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Lyophilisation and resuscitation of sporozoites of *Theileria parva*: preliminary experiments

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Abstract

Lyophilisation of *Theileria parva* sporozoite stabilates used for immunisation of cattle against East Coast fever would greatly improve vaccine storage and delivery. We report three attempts to lyophilise and resuscitate the sporozoites of *T. parva*. Sporozoites survived lyophilisation and were effective for immunisation. Lyophilised stabilate survived for 2 weeks at 5 °C and for 12 weeks at −20 °C. Although the viability of the stabilates was severely reduced during lyophilisation, this work suggests that this method has potential and should be considered for other Apicomplexan parasites such as *Babesia* sp. or *Plasmodium* sp.
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1. Introduction

Live *Theileria parva* sporozoites have been used widely in sub-Saharan Africa to immunise cattle against East Coast fever. *T. parva* belongs to the Phylum Apicomplexa and is transmitted to cattle by the tick, *Rhipicephalus appendiculatus*. In cattle, the sporozoites rapidly enter lymphocytes where the parasites develop into schizonts and transform the cells causing them to proliferate. The uncontrolled multiplication of lymphocytes and their subsequent destruction are responsible for the pathology of the disease. Recovered cattle show a strong cellular immunity that is difficult to induce with dead or recombinant antigen vaccines [1]. The only currently available method of immunisation against *T. parva* is by "infection and treatment" in which living cryopreserved sporozoites and oxytetracycline are inoculated simultaneously [2], conferring a lifelong immunity against homologous challenges [3].

The cryopreservation of sporozoites of *T. parva* [4] was crucial to many of the important discoveries in the biology and control of the parasite. However, throughout east and central Africa where the disease is a major cause of mortality and lost production, the requirement for an ultra low cold chain (<−70 °C) seriously restricts the application of vaccination [3]. In this context, the ability of lyophilisation to preserve the viability of *T. parva* sporozoites was assessed.

Lyophilisation stabilises biological material by desiccation. It is achieved using two overlapping drying procedures, primary drying by sublimation of the ice crystals from frozen material and secondary drying or desorption by evaporation of the free water adsorbed into the dried product [5]. Primary drying requires temperatures lower than the collapse temperature of the formulation so that the matrix of the product remains rigid, as collapse might result in a loss of solubility and potency of the product. Secondary drying requires higher temperatures to rid the product of adsorbed water. Desorption increases the stability of the product but if it is too severe, it leads to the removal of bound water and a loss of viability of the lyophilised cells. There is only one report of the successful lyophilisation of protozoan parasites, that of the spores of *Octosporea muscadomesticae* and *Nosema* sp. by Teetor-Barsch and Kramer [6]. Du Plessis et al. [7] reported the lyophilisation of the rickettsial tick-borne organism, *Ehrlichia* (*Cowdria*) *ruminantium,* and its subsequent use as a live vaccine against heartwater in ruminants. However, *Ehrlichia* is a prokaryote while the more complex eukaryotes, which include the protozoa, are much less

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amenable to lyophilisation. This paper describes the successful lyophilisation and resuscitation of the sporozoites of *T. parva*. Ultimately, lyophilisation could be applied for live vaccines against other protozoan parasites such as *Babesia* sp. [8,9] or *Plasmodium* sp. [10,11], with a great potential impact for the control of these world-wide animal and human pathogens.

2. Materials and methods

2.1. Theileria parva sporozoite stabilate

Three sporozoite stabilates of the *T. parva* Katete stock (A–C) with a concentration of 10 tick-equivalents per ml were produced as described by Marcotty et al. [12]. Engorged nymphal *R. appendiculatus* ticks were harvested from experimentally infected cattle. Ten to 13 weeks after engorgement, the resulting adult ticks were applied on rabbits for 4 days to induce the maturation of the sporoblasts. Samples of tick salivary glands were dissected and stained to estimate the *T. parva* abundance [13] (Table 1). The remaining ticks were triturated for 20 min using a pestle and a mortar containing glass fragments and medium. The stabilate medium was composed of Eagle's minimum essential medium with Hepes buffer, bovine serum albumin and antibiotics. The pH was adjusted to 7.2. The resulting tick material was centrifuged at $49 \times g$ for 5 min and the supernatant containing the sporozoites harvested.

2.2. Experimental setup

The cryopreservation and the lyophilisation of two of the stabilates (A and B) was done using 0.1 mol trehalose (Sigma # T 0167) as protectant. One millilitre of the cryoprotected sporozoite suspension was dispensed in glass vials allowing for a depth of fill of about 4 mm and frozen at −70 °C. The stabilates were lyophilised separately in a Unitop 600 SL® (Virtis) for 24 h. During the first 3 h, the shelves were kept at a temperature of −40 °C then cooling was switched off and the shelf temperature was left to the equilibrium between the thermal capacity of the system and the product sublimation. Throughout the 24 h, the condenser coil had a temperature of −56 °C. After lyophilisation, vials were closed under nitrogen gas, sealed and stored at −20 °C for 3–4 weeks.

