reaction category to homo- logous challenge
	Day of onset	Duration in days	Maximum temp °C			
1	16	9	40,6	d4(19)[1]	1280	IV
2	16	6	40,3	d4(19)	1280	IV
3	—	—	—	16 days p.i.[2]	80	IV
4	15	2	40,1	d2(16)	20	IV
5	17	9	39,7	16 days p.i.	80	IV
6	16	9	40,7	NT[3]	320	IV
7	16	12	40,5	d2(17)	320	IV
8	16	8	40,9	d4(19)	80	IV
9	17	7	40	16 days p.i.	20	IV
10	—	—	—	16 days p.i.	20	IV
11	13	8	40,2	d2(17)	320	IV
12	—	—	—	16 days p.i.	−ive	II
13	19	6	41,3	NT	1280	IV
14	—	—	—	16 days p.i.	−ive	I
15	16	7	40,3	d4(19)	80	IV
16	18	8	41	NT	20	IV
17	16	5	40,7	d3(18)	20	IV
18	16	6	41,1	d4(19)	80	IV
19	16	4	40,2	d2(17)	20	IV
20	19	5	40,3	NT	320	IV
21	14	6	41,4	d4(17)	320	IV
22	Control					III
23	Control					I Died

[1] d4(19) = 0× 1 was treated on day 4 of the febrile reaction, 19 days after infection

[2] 0× 3 was treated 16 dates after infection

[3] NT = not treated

with Ball 3 stock, reacted and died, one of which (Sheep 6) after the sample had been stored at −28 °C for 220 days. Two other sheep (Sheep 8 & 9), however, inoculated with undiluted samples directly after freeze-drying failed to react, whereas the control animal (Sheep 7), injected with the infected sheep blood diluted 1:10 prior to lyophylization, reacted and died. The only difference between the freeze-dried samples inoculated into Sheep 2–6 and those injected into Sheep 8 and 9 was that the former had been freeze-dried over a cycle of 30 h and the latter over 53 h. The Welgevonden stock-infected sheep blood similarly freeze-dried over 53 h and stored at −28 °C for 60 days, elicited severe reactions (Sheep 10–14), even at a dilution of 1:10.

Immunization of calves and oxen

Only 2 out of 20 calves showed mild febrile reactions to the lyophilized sheep blood, while the other 18 failed to show any febrile reaction. Two out of these 18 calves, however, had severe reactions when they were challenged 6 months later, and one of them died. The other 16 and the 2 that had shown mild febrile responses when they were immunized, were immune to challenge.

The reactions of the oxen both to immunization and homologous challenge are shown in Table 4. Their reactions to challenge were arbitrarily divided into 4 categories as previously described (Du Plessis & Bezuidenhout, 1979). The 2 animals in Categories I and II were considered susceptible and the other 19 in Category IV immune.

It can also be seen from Table 4 that 4 out of 6 oxen treated 16 days p.i. failed to show any febrile reaction and, when they were challenged, 2 of them were susceptible. It is noteworthy that both these animals were serologically negative a month after infection.

All 4 animals that were not treated had moderate febrile reactions, were serologically positve a month p.i. and immune to challenge. The other 11 oxen

were treated on either the 2nd, 3rd or 4th day of the febrile reaction, which corresponded with days 16–19 p.i. They all recovered, were serologically positive and resistant to challenge.

DISCUSSION

These experiments have shown that homogenized mouse tissue and sheep blood, infected with either the Kümm, the Welgevonden or the Ball 3 stocks of *C. ruminantium*, can be freeze-dried successfully. Although there is some loss of infectivity during the process of lyophilization, freeze-dried tissues stored at room temperature, 4 °C and −28 °C remain infective for 3, 90 and 730 days, respectively. This variation in maintenance of infectivity appears to be directly related to the temperature at which the product is stored.

There is some indication, however, that the duration of the freeze-drying cycle played a role in the case of Ball 3-infected sheep blood. Whereas blood lyophilized for 30 h remained infective, blood freeze-dried over 53 h lost its infectivity to sheep. Sheep blood infected with the Welgevonden stock, however, on 2 occasions retained its infectivity after lyophilization over 53 h. This is merely a preliminary observation based on a single experiment that must be repeated.

