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Xerovac: an ultra rapid method for the dehydration and preservation of live attenuated Rinderpest and Peste des Petits ruminants vaccines

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Abstract

The accepted procedure for the long-term preservation of live viruses and bacteria in vaccines has been lyophilisation. We show that thermolabile viruses can be dehydrated in vitro, within 18 h, in an excipient containing trehalose. We further demonstrate that in the resulting dehydrated state, where the viruses are captive in a metastable glass composed of trehalose, they are capable of resisting 45°C for a period of 14 days with minimal loss of potency. The degree of thermotolerance achieved matches that of current 'thermostable' lyophilised vaccines, but with the distinct advantage of a shorter, cheaper and simpler process. The development and utilisation of this process can make significant improvements in current live virus vaccine production. It presents a further step away from dependence on mandatory low temperature refrigerated storage and could lead to greater confidence in vaccine stability, potency and efficacy.

Keywords: Anhydrobiosis; Trehalose; Dehydration

1. Introduction

The preservation of biodegradable materials by dehydration and osmoconcentration is a familiar and ancient technology. When the task of preserving sensitive biomolecules became necessary, simple drying by dehydration failed, as structural water was removed, causing subsequent denaturation and loss of vital activity. Cryopreservation in liquid nitrogen and lyophilisation have become the accepted methods for the long term preservation of sensitive biomolecules, the latter method being used extensively for the preservation of live attenuated vaccines.

Improved thermotolerance of freeze dried Rinderpest vaccine has been achieved by extending the secondary drying cycle, in order to reduce residual moisture (RM) levels to around 1–2%. This entails long and high energy consuming operational cycles of up to 72 h. Vaccines produced by this method are distinguished from the standard vaccine, by the name Thermovax®[1].

Rinderpest is a devastating disease of cattle that has been controlled but not yet eradicated by a very successful international vaccination campaign throughout the 1960s, 1970s and 1980s. The disease has a high economic impact, with high mortality, reduced productivity and loss of income due to the imposition of non-tariff trade barriers on cattle imports from endemic areas.

The FAO Animal Health Service in collaboration with the OAU/Inter–African Bureau for Animal Resources, OIE, and EU, has declared a Global Rinderpest Eradication Programme linking vaccination campaigns in Africa and Asia with the declared intention of global eradication by the year 2010.

Live attenuated veterinary and human vaccines can and do suffer serious deterioration in vaccination campaigns conducted in tropical and subtropical environments. The cause in most cases is due to the difficulty of maintaining the cold chain, which inevitably results in loss of potency, therefore a process that improves the

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thermotolerance of the vaccine exposed to difficult conditions will further disease eradication goals.

During evolutionary natural selection, certain species of plants and animals acquired the remarkable and elegant ability, to tolerate extreme dehydration, remaining dormant in hostile environments for very long periods of time, yet able to assume complete vital activity on rehydration. The resurrection plant *Selaginella lepidophyla*, the brine shrimp *Artemia salina* [2], the yeast *Saccharomyces cerevisiae* [3] and the tardigrade *Macrobiotus hufelandi* [4] are well known examples. Such organisms are termed cryptobiotic and the process by which they survive is known as anhydrobiosis. All species of animals and plants that display this ability contain the disaccharide trehalose (α-D-glucopyranosyl-α-D-glucopyranoside). Its presence generally in the order of 0.2 g/g dry cell weight in most cryptobionts enables them to resist extreme dehydration, high temperatures, X-rays, and also in some species of tardigrades, pressures as high as 600 MPa [5].

Dehydration as a method of preservation for biologicals, has obvious technical and economic advantages. However, freezing and drying are fundamentally different stress vectors [6]. Dimethyl sulphoxide (DMSO) is an excellent cryoprotectant but causes total denaturation following dehydration. Freezing water expands and causes damaging crystallisation. In a terrestrial environment, microorganisms are more frequently exposed to dehydration and trehalose has become nature's dessiprotectant.