Table 1
Protectants used and abundance of infected acini in the salivary glands of the ticks used for the production of the tick-derived *T. parva* sporozoite stabilates

Stabilate	Cryoprotectant used	Lyoprotectant used (mol)	Number of ticks dissected	Abundance of infected acini in tick salivary glands
(A)	Trehalose	0.1	56	51
(B)	Trehalose	0.1	59	18
(C)	Sucrose	0.3	65	51

To assess the viability of the sporozoites, lyophilised stabilates were reconstituted at ambient temperature (20–25 °C) by adding distilled water up to the initial volume of the stabilate and shaking the vials gently, and inoculated immediately into susceptible calves (4 and 10 ml for stabilates A and B, respectively).

To test a cheaper molecule than trehalose, 0.3 mol sucrose (Sigma # S 1888) was used as protectant for a third stabilate (C). Three millilitre of fresh supernatant stabilate were dispensed in 50 ml glass bottles resulting in a depth of fill of about 3 mm, frozen at −70 °C and lyophilised using a Modulyo-4 K® lyophiliser (Edwards). Prior to the application of the vacuum, the shelves of the lyophiliser were cooled for 30 min in a freezer at −20 °C. Lyophilisation lasted 10 h with a coil temperature of −45 °C and a pressure ranging between 300 and 80 Pa. The interface ice–vapour temperature corresponding to a saturation vapour pressure equivalent to the measured chamber pressure was calculated according to Jennings [5] (Fig. 1). Vials were closed and sealed in a vacuum and stored either in the gas phase of liquid nitrogen or in a −70 °C freezer. The stabilate was reconstituted in a similar manner to stabilates A and B. Three millilitre were inoculated into one calf to assess its viability. To determine the effect of storage on viability, vials of lyophilised stabilate C were kept for 7 days at ambient temperature (approximately 20 °C), for 14 days in a fridge (5 °C) and for 2, 5, 8, 10 and 12 weeks in a domestic freezer (−20 °C). Then, 3 ml of neat reconstituted material from each storage temperature or time were injected into one experimental animal. Finally, immunisation by the infection and treatment method using lyophilised stabilate C was attempted in two calves. These animals were injected with 20 mg/kg body weight of long-acting oxytetracycline (Oxyject LA®, Dopharma) at the same time as the stabilate was inoculated.

2.3. Clinical and parasitological monitoring of experimental animals

All experimental animals were Friesian calves of 2–12 months of age. They contained no antibodies to *T. parva* as determined by the immunofluorescent antibody test [14] and were free of ticks and tick-borne parasites. Stabilates were inoculated subcutaneously below the ear. The calves were monitored daily and clinical reactions were classified according to the criteria proposed by Morzaria et al. [15]. Needle biopsy samples taken from swollen lymph nodes and blood smears were stained with May-Grünwald/Giemsa's stain and were examined for *T. parva* schizonts and piroplasms, respectively. All the infected calves, together with at least one susceptible control, were challenged using a lethal dose of homologous stabilate. Experimental animals were maintained and treated according to the Guidelines of the Ethical Committee for Experimental Animals of the Institute of Tropical Medicine of Antwerp (Belgian registration number LA 1100120).

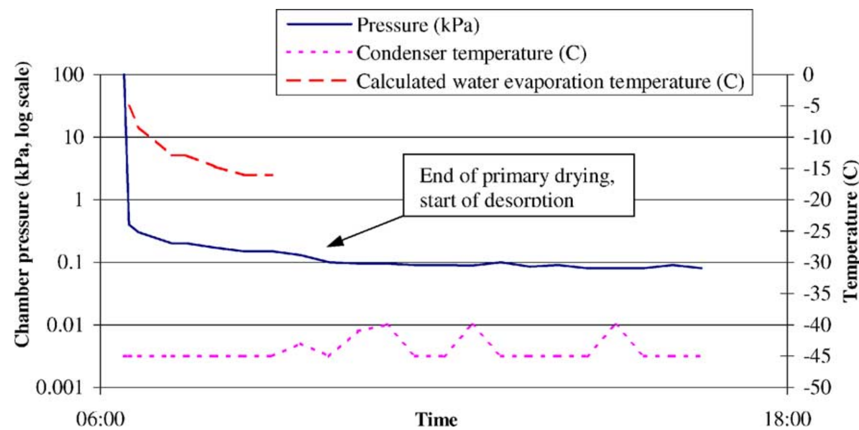

Fig. 1. Lyophilisation parameters of stabilate C.