Bearing in mind the extreme fragility of the heartwater agent and, comparing e.g. the loss of infectivity within 96 h of tissue in an unfrozen state left at 4 °C (Logan, 1987), the process of lyophilization promises to have great value in the distribution and storage of heartwater vaccine. The present vaccine has at all times to be stored at temperatures below −70 °C, entailing considerable cost not only to the manufacturing laboratory but also to distributing depots of the vaccine. The dispatch of the vaccine in dried ice is also costly and the farmer drawing his supply from a depot is obliged to transport the vaccine on dry ice or in liquid nitrogen.

Since it was found that the lower the storage temperature the better the maintenance of infectivity, the lyophilized product, both in the laboratory and at the depot, should preferably be stored at temperatures below −28 °C. Since, however, it was found that freeze-dried sheep blood stored at −28 °C had lost none of its infectivity after 6 months, for practical purposes this teperature appears to give satisfactory results.

The farmer should, however, derive the greatest benefit from freeze-dried vaccine. He would be able to transport his vaccine requirements for 6 months either from the laboratory or the depot in a cool bag on ice and store it in a freezer at −18 °C, probably the temperature of the average household freezer. It would be important to ascertain the temperature of the average household freezer and determine exactly how long freeze-dried vaccine can be stored at the higher range of this temperature. The easier storage of vaccine should lead to the vaccination of calves, lambs and kids on a much larger scale. In general, calves in beef cattle ranching areas where heartwater is endemic are born over a period of 2–3 months. The bothersome task of frequently obtaining small quantities of the present vaccine in a deep-frozen state and the absolute necessity that it must be used within hours of its arrival on the farm and cannot be stored for any considerable period of time at 4 or −18 °C, limits its use. This is even more so in the case of newborn lambs and kids where the period of natural resistance does not exceed 8–10 days (Van der Merwe, 1987). The fact therefore that throughout the cold chain, from the moment of production to the intravenous inoculation of the vaccine, the lyophilized product is so much less vulnerable than the deep-frozen product, is a definite advantage and places a much more reliable product in the hands of the average farmer.

Although it is unlikely that the Ball 3 stock will be replaced by the Welgevonden stock as a vaccine (Du Plessis, Van Gas, Olivier & Bezuidenhout, 1989; Du Plessis, Potgieter & Van Gas, 1990), the opportunity was used on one hand to ascertain whether a lyphilized vaccine can be used to vaccinate cattle and, on the other hand, to further characterize the behaviour of the Welgevonden stock in cattle.

Nineteen out of 21 oxen immunized with freeze-dried Welgevonden infected sheep blood were fully resistant to homologous challenge 6 months later. The other 2 oxen, both treated 16 days p.i. before showing a rise in body temperature, were susceptible to challenge. Treatment on this day is in conformity with the recommendations in the case of the block method of vaccination (Du Plessis & Malan, 1987b), but it must be borne in mind that these recommendations apply to the Ball 3 stock. This illustrates one of the shortcomings of this method and confirms the observation that the severer the reaction to infection the better the immunity (Du Plessis & Malan, 1987b). The absence of antibody demonstrable with the IFA test in these 2 oxen on one hand and the high titres recorded in the case of animals that had shown moderate to severe reactions on the other, are consistent with earlier observations (Du Plessis & Malan, 1987a).

Regarding the infection and treatment method of vaccination of cattle, the day of the febrile reaction on which treatment should be given, is a much debated point. In the present study, 6 animals, treated once only as late as the 4th day of the febrile reaction, made an uneventful recovery. The natural resistance of the oxen used (Du Plessis & Malan, 1987c) in all probability played an important role. The non-specific resistance of Bonsmara cattle is well known, but then at a later age (Du Plessis, 1985b). Friesland calves 11–14 days old, however, were also only partially susceptible to the Welgevonden stock (Du Plessis & Malan, 1988). The observation in the present study that 4 oxen even survived without treatment, suggests that the older experimental animals as a group had a reasonable level of non-specific resistance, even at the age of 13–14 months.

The behaviour of the Welgevonden stock in newborn calves was similar to that of Ball 3 (Neitz & Alexander, 1945; Du Plessis, Bezuidenhout & Lüdemann, 1984). Only 2 out of 20 reacted mildly to the infected freeze-dried sheep blood and, with the exception of 2 calves that were susceptible to challenge 6 months later, they were immune to homologous challenge.

Nevertheless, contrary to an earlier view (Du Plessis & Van Gas, 1989) the present observations suggest that, whereas the Welgevonden stock is highly pathogenic to sheep (Du Plessis & Van Gas, 1989), its pathogenicity to Bonsmara cattle is only moderate and not much different from that of the Ball 3 stock.

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