In a dehydrating environment, trehalose dries as a transparent glass and the resulting vitrification prevents the expansion of fluids, thus preventing cells from disruption. This is thought to provide a micro scaffold, which supports the tertiary structural integrity of biomolecules and reduces degrading molecular reactions to insignificant levels [7]. Trehalose does not participate in the harmful Maillard reaction found with other reducing sugars [8,9]. It is inert, non-toxic, non-hygroscopic, and redissolves exhibiting flash solubility.

It was generally thought that the intracellular synthesis of trehalose, under genetic control, was essential for anhydrobiosis. However, studies have shown that the mere addition of trehalose to a suspension of biomolecules such as sensitive DNA restriction enzymes will confer thermostability during dehydration, such that they retain full activity even after exposure at 70°C for 35 days [10].

A number of trehalose stabilised pharmaceuticals, and diagnostic reagents, reported in the literature, have been dried without freezing, using relatively small micro litre volumes under atmospheric pressures and at temperatures ranging from 20 to 37°C for periods of 20–36 h [10].

To meet the operational requirements of large scale vaccine production, where unit volumes from 1.0 ml upwards, and production batches of 20 l are the norm, a different strategy is required to remove that volume of water, in an economically acceptable time. Drying at atmospheric pressure even at the highest physiologically tolerated temperatures, would require an unacceptably long time to remove water quickly enough, from partially stoppered vaccine vials, and would inevitably result in denaturation and loss of potency. In order to encourage wide acceptance of the method, it was essential to avoid expensive retooling and concentrate on the use of existing equipment. Thus the use of a freeze dryer, as an aid in faster removal of moisture during the drying process, was a convenient solution.

The following method shows that by suspending live attenuated strains of Rinderpest virus (RP) and Peste des Petits ruminants (PPR) virus in an excipient consisting of half strength Hanks + 1% FCS and 2.5%w/v trehalose dihydrate, it is possible to dehydrate them using a freeze dryer, at a product temperature of 37°C and at reduced pressure to give a 'thermostable' vaccine within 18 h. The success of this method is achieved by careful adjustment of the freeze dryer, to prevent freezing of the product, and by careful control of heat input and pressure. Maintaining a pressure gradient, from the product surface to the refrigerated condenser, positively drives out the water vapour rapidly from product to the condenser. This removes 90% of the water within 60 min. The remaining water being removed down to a residual moisture of less than 2% by extending the drying at 37°C for a period of a further 17 h, reaching a final product temperature of 42.4°C.

2. Materials and methods

2.1. Preparation of the RP and PPR vaccine cultures

Peste des petits ruminants (PPRV 75/1) and Rinderpest (RBOK) strains were grown initially using vero cells in Glasgow modification Eagles medium [GMEM] supplemented with 10% tryptose phosphate broth (TPB, Difco) and 10% foetal calf serum (FCS) as follows:

Vero cells were seeded into 5×150 cm^2 plastic flasks at a cell concentration of 287 000 cells/ml, 60 ml per flask. Two flasks were inoculated with 0.5 ml PPR virus suspension at a multiplicity of infection of 0.03 virus particles/cell. Two flasks were similarly inoculated with RP virus. One flask remained uninoculated as a control.

The flasks were incubated at 37°C in 5% CO_2 and cells examined daily for development of cytopathic effects(cpe). On day 4 the GMEM medium was replaced with Hanks lactalbumin yeast extract (Hanks LYE), containing 2% FCS and 0.1% trehalose dihydrate. On day 6 the cpe was approximately 80% and the virus harvests were pooled without clarification,

frozen,and stored at −20°C. The control flask remained in the incubator for 10 days, and proved to be free from contamination or cell degradation, with no obvious sign of adventitious agents.

2.2. Dehydration procedure

The dehydration procedure used can be considered to consist of two main components similar to lyophilisation: primary drying and secondary drying. The fundamental difference from lyophilisation is that the product is not frozen and drying is by simple dehydration, not sublimation from ice.