3. Results

The lyophilised products showed a light and reticulated structure without signs of matrix collapse. Stabilates lost 85% of their weight during lyophilisation and therefore were reconstituted by adding 0.80–0.85 ml of distilled water per ml of initial volume.

All three lyophilised stabilates induced clinical reactions with fever and parasite development in susceptible calves (Table 2). Lyophilised stabilate (batch C) kept for 7 days at 20 °C failed to induce a reaction suggesting that the sporozoites either did not survive or not in sufficient numbers to induce a patent infection or a protective immune response. However, lyophilised sporozoites stored for 2 weeks at 5 °C and for up to 12 weeks at −20 °C survived and were still capable of inducing moderate to severe clinical reactions in most animals (Table 3). Immunisation by the infection and treatment method using 3 ml of 0.3 mol sucrose lyophilised stabilate induced a mild clinical response in the two calves. These animals survived a challenge lethal for susceptible animals, in spite of one of them having developed a clinical response classified as severe [15] accompanied by minor loss of condition.

Table 2
Assessment of the viability of lyophilised stabilates in susceptible calves

Stabilate	Volume inoculated (ml)	Clinical reactions	*T. parva* stages observed
A (trehalose)	4	Severe	Schizonts and piroplasms
B (trehalose)	10	Mild, non-immunogenic	Schizonts
C (sucrose)	3	Severe	Schizonts and piroplasms

Table 3
Assessment of the viability of the sucrose lyophilised stabilate (C) after various conditions of storage: responses to the inoculation of 3 ml of stabilate in susceptible calves

Temperature of storage (°C)	Duration of storage (days)	Clinical reactions[a]	*T. parva* stages observed
Room temperature (20)	7	None	None
Fridge (5)	14	Moderate	Schizonts and piroplasms
Domestic freezer (−20)	35	Severe	Schizonts and piroplasms
Domestic freezer (−20)	56	Moderate	Schizonts and piroplasms
Domestic freezer (−20)	70	None	None
Domestic freezer (−20)	84	Severe	Schizonts and piroplasms

[a] All clinical reactions were immunogenic.

4. Discussion

The detection of schizonts and piroplasms in animals inoculated with lyophilised sporozoite stabilates showed that *T. parva* survived lyophilisation. It is very likely that a large number, if not the majority of sporozoites were destroyed during the freeze–drying process since similar high doses stored by conventional cryopreservation was potentially fatal (unpublished data). However, the sporozoites that survived showed a better storage stability between −20 and 5 °C than non-lyophilised sporozoites kept under the same conditions (unpublished data). The variability in the responses induced by lyophilised stabilate kept at −20 °C (Table 3) could be due to a heterogeneous survival among individual bottles. It could also be due to the fact that low numbers of live sporozoites can induce a wide range of clinical reactions [16]. Based on the parasitological reactions in cattle and the lower morbidity induced by lyophilised stabilates as opposed to non-lyophilised ones, we estimate that 0.1–1% of the sporozoites survived the lyophilisation and resuscitation process. In vitro infectivity [17], which should replace in vivo infections for assessing viability and infectivity, was not sufficiently sensitive to appraise lyophilised stabilates, probably because of the low number of sporozoites that

survived. Despite of this, in vitro infection of lymphocytes was once successful using a different lyophilised stabilate (unpublished data). This additional proof that *T. parva* sporozoites can survive lyophilisation is an encouragement for refining the in vitro infectivity for the evaluation of lyophilised stabilates.

The successful lyophilisation and resuscitation of *T. parva* sporozoites opens the way for a more detailed investigation of the potential of freeze–drying for the preservation of sporozoites and its use in immunisation by the infection and treatment method with great practical potential for the control of East Coast fever. The sporozoite survival may be improved by identifying a more appropriate lyoprotection system and by developing a more suitable protocol for lyophilisation. In our third lyophilisation attempt, lyophilised sporozoites survived a primary drying at a relatively high temperature (−8 °C after 15 min and between −13 and −16 °C for most of the primary drying) and a secondary drying of 6.5 h at least at a temperature of about 25 °C and a pressure of 80 Pa (Fig. 1).

The lyophilisation technique might be useful for the preservation of other apicomplexan parasites, especially those with a potential for using as live vaccines, such as *Babesia* and *Plasmodium*. Sporozoites of *Plasmodium* species infecting humans are expected to suffer more from the lyophilisation process because of their large cell volume. They have a narrow and slender shape and measure 10–14 μm long [18] whereas *T. parva* sporozoites are spherical and measure only about 1.5 μm [19]. Erythrocytic stages of *Babesia* or *Plasmodium* might survive lyophilisation too and be used as live vaccines.

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