

Infectivity of *Theileria parva* sporozoites following cryopreservation in four suspension media and multiple refreezing: Evaluation by *in vitro* titration

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ABSTRACT

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Theileria parva sporozoite stabilates are used for immunizing cattle against East Coast fever and *in vitro* sporozoite neutralization assays. In this study, we attempted to identify a cheaper freezing medium and quantified the infectivity loss of sporozoites due to refreezing of stabilates, using an *in vitro* technique. Pools of stabilates prepared using Minimum Essential Medium (MEM), Roswell Park Memorial Institute (RPMI 1640), foetal calf serum (FCS) and phosphate-buffered saline (PBS) were compared. All were supplemented with bovine serum albumin except the FCS. RPMI 1640 was as effective as MEM in maintaining sporozoite infectivity while the infectivity in PBS and FCS reached only 59% and 67%, respectively. In a second experiment, a stabilate based on MEM was subjected to several freeze-thaw cycles including various holding times on ice between thawing and refreezing. Refrozen stabilate gave an average sporozoite infectivity loss of 35% per cycle. The results indicate that RPMI can be used as a cheaper freezing medium for *T. parva* stabilates and that refrozen stabilate doses need to be adjusted for the 35% loss of infectivity.

Keywords: Cryopreservation, culture media, *in vitro*, refreezing, sporozoites, *Theileria parva*, ticks

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INTRODUCTION

The inoculation of *Theileria parva* sporozoites into cattle usually causes East Coast fever (ECF), an acute and often fatal lymphoproliferative disease of major economic importance in Eastern, Central and Southern Africa (Young, Grocock & Kariuki 1988). This obligate intracellular parasite is transmitted mainly by the three-host tick, *Rhipicephalus appendiculatus*, from which sporozoites can be extracted. These sporozoites extracts are then cryopreserved as stabilates and used for immunization by the infection and treatment (I&T) method, challenge of immune or vaccinated animals, *in vitro* testing and research investigations. The I&T method of immunization (Radley, Brown, Burrige, Cunningham, Kirimi, Purnell & Young 1975) is the only means currently available

for immunizing cattle against homologous challenge (Uilenberg 1999). The technique requires simultaneous inoculation of *T. parva* sporozoites and a long acting tetracycline. It is widely used in several provinces of Zambia and other countries in the region (Uilenberg 1999; Marcotty, Billiouw, Chaka, Berkvens, Losson & Brandt 2001; Fandamu, Thys, Duchateau & Berkvens 2006). Univalent stabilates are used in Zambia but stabilates containing several *T. parva* stocks are required elsewhere, e.g. in Tanzania (Morzaria, Nene, Bishop & Musoke 2000). The extraction of *T. parva* sporozoites into different media has been described for both experimental and field-use stabilates (Cunningham, Brown, Burridge, Joyner & Purnell 1973a; Cunningham, Brown, Burridge & Purnell 1973b; Purnell, Brown, Cunningham, Burridge, Kirimi & Ledger 1973; Kimbita, Silayo & Dolan 2004). These media include bovine serum and Eagle's Minimum Essential Medium (MEM) supplemented with bovine serum albumin (BSA). The stabilates for field immunization are routinely produced using MEM supplemented with BSA.

Well-characterized and homogeneous stabilates need to be available for immunizing cattle against East Coast fever, use in *in vitro* sporozoite neutralization assays and research in general. For developing countries, they should be cheap and easy to produce. The powdered formulation of MEM is much cheaper to import into Africa than the liquid form but is not always readily available. Therefore, other media for their efficiency in maintaining sporozoite infectivity were evaluated. Two media to test and compare with MEM were selected, namely the powder formulation of Roswell Park Memorial Institute (RPMI 1640) and phosphate-buffered saline (PBS). RPMI 1640 is the most common medium used for cell culture while PBS, which does not contain nutrients, is a very basic buffer solution found in most laboratories. BSA, on the other hand, is expensive. Hence, the possibility of using FCS instead of MEM with BSA was investigated.

