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Research paper

An alternative cold chain for storing and transporting East Coast fever vaccine

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ABSTRACT

East Coast fever (ECF) is an often fatal, economically important cattle disease that predominantly affects eastern, central, and southern Africa. ECF is controlled through vaccination by means of simultaneous injection of oxytetracycline and cryogenically preserved stabilate containing live, disease-causing parasites. Storage and transportation of the stabilate requires liquid nitrogen, a commodity that is commonly unreliable in low-resource settings. Here we show that storage of conventionally prepared stabilate at −80 °C for up to 30 days does not significantly affect its ability to infect cultured peripheral blood mononucleated cells or live cattle, suggesting an alternative cold chain that maintains these temperatures could be used to effectively manage ECF.

1. Introduction

East Coast fever (ECF) is an acute, often fatal, lymphoproliferative, cattle disease caused by the protozoan parasite *Theileria parva* and transmitted by the tick *Rhipicephalus appendiculatus*. ECF predominantly affects eastern, central, and southern Africa and is estimated to be responsible for over $300 million in losses and one million cattle deaths per year (McLeod and Kristjanson, 1999). High-producing breeds are particularly susceptible to ECF-related mortality, impeding the success of many programmatic measures aimed at increasing farmer livelihoods by introducing these genetic lines as full or cross breeds (Giulio et al., 2009; Mckeever, 2006; McLeod and Kristjanson, 1999).

Control of ECF has been attempted by treating cattle with pesticides, such as acaricides. Issues associated with costs, logistics, acaricide-resistance, and contamination of food sources prevented widespread adoption of this approach (George et al., 2004). Immunization using subunit vaccines have also proven suboptimal thus far (Nene et al., 2016). Consequently, ECF is predominantly prevented using the Infection and Treatment Method (ITM) (Radley et al., 1975; Uilenberg, 1999).

Vaccination by ITM involves concurrent injections of live *T. parva* parasites and the antibiotic oxytetracycline. Oxytetracycline suppresses the otherwise highly lethal *T. parva* infection through a largely unknown mechanism that may involve inhibition of sporozoite maturation (Spooner, 1990). ITM results in mild or no symptoms and lifelong ECF immunity.

Live parasites for ITM are extracted from infected ticks, processed, and cryopreserved in liquid nitrogen (LN) or LN vapor until the day of use (Cunningham et al., 1973). As these stabilates are generally formulated infrequently and in large batches at centralized facilities, LN tends to be available for their preparation and transportation to distribution hubs. However, in much of the African continent, including areas of high ECF endemicity, LN is unreliable or absent, which can dramatically impede or preclude ITM program efficacy by constraining ITM outreach (Perry, 2016). Pressurized liquid carbon dioxide (LCO_2), on the other hand, is often widely distributed in low-resource settings such as these due primarily to its use in the carbonated beverage industry (Foster, 2008; Hayford et al., 2004; Linnander et al., 2017).

Like ITM, bovine artificial insemination relies on a LN cold chain. We previously described an alternative cold chain for artificial insemination

Abbreviations: ECF, East Coast Fever; ITM, Infection and Treatment Method; LCO_2, liquid carbon dioxide; LN, liquid nitrogen; FCS, fetal calf serum; PBMC, peripheral blood mononuclear cells; TE, tick equivalents.

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outreach that utilizes on demand dry ice production from commonly-distributed LCO_2 tanks (Kuiper et al., 2020). This cold chain or another involving dry ice could also be used for ITM so long as the stabilate maintains efficacy when transferred from the long-term storage temperature of LN (−196 °C) to dry ice temperatures (−79 °C). It is not known if storage in dry ice would affect the preservation of stabilate vials. This information is critical to assure the quality of a vaccination scheme relying on this type of alternative cold chain.

2. Materials and methods

2.1. Reagents

For *in vitro* studies, the stabilate batch number MCL0201 was purchased from the Centre for Ticks and Tick-Borne Diseases in Lilongwe, Malawi. RPMI-1640, HEPES, fetal calf serum (FCS), gentamycin, 2-mercaptoethanol, and L-glutamine were purchased from Sigma, 96-well flat-bottom microplates were purchased from Nunc, and Polymorphic Immunodominant Molecule recombinant antigen ELISA Kit was purchased from the International Livestock Research Institute, Nairobi, Kenya.