The pooled virus suspensions were thawed and diluted 1:1 with a sterile 5% w/v aqueous solution of trehalose dihydrate, thus giving a final concentration of 2.5% w/v trehalose in the mixture. One-ml volumes were distributed into each of 5-ml vaccine vials and partially sealed with dry vented butyl rubber inserts. This operation was carried out at room temperature, in a laminar airflow biohazard cabinet and observing strict aseptic precautions.

2.2.1. Primary drying

The dehydration process was carried out using an Edwards Supermodulyo freeze dryer with precise control over chamber pressure, condenser pressure, shelf and product temperatures. The freeze dryer was prepared in advance before loading the shelf chamber with the vials containing the product. The shelf temperature was raised to 40°C, and the condenser temperature was allowed to reach the operational limit of −40°C. Vials were then placed on the shelves and the contents allowed to reach 35°C. The chamber door was closed with the macro and micro air admittance valves fully opened and the vacuum pump switched on with full gas ballast. The pressure in the chamber was adjusted to 800 mbar by carefully closing the macro air admittance valve. The pressure in the condenser was maintained at 500 mbar in order to produce a pressure gradient between the chamber and condenser and this provided the driving force to induce water vapour to flow from the product surface to the condenser. Partial closure of the vials with the stoppers also had the beneficial effect of throttling the aperture thus increasing the pressure still further at the product surface. It was noticed that the partially closed vials dried quicker than the fully open ones containing the temperature recording thermocouples.

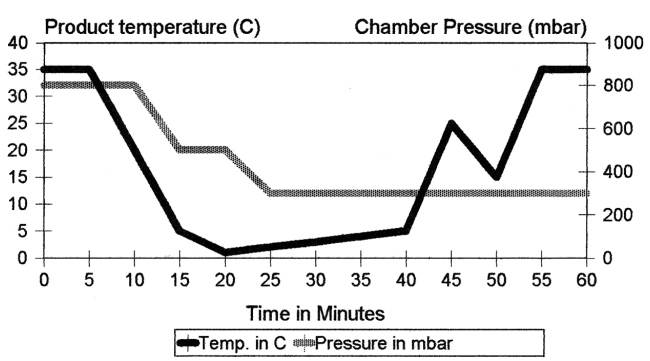

Fig. 1. Primary drying process chart.

Evaporation started immediately as indicated by the fall in product temperature. The product temperature was controlled primarily by carefully closing the macro air admittance valve during the first 15 min, and thereafter by manipulation of the micro air admittance valve, making sure not to allow the product to freeze. Maintaining a temperature around 1–2°C caused by evaporative cooling, increased the evaporation rate so that 90% of the water had evaporated within 1 h and the product temperature began to rise to match the shelf temperature. As dehydration proceeded a critical point was reached after 40 min when there was a sudden rapid rise, of 20°C followed within seconds, by a sudden fall in product temperature of 10°C. This was accompanied by a dramatic bubbling of the product (Fig. 1) with the production of a foamy glasslike matrix.

2.3. Residual moisture determination

The residual moistures were determined using a thermo gravimetric method estimating the mean weight of ten vials from each vaccine after drying for 20 h at 80°C and expressing the weight of water lost from the dried vaccine as a percentage.

2.3.1. Secondary drying

A further batch of RP *Xerovac* (batch 2) was prepared and subjected to primary drying of 1 h, as in the first experiment followed by a period of secondary drying, where the temperature was raised over a period of a further 17 h to a final product temperature of 42.4°C and a pressure of 0.06 mbar, with gas ballast fully closed. This had the effect of reducing the RM to approximately 0.72%.

Another batch of PPR *Xerovac* (batch 2) was prepared where the secondary drying was stopped after a short 2-h period at a product temperature of 42.8°C and a chamber pressure of 0.06 mbar. This resulted in a higher residual moisture level of 5.36%.

A further batch of PPR *Xerovac* (batch 3) was prepared and the secondary drying continued for 24 h in which differing concentrations of trehalose were added to the same excipient. This was to see if the extended drying had the same effect as was seen in the RP virus and to determine if the PPR required more trehalose to improve thermotolerance.