The objective of the second study was to quantify the loss of infectivity for stabilates undergoing a refreezing step after production. This technique is used in the production of polyvalent ECF vaccines to allow titration of individual components before mixing them. It is also envisaged that, with the onset of veterinary services privatization in Zambia and several other countries in the region, stabilate refreezing may be considered by some animal health service providers in an attempt to salvage left-over doses after an immunization campaign. Refreezing may also be useful in cases where homogeneous stabilate needs to

be used at different time periods for research work. Ensuring homogeneity of the stabilates by pooling and refreezing aliquots for use in particular sets of experiments is one practical method of removing variability in infectivity seen in stabilate taken from different storage vials (V. Mbaio, unpublished observations 2005). From immunization protocol using refrozen stabilates (Njuguna & Musisi 1996), it is known that *T. parva* sporozoites do survive refreezing cycles and it is expected that they lose some viability at each cycle. It is, however, not known to what extent. There is therefore need to have empirical data on the effect of such a process for quality assurance. In this study, sporozoite infectivity was evaluated after single and multiple refreezing cycles. Multiple cycles were included to amplify any effects there might be, and to aid in calculating the average loss per cycle.

Titration of stabilates was done *in vitro*. Equivalence testing (Mbaio, Speybroeck, Berkvens, Dolan, Dorny, Madder, Mulumba, Duchateau, Brandt & Marcotty 2005) was used to calculate the effect of alternative media on sporozoite viability compared with the standard medium (MEM/BSA) and quantify loss of sporozoite infectivity due to refreezing. A random effect model was applied in view of the levels of confounders at tick batches, grinding pools and storage vials.

MATERIALS AND METHODS

Media

All media and additives were obtained from Invitrogen (Carlsbad, California), unless otherwise stated. The following media were used for preparation of the sporozoite suspensions: MEM (with Earle's salts), RPMI-1640 (powder formulation), PBS and heat inactivated FCS. The MEM, RPMI and PBS solutions were supplemented with BSA (Acros Organics, Belgium) at 35 g/l and all of them, including FCS, with L-Asparagine (BDH Biochemical, UK) at 100 mg/l, HEPES (25 mM/l), Penicillin-Streptomycin at 100 iu/ml and Kanamycin at 100 µg/ml. The pH of media was adjusted to 7.0–7.2 using sodium bicarbonate.

Stabilate preparations

Three batches of nymphal *R. appendiculatus* ticks were infected at different times and locations with the *T. parva* Katete stock and allowed to moult to adults in an incubator at 22 °C and 85% relative humidity. Six to 8 weeks after engorgement as nymphs, the adult ticks were fed on rabbits for 4 days to in-

duce sporogony of *T. parva* (FAO 1984) and removed. The ticks in Batches 1 and 2 were divided randomly into groups as shown in Table 1 and ground separately in each of the four media in the volumes shown.

For Batch 1, eight groups were ground (Table 1) using an Omni-mixer Homogeniser® (Omni International, USA, model 17106) following the standardized international protocol (OIE 2005) with some modifications (Mbao *et al.* 2005). The Batch 2 ticks were ground using an Ultraturax® tissue homogeniser (Janke & Kunkel KG, Staufen, Germany, model TP18/2). Batch 3 ticks were ground manually using a mortar and pestle for 15 min (Cunningham *et al.* 1973b). The different methods of grinding were used due to different laboratory set-ups in the three places where the stabilates were produced.

An equal volume of chilled medium with 15% (w/v) glycerol was added drop-wise to the ground up tick supernatant (FAO 1984). The extracts were stirred continuously in an ice bath. The 13 suspensions containing the numbers of ticks per ml (tick equivalents [t.e.]) are shown in Table 1. All suspensions were aliquoted into 1.5 ml cryogenic vials (Nalgene) (1 ml per vial), cooled in an ultra freezer at -80°C for 24 h and then stored in liquid nitrogen.

Refreezing cycles

The stabilate from Batch 3 was used for the stabilate refreezing experiment. A 'cycle' was defined as a refreeze and subsequent thaw process. Vials were thawed by placing them in a water bath at 37°C for 5 min. Before the first refreeze cycle, thawed vials were pooled to ensure homogeneity, centrifuged (400 g for 10 min) to remove fungi and yeasts (Marcotty *et al.* 2004) and supernatants re-aliquoted. In a first step, two titration sessions were set up to compare control and single-refreeze stabilates. Aliquots of the thawed, pooled and centrifuged stabilate material were kept on ice (control material, not for re-

freezing) while the rest was refrozen. Refreezing was for 1.5 h in a -80°C freezer. In a second step, five groups of vials from the same homogeneous pool were subjected to several cycles. All groups were refrozen once. After 1.5 h, four of these groups were thawed and refrozen. After a further 1.5 h, three groups from the previous four were refrozen and this continued until the last group had undergone a fifth cycle. Each group had been subdivided into three subgroups and each of these subgroups was held on ice for 5, 30 and 60 min before a subsequent refreezing.