2.2. In vitro infectivity

Infectivity of PBMCs was performed as previously described (Marcotty et al., 2004; Mbao et al., 2005), with minor modifications. Peripheral blood mononuclear cells (PBMC) were isolated from the blood of a Friesian heifer by density gradient. Cells were suspended at 6×10^6 cells/mL in culture medium containing RPMI-1640 supplemented with 25 mM HEPES, 15 % FCS, 50 mg/mL gentamycin (50 mg/mL), 50 μM 2-mercaptoethanol, and 0.2 M L-glutamine. The following day, *T. parva* stabilates were thawed and diluted serially with cell culture medium in 96-well flat-bottom microplates. Then 50 μL of PBMC was added to 50 μL of sporozoite suspension in each well. The plates were incubated for 1 h at 37 °C in an atmosphere containing 5 % CO_2, centrifuged (200 *g* for 10 min), and the excess medium was decanted. Then 150 μL of fresh culture medium was added to each well and the plates incubated for 10 days under the same conditions. On day 10 samples were prepared for cyto-centrifugation before being stained. Briefly, slides were labeled and mounted on slide holders with a filter card and slotted in a cytospin machine (Cytospin 4™ Thermo Fisher Scientific). 100 μL of each sample was added to a slide and centrifuged at 1000 rpm for 5 min. Slides were removed and allowed to air dry before being fixed with methanol for 2 min and incubated with Giemsa's stain for 20 min. Slides were then washed under tap water and allowed to dry. The presence or absence of schizonts on each slide was then determined by light microscopy.

2.3. In vitro assay design

Twenty *in vitro* titration plates were used in five sessions to compare the potency of two test stabilates each stored at −80 °C or LN for 10 and 30 days, respectively. Stabilate stored for 10 days was compared to the control in twelve plates in three sessions, with half the plate rows used for the test and half used for the LN control stabilate. Similarly, half the plate rows were used for the test and the LN control stabilates in sessions comparing storage for 30 days to the control in eight plates in two sessions.

2.4. In vivo infectivity

MCL0201 stabilate straws were transferred to a −80 °C freezer or maintained in LN. After 10 or 30 days 18 or 20 straws, respectively, were removed from cold storage and thawed by rubbing gently between the technician's palms. Stabilate material was pooled for each treatment, mixed thoroughly, and maintained on ice for up to 1 h before inoculation.

Friesian cattle were divided into groups based on similar weight ranges and or average weight. Cattle ranged from 51 to 90 kg or 70–130 kg for 10- and 30-day time points, respectively. A 1 mL dose of the neat stabilate was administered subcutaneously over the right parotid gland in all animals except for animal number B0149 in the test group that received 0.8 mL. Animals were then monitored daily to follow the course of ECF manifestation.

2.5. Detection of schizonts and piroplasms

Schizonts and piroplasms were detected as previously described (Lawrence et al., 1992). Briefly, biopsies were obtained from the prescapular lymph node, blood was collected from the jugular vein, and thin smears were made on slides for schizonts and piroplasms, respectively. Slides were placed in a racked staining jar and fixed in methanol for 2 min, air-dried for 10 min, then stained with Giemsa for 25 min and 30 min for schizonts and piroplasms, respectively. Slides were washed in running water for 1 min and air dried for 30 min. Each blood smear was examined by microscopy for theilerial piroplasms in the red blood cells and schizonts in lymphocytes using oil immersion and a 100x objective.

2.6. Ethical clearance

Animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) of the Faculty of Veterinary Medicine at Lilongwe University of Agriculture and Natural Resources (protocol number IAE/LUANAR/07/2017). All animals were monitored closely by a registered veterinarian for symptoms of infection and general discomfort and treated accordingly.

2.7. Statistics

Stata 11 (StataCorp) was used for *in vitro* assay analysis as previously described (Mbao et al., 2005). Logistic regression was applied on the data using the stabilate (control, test 10 day, and test 30 day) and the logarithm of the dose as explanatory variables. The likelihood ratio test was used to evaluate alternative models using the number of days of storage *i.e.* 0, 10, or 30 as continuous variable or pooling data of 10- and 30-day storage. The cluster effect attributed to the five titration sessions was then taken into account in a robust model (clusters). Effective doses for 50 % positive wells (ED_{50}) were calculated using non-linear combinations of estimators. ED_{50} ratios, estimating the viability loss compared to the control, were calculated in a similar way.

In vivo data were analyzed in Poisson regressions using time of storage at −80 °C and experiment (1 or 2) as categorical explanatory variables. Likelihood ratio tests were performed to evaluate whether regressions using storage at −80 °C as single binary response were significantly different. The goodness of fit of the data to the Poisson distribution was verified for all regressions. Missing values were ignored.