Table 1
Thermostability test of Rinderpest *Xerovac* batch 2

Day of incubation	Virus titre after storage at various temperatures			
	4°C	25°C	37°C	45°C
0	4.97	4.97	4.97	4.97
3	4.83	4.70	4.10	3.80
7	4.83	4.63	4.17	3.37
10	4.80	4.57	4.10	3.30
14	4.87	4.30	3.83	3.03

Table 2
Thermostability test of PPR *Xerovac* batch 2

Days of incubation	Virus titre after storage at various temperatures			
	4°C	25°C	37°C	45°C
0	5.40	5.40	5.40	5.40
3	5.33	4.80	4.57	3.90
7	5.33	4.77	4.40	2.70
10	5.40	4.77	3.97	2.50
14	5.27	4.50	3.60	0.00

Table 3
Rinderpest virus titration results after primary drying

Substance tested	Test 1	Test 2	Mean
Virus pool+2.5% trehalose	5.7	5.9	5.8
Virus pool+2.5% trehalose dried at 37°C for 1 h	5.0	5.7	5.35
Lyophilised reference vaccine	4.8	4.8	4.8

Table 4
Peste des petits ruminants virus titration results after primary drying

Substance tested	Test 1	Test 2	Mean
Virus pool+2.5% trehalose	5.0	4.9	4.95
Virus pool+2.5% trehalose dried at 37°C for 1 h	4.9	4.7	4.8
Lyophilised reference vaccine	4.9	4.9	4.9

2.4. Virus titrations

These were carried out to determine the efficiency of the ultra rapid 1-h dehydration to assess the degree of protection induced by the trehalose glassy foam formed during the primary drying phase and subsequently to monitor the effect of further dehydration for 17 h during the secondary drying phase. The virus titrations were performed as described in the standard operating procedures for the quality control testing of Rinderpest cell culture vaccine [11]. Briefly the titration of virus was carried out using 10-fold dilutions from 10^{-1} to 10^{-8} in cell growth medium using 96-well flat bottomed tissue culture grade microtitre plates ten wells for each dilution 100 μl/well followed by 100 μl of a vero cell suspension containing 250 000–300 000 cells/ml to each well. The resulting monolayers were examined daily for evidence of cytopathic effect and the titre calculated using the Spearman–Karber formula the titre expressed as the 50% median effective dose $TCID_{50}$/ml.

2.5. The test for thermostability

Samples from batch 2 of each of PPR and RP *Xerovacs* were placed at 4, 25, 37 and 45°C. Three vials from each storage temperature were taken for virus titration on days 0, 3, 7, 10 and 14-post incubation. The geometric mean of the three vials was considered as the residual virus titre for each *Xerovac* batch type at each temperature for the specified period of incubation (Tables 1 and 2).

Samples from PPR batch 3 were incubated at 45°C for 3, 5, 8, and 14 days and three vials from each trehalose level were taken for virus titration. The geometric mean of the three vials was considered as the residual virus titre remaining after exposure to 45°C for the indicated times.

3. Results and discussion

The results obtained from paired samples of RP and PPR vaccine dried for 1 h, using the method described above, in comparison with a control sample of lyophilised vaccine, and also with the parent untreated virus pool containing 2.5% trehalose were as depicted in Tables 3 and 4. The excipient containing 2.5% trehalose gave good protection of RP virus following the 1-h rapid dehydration at 37°C under conditions of reduced pressure at 800 mbar with the loss following drying of 0.45 $\log_{10}$ $TCID_{50}$/ml.

The protection of the PPR virus in the same excipient was excellent with only the loss of 0.15 $\log_{10}$ $TCID_{50}$/ml following drying.

The inclusion of a lyophilised reference vaccine of known titre was merely included as a check on the virus titration procedure.

The incorporation of a secondary phase in the dehydration process clearly has a marked effect on the thermotolerance of the product. This is demonstrated by the fact that the RP *Xerovac*, which was subjected to 17 h of secondary drying, lost only 1.9 $\log_{10}$ $TCID_{50}$ after 2 weeks at 45°C.