In vitro titrations

The protocol for *in vitro* titration of *T. parva* tick-derived stabilates that was used is that described by Marcotty, Speybroeck, Berkvens, Chaka, Besa, Madder, Dolan, Losson & Brandt (2004) and modified by Mbao *et al.* (2005). Briefly, test stabilate is serially diluted in 96-well microtitration plates and then bovine Peripheral Blood Mononuclear Cells are added to the wells. The plates are incubated for 10 days at 37°C in 5% CO_2 after which cyto-centrifuged samples are taken, giemsa stained and microscopically examined for *T. parva* macroschizonts.

Titrations for media comparison were conducted in two stages. The first stage comprised six sessions in which all four media were titrated in parallel. There were four sessions for tick Batch 1 stabilates and two for tick Batch 2 stabilates. In the second stage, only MEM and RPMI 1640 stabilates were compared in six sessions: four sessions for tick Batch 1 stabilates and two for tick Batch 2 stabilates. The total number of microtitration wells read for the first and second stages were 855 and 574, respectively. Stabilates from Batch 1 were diluted serially six times (1.5 times per dilution step) and those from the second (Batch 2) 12 times (1.5 times per dilution step). This was necessitated because the first batch had lower infectivity, as determined by a preliminary *in vitro* titration.

TABLE 1 Number of ticks ground per batch per medium

Medium	Batch 1		Batch 2	Batch 3
Vol* (ml)	20	20	25	50
Final conc. (t.e./ml)	5	5	10	10
MEM	200	200	500	1 000
PBS	200	200	500	
RPMI	200	200	500	
FCS	200	200	500	

* Volume of medium per group

TABLE 2 Ratios of effective doses (ED_{MEM}/ED_x) with lower and upper 95 % confidence limits

Medium	Estimate*	Lower limit	Upper limit
PBS	0.59	0.31	1.14
RPMI	1.03	0.63	1.67
FCS	0.67	0.35	1.29

* This ratio is an indicator of the relative infectivity of stabilates in comparison to the reference (MEM)

MEM = Minimum essential medium

X = Test stabilate medium (PBS, RPMI or FCS)

TABLE 3 Percentage loss of sporozoite infectivity (1- ED ratio) after several cycles of refreezing with lower and upper 95 % confidence limits

Cycle	Estimate	Lower limit	Upper limit
Single	32 %	5 %	52 %
Multi	35 %	28 %	40 %

$$ED \text{ (effective dose) ratio} = ED_{\text{cycle}}/ED_{\text{cycle} + 1}$$

The multiple frozen and thawed stabilates (Cycles 1–5) were titrated in parallel a day after the refreezing cycles. Six and two titration sessions for the multiple freezing and control experiments, respectively were conducted. A total number of 1115 and 335 microtitration wells were read for multiple freezing and control experiments, respectively.

Statistical analysis

Data from the two experiment stages of media comparison (i.e. all four media and MEM vs RPMI) were pooled. Wells either scored positive or negative. The binary results were analysed by logistic regression using Generalized Linear Latent and Mixed Models (GLLAMM) in Stata8/SE® (StataCorp, 2003). The proportion of positive wells was the response variable. The three explanatory variables were: the natural log of the stabilate concentration in tick equivalents (*ln* t.e.), the experiment stage and the stabilate medium. Several random effects that could affect the model were identified, namely the tick batch, grinding pools and stabilate storage vials. Random effects are factors or hidden variables that do not interest us but still have an effect on the variability of the data and as such should not be ignored during the analysis.

Comparison of respective infectivities of test stabilate media to those of MEM stabilates were conducted by calculating the ratios of effective doses $[ED_{MEM}/ED_x]$ where ED = the effective dose, in tick equivalent, of stabilate that results in a given propor-

tion of wells to be positive and *x* = test media. The ratios were calculated by use of non-linear combination of estimators (nlcom) in Stata8/SE® (Mbao *et al.* 2005) which also fits 95 % confidence intervals around the estimates (ratios). The level of significance was set at 5 %.

The same model was used for the assessment of the effect of multiple freezing. The natural log of the stabilate concentration (*ln* t.e.), the cycle number and the holding time were continuous explanatory variables. The cycle was regarded as a continuous variable so as to enable estimation of a loss of infectivity per cycle. The response variable was proportion of positive wells and ‘session’ of titration was a random effect. Ratios of effective doses, $[ED_x/ED_{x+1}]$ where *x* = cycle number or holding time, were calculated to compare sporozoite infectivities at each cycle and holding time. To express this as infectivity losses between subsequent cycles, the calculated ratios were subtracted from unit (1-ratio) e.g. a ratio of 0.99 per cycle is actually 1 % loss of infectivity per cycle.