3. Results

3.1. In vitro assessment

T. parva Mugugu cocktail stabilate was maintained in LN or transferred to −80 °C for 10 or 30 days. Potency was then assessed by titrating and measuring the abilities of the differentially stored stabilates to infect cultured PBMCs (Fig. 1). ED_{50} derived from the coefficients of a robust logistic regression using stabilate dose and 10 and 30 days of storage at −80 °C as explanatory variables ranged between 0.0005 and 0.0008 tick equivalents (TE), corresponding to high potency for all three storage conditions that were tested (Mbao et al., 2006, 2005) (Fig. 1D). According to the model, storage for 30 days at −80 °C did not reduce the stabilate viability compared to 10 days ($p = 0.59$). However, the model using time of storage as the continuous explanatory variable was

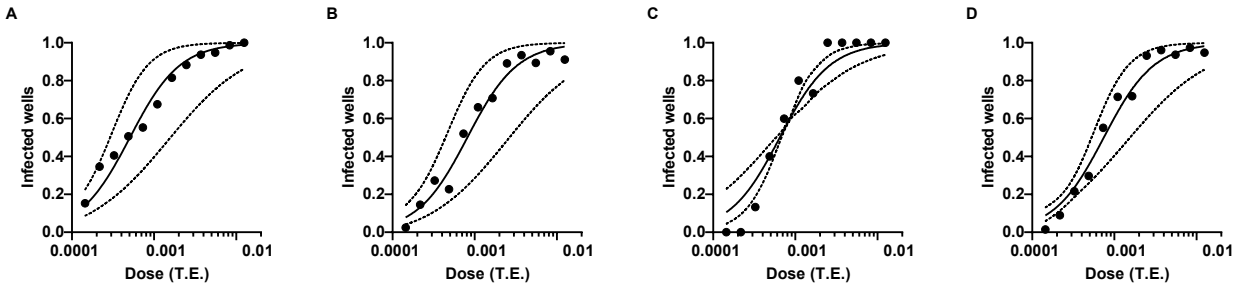

Fig. 1. Titration curves for stabilates stored for (A) 0 (control), (B) 10 days at −80 °C, and (C) 30 days at −80 °C with 95 % confidence intervals (robust logistic regression). (D) Data from (B) and (C) were merged and reanalyzed.

significantly different from the model using time as categorical explanatory variable (likelihood ratio test $p < 0.001$), possibly due to lack of linearity of the time effect.

Storage data for 10 and 30 days were merged for the final model, which did not differ significantly from the first model with three time categories (likelihood ratio test $p = 0.08$). The merged data (Fig. 1D) overcome an artifact caused by lack of observations that incorrectly suggests high precision of fifth data point for the 30 day −80 °C hold data (Fig. 1C). The merged model was used to calculate ED_{50} ratio for equivalence testing between stabilate stored at −80 °C and the control. To this effect a second robust logistic regression gave 0.74 (95 % CI: 0.39–1.43) as ED_{50} for the stabilate stored at −80 °C and 0.51 (95 % CI: 0.18–1.43) for the control. The calculated ED_{50} ratio was 0.68 (95 % CI: 0.43–1.08). The lower limit of this 95 % confidence interval can be used for equivalence testing: with 95 % confidence, it can be stated that the stabilate stored at −80 °C had a potency at least 43 % of the control. In other words, it can be stated with 95 % confidence that storage at −80 °C for 10–30 days caused a viability loss not exceeding 57 %.

3.2. *In vivo assessment*

To assess efficacy *in vivo*, stabilate was maintained in LN or transferred from LN to −80 °C. After 10 or 30 days, 14 cattle were infected with a 1–100 dilution of stabilate and 13 were used as positive controls (stabilate stored in LN). Cattle were monitored for signs of ECF manifestation (Fig. 2 and Table 1). Two animals infected with stabilate stored in LN for 30 days did not react based on schizont score. Their temperature profiles were also inconsistent, suggesting previous *T. parva* infection. Data from these animals were excluded from all analyses except onset and duration of fever. All other animals in the study demonstrated clinical signs of ECF infection.

Animals infected with stabilate stored at −80 °C showed no statistically relevant delays—indicative of less potent infection—for onset of fever, schizonts, piroplasm, swollen right or paratid gland or duration of fever, schizonts, or piroplasms either when 10 and 30 day hold times were analyzed individually or in aggregate (Fig. 2, Table 2). The duration of piroplasms ($P = 0.45$, $N = 24$) and days to death ($P = 0.23$, $N = 11$) were different between −80 °C and LN storage. In all cases death of animals was due to acute ECF.

4. Discussion

LN is the standard medium for storing and transporting *T. parva* stabilate for ITM. In an attempt to skirt the costs and logistics associated with ITM using LN, a group previously attempted to thaw *T. parva* stabilate and maintain on water ice until use (Marcotty et al., 2001). Due to the significant loss in infectivity, the authors concluded that this method compromised ITM to too great of an extent to offer an effective alternative.

Another investigation found that transferring stabilate from LN to −70 °C does maintain efficacy over a six month period, albeit with a slight time-dependent decrease in potency (Mbassa et al., 1998). While encouraging, the study focused on approaches to reduce the use of oxytetracycline and monitoring, and it is not clear how ITM outreach could be implemented at these temperatures.

We previously described a transport device capable of housing bovine semen straws in dry ice produced from commonly distributed LCO_2 tanks (Kuiper et al., 2020). Noting that stabilate and semen straws are the same dimensions, we postulated that the same or similar cold chain could be used to transport *T. parva* stabilate for ITM outreach.

Here we show that transferring *T. parva* stabilate to −80 °C for up to 30 days does not significantly affect its ability of infect cattle or cultured PBMCs, suggesting that dry ice is an effective alternative cold chain for ITM in last mile applications. Equivalence analysis of *in vitro* data found

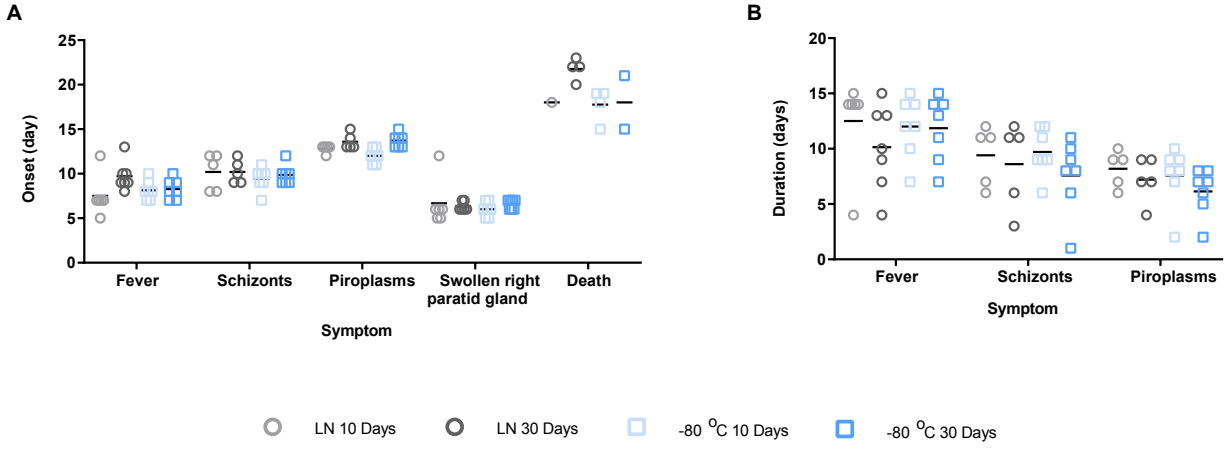

Fig. 2. Summary of clinical signs of ECF infection. Calves were injected with *T. parva* stabilate that had been maintained in LN or transferred to −80 °C for 10 or 30 days. The day of onset (A) and duration (B) of symptoms associated with ECF manifestation are displayed.

Table 1
Clinical signs of ECF infection.