On the other hand a PPR *Xerovac*, which underwent only 2 h of secondary drying, had no detectable virus after 14 days of incubation at 45°C.

Table 5
Thermostability test of PPR *Xerovac* batch 3 stored at 45°C

% Trehalose concentration	Liquid virus pool titre + trehalose	Residual virus titre of the dried vaccine post incubation					
		Day 0	Day 3	Day 5	Day 8	Day 14	RM%
0	0.0	4.35	0	0	0	0	0.98
1	5.95	5.20	4.25	4.0	2.15	<1.5	1.04
2	5.70	5.10	4.55	4.40	3.30	2.6	1.8
4	5.70	5.20	4.80	4.75	3.45	2.75	2.0
8	5.50	5.00	4.75	4.85	4.15	3.10	1.3

Table 5 shows clearly the protective effect of trehalose on PPR virus dried in its presence for a total of 24 h indicating that the poor result in Table 2 is due to higher residual moisture and not the stability difference between RP and PPR virus. It would appear however that PPR virus might require higher levels of trehalose than required by RP virus to give the same degree of thermotolerance.

The dehydration of Rinderpest and PPR viruses using the anhydrobiotic procedure described, produced a glass-like, honeycombed structure of approximately 10% residual moisture within 1 h. It is hypothesised that the observed exotherm after 40 min drying, might indicate the glass transition temperature of the trehalose excipient under reduced pressure, where the trehalose changes from a liquid and forms a metastable glass [12]. The product in this state with a residual moisture content of about 10% had a micro-crystalline structure and exhibited dramatic 'flash solubility' on rehydration with diluent. Accelerated thermostability tests on the product at 5.36% RM caused unacceptable deterioration as evidenced by huge loss of virus titre (Table 2).

The excipient containing half strength Hanks LYE, 1% FCS and 2.5% w/v trehalose was sufficient to protect both RP and PPR viruses during the 1-h ultra rapid dehydration, when the residual moisture (RM) content was rapidly reduced to 10% (Tables 3 and 4).

Exposure to 45°C for 14 days at 5.36% moisture destroyed the virus (Table 2). However, extension of the secondary dehydration for 17 h had the expected effect of further reduction of the residual moisture (to less than 1%), thereby conferring increased thermostability (Table 1).

The drop in titre of 1.9 $\log_{10}$ $TCID_{50}$/ml, after exposure to 45°C for 14 days, whilst maintaining a minimum titre of 3.03 $\log_{10}$ $TCID_{50}$/ml compares favourably with the expected fall in titre found in the current lyophilised 'thermostable' (Thermovax) vaccines. The complete loss of virus in PPR *Xerovac* batch 2, which underwent only 2 h of secondary drying and then similarly exposed to 45°C, highlights the damaging effect of a high residual moisture content and emphasises the necessity of extending the secondary drying to ensure a low RM.

The effect of different concentrations and extended drying times for PPR virus as described in Table 5 also illustrates the protective effect of trehalose and confirms that low residual moisture is necessary with both PPR and RP virus in order to secure thermostability.

4. Conclusion

The preparation of the vaccine and operating procedure using a freeze dryer for the desiccation of Rinderpest and Peste des Petit ruminants vaccine, without the lyophilisation step of sublimation from ice, exploits the unique property of the disaccharide trehalose to protect tertiary macromolecules during desiccation.

Compared to conventional freeze drying the *Xerovac* method offers the following benefits:

It provides a high level of virus protection, employing a very short, simple, 18-h production cycle, thus reducing production cycle time and energy costs. Basic drying equipment is all that is required, although sophisticated microprocessor controlled freeze dryers can also be used, but are not strictly essential.

The method is tolerant of power interruption, unlike lyophilisation where even a short power failure can cause product melting, leading to unacceptable loss of virus.

The results presented here are the outcome of preliminary investigations, and it is envisaged that shorter drying times and higher levels of thermostability can be achieved. Further experimentation is continuing, the results of which will be presented at a later date.

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