RESULTS

Differences in the infectivity of stabilates prepared and stored in PBS (*n*=215), RPMI (*n*=502) and FCS (*n*=212) were not statistically significant (*P* > 0.05) when compared to MEM (*n*=500). Estimates of ED ratios were 0.59, 1.03 and 0.67 for PBS, RPMI and FCS, respectively (Table 2). The regression curves of predicted values, comparing infectivities, were

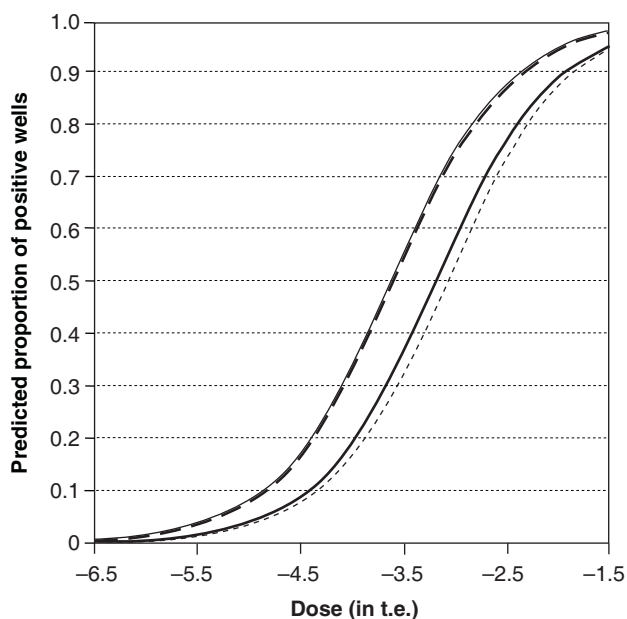


FIG. 1 Titration curves of RPMI (thin continuous line), MEM (thick broken line), FCS (thick continuous line) and PBS (thin broken line) based sporozoite stabilates

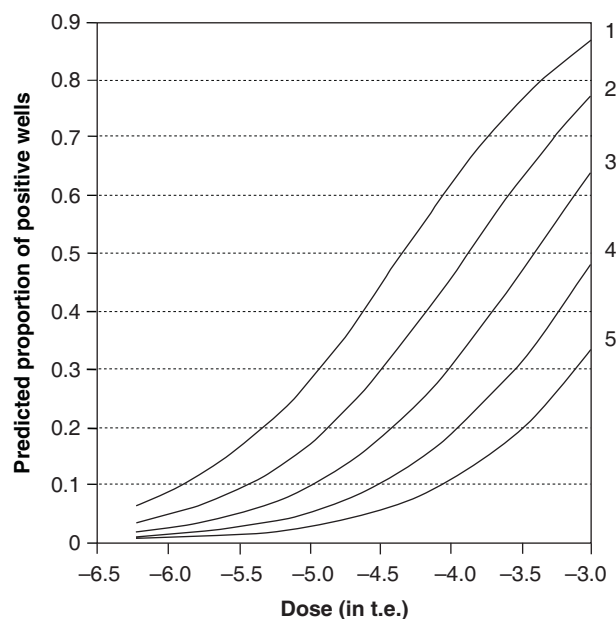


FIG. 3 Titration curves of multiply frozen stabilates (Cycles 1–5)

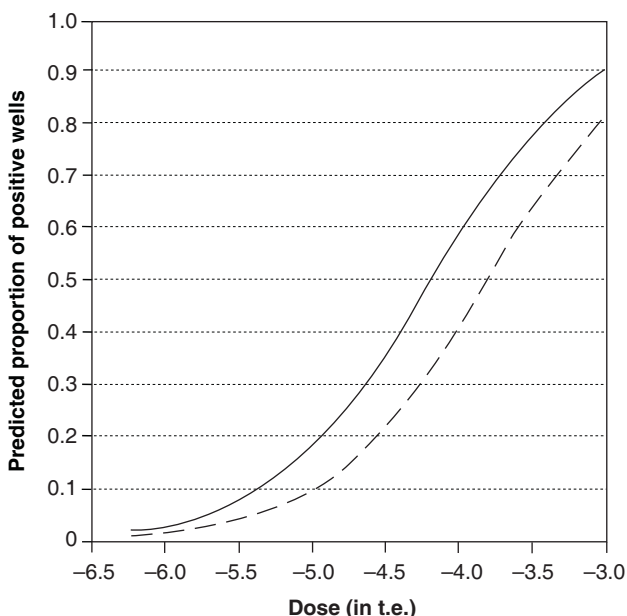


FIG. 2 Titration curves of stabilate that underwent a single re-freeze/thaw cycle (broken line) and control stabilates (1.5 h storage on ice) (solid line)

very close, with MEM and RPMI showing a superimposition of their curves (Fig. 1). While the dose (*In t.e.*) was significant ($P < 0.001$), the stage did not significantly influence the outcome ($P = 0.10$).