Animal ID	Cyro-preservant	Hold time (days)	Days to fever	Duration of fever	Days to schizonts	Duration of schizonts	Days to piroplasms	Duration of piroplasms	Days to swollen right parotid gland	Days to euthanasia
B0141	LN	10	5	14	8	11	13	6	5	18
B0162	LN	10	7	14	8	12	13	9	6	–
B0195	LN	10	7	14	12	6	13	9	6	–
B0148	LN	10	7	14	12	7	13	7	5	–
B0167	LN	10	7	15	11	11	12	10	6	–
B0278	LN	10	12	4	–	–	–	–	12	–
C0104	LN	30	10	10	–	–	–	–	6	–
C0152	LN	30	8	9	9	11	13	7	6	20
C0187	LN	30	13	7	–	–	–	–	6	–
C0124	LN	30	9	13	9	12	13	9	6	22
C0182	LN	30	10	4	10	3	15	4	7	–
C0161	LN	30	9	13	11	11	13	9	6	22
C0111	LN	30	9	15	12	6	14	7	7	23
B0191	−80 °C	10	8	14	9	9	12	10	6	–
B0139	−80 °C	10	7	12	7	12	11	8	5	18
B0142	−80 °C	10	10	10	11	9	13	7	7	19
B0175	−80 °C	10	7	15	10	12	13	9	5	–
B0122	−80 °C	10	9	7	10	6	12	2	6	15
B0216	−80 °C	10	8	12	9	11	11	9	6	19
B0149	−80 °C	10	8	14	10	9	12	8	7	–
C0158	−80 °C	30	10	9	10	1	14	5	6	–
B0474	−80 °C	30	8	15	9	8	13	8	7	–
C0168	−80 °C	30	9	14	10	10	15	7	7	–
C0112	−80 °C	30	7	14	9	11	13	8	6	21
C0167	−80 °C	30	9	11	12	8	14	7	7	–
C0116	−80 °C	30	7	7	9	6	13	2	6	15
C0155	−80 °C	30	8	13	10	9	14	6	7	–

Table 2
Statistical Analysis of *in vivo* data. Results of Poisson regressions applied to different response variables. Explanatory variable was storage at −80 °C for 10 to 30 days. The difference between 10 and 30 days was significant in none of the regressions. The goodness of fit was significantly different from a Poisson regression in none of the regressions. Bold figures indicate values that should be considered to evaluate stabilate viability loss, assuming storage at −80 °C can only reduce the severity of clinical reactions.

Response variable	N. obs.	Ratio (−80 °C/ LN)	p	Lower	Upper
Days to fever	27	0.95	0.67	0.73	1.23
Duration of fever	27	1.06	0.60	**0.85**	1.33
Days to schizonts	24	0.95	0.67	0.73	**1.22**
Duration of schizonts	24	0.96	0.77	**0.73**	1.26
Days to piroplasms	24	0.97	0.82	0.78	**1.22**
Duration of piroplasms	24	0.89	0.45	**0.66**	1.20
Days to swollen right parotid gland	27	0.97	0.86	0.72	**1.31**
Days to euthanasia	11	0.85	0.23	0.65	**1.11**

with 95 % confidence that viability loss did not exceed 57 %. As ITM injections contain vastly larger numbers of live parasites than are needed for immunization (Patel et al., 2016), these data suggest that any loss in potency would not result in loss of protection *in vivo*. Indeed, *in vivo* infectivity—albeit of a small data set—did not show evidence that stabilate stored at −80 °C for up to 30 days lost infectivity. However, use of −80 °C storage in practice might benefit from dose adjustment.

It should be noted that two animals injected with stabilate stored at LN showed signs of previous infection and were thus excluded from further analysis except for onset of fever. Any bias caused by this change would be more conservative as replacement of missing values would be expected to reduce the reaction severity of LN controls and thus the ratio.

For our study we utilized −80 °C freezers rather than dry ice (−79 °C). While we previously showed that the top, middle, and bottom portions of semen straws maintain dry ice temperatures in a dry ice cold chain (Kuiper et al., 2020), it is possible that that the use of a dry ice cold chain could lead to different outcomes than we observed here.

Our data are highly suggestive that a dry ice cold chain would be effective for ITM. Should the temperature alteration lead to a larger decrease in potency than our *in vitro* analyses found such that *in vivo* protection is compromised, the stabilate concentration could be adjusted accordingly.

A dry ice cold chain could be used to for both artificial insemination and ITM delivery to synergistically improve famer wealth by introducing high producing genetics without the increased risk of animal death by ECF. Efficiencies of using shared equipment and or personnel are also possible. Additional validation will be required to demonstrate the use of a device under realistic field conditions.

5. Conclusion

We investigated the effects of transferring conventionally prepared *T. parva* stabilate to −80 °C for limited durations that are compatible with ITM outreach. Infectivity of both cultured peripheral blood mononucleated cells (PBMCs) and live animals showed no significant effects as compared to LN controls. These results provide foundational justification for the use of a dry ice cold chain system for live vaccination against ECF when LN is scarce.

CRediT authorship contribution statement

David Kalenzi Atuhaire: Investigation, Project administration, Writing - review & editing. **Daniel Lieberman:** Conceptualization, Project administration, Writing - review & editing. **Tanguy Marcotty:** Formal analysis, Writing - original draft, Writing - review & editing. **Antony Jim Musoke:** Investigation. **Damian Madan:** Conceptualization, Project administration, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

the work reported in this paper.

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