A single re-freezing cycle resulted in a significantly lower infectivity ($P = 0.03$) than the control kept on ice for 1.5 h (Fig. 2). The ED ratio was 0.68 (95% CI: 0.48–0.95) and the estimated loss in infectivity

was 32%. The loss during multiple freeze cycles was also significant ($P < 0.001$). On average, the ED ratio was 0.65 (95% CI: 0.60–0.72) giving an estimated loss of 35% per cycle (Fig. 3). In both single and multiple refreezing cycles, dose effect was significant ($P < 0.001$). These results are shown in Table 3. Holding times were not significant ($P = 0.88$) nor was the random effect of session ($P = 0.34$). A stabilate kept on ice for 1 h did not lose more than 30% of its infectivity.

DISCUSSION

Sporozoite infectivities for stabilates prepared in PBS, RPMI and FCS when compared to MEM were not statistically significantly different. However, the RPMI stabilates were closest in infectivity to MEM stabilates as shown by the equivalence test estimate (minimum 0.63 times). This observation agrees with the findings of Kimbita *et al.* (2004) when they compared different media including Leibovitz-15, Optimem and Iscove's MEM. In their study, RPMI and MEM stabilates showed similar infectivity when compared to L-15. Gray & Brown (1981) showed that neat serum could have inhibitory effects on sporozoite infectivity and this could explain why the ED of FCS was lower than that of MEM. However, the effect may not be very pronounced as the confidence interval is wide. FCS is expensive (about €107/ℓ) due to the stringent standards required for international distribution. If it could be produced locally in existing

regional laboratories with nationally or regionally accepted quality control standards from known naive dams, it could be very much cheaper than international grade FCS. Newborn calf serum might also be used (€67.4/ℓ) and would be much cheaper from local sources. The cost of using locally produced reagents would then be lower than commercial media supplemented with BSA (1 ℓ of RPMI with BSA at 3.5% costs €72).

The PBS stabilates were also less infective than MEM stabilates but still showed a reasonably high infectivity (59% of that of MEM). It could be that the nutritive value of the defined medium components may not be as critical as their buffering and cryoprotective qualities during storage. If this is the case, PBS supplemented with BSA has the potential to be the cheapest alternative medium (€62/ℓ). Although PBS and FCS were found to maintain a high proportion of the infectivity of the sporozoite dose, their suitability needs further investigation.

One refreezing cycle significantly decreased the infectivity of sporozoites in comparison to storage for 1.5 h on ice. The latter should not affect the infectivity as Marcotty *et al.* (2001) found that keeping a stabilate on ice for up to 6 h did not reduce sporozoite infectivity significantly. Although the actual freezing temperature was not measured in these experiments, 1.5 h was found to be sufficient to freeze the vial contents to -60°C (Njuguna & Musisi 1996). This is below the critical temperature range (-20 to -50°C) at which ice crystals form and raise extra-cellular solute concentrations, a process that is detrimental to cell integrity in cryopreservation (Farrant 1970). Subsequent refreezing cycles caused similar losses in infectivity.

Refreezing of stabilates seems to induce considerable loss in infectivity. Attempts to refreeze stabilates left over from field immunization would result in low quality stabilates that may not be protective upon inoculation. It would be helpful to have this information in the extension packages for stabilate delivery especially for the private sector involved in ECF immunizations. A re-titration of such stabilate may be necessary to determine appropriate immunizing doses. This would be beyond the expertise of the service provider. Moreover, they may not have appropriate equipment to undertake a standard refreezing process. In unavoidable situations, e.g. polyvalent ECF vaccine production, it would be advisable that the infectivity loss is considered and immunizing doses adjusted accordingly. The process of refreezing may be useful in research work for reducing variability arising from different storage vials. This

involves thawing the stabilate, pooling the contents of vials and refreezing for later use.

Holding the stabilates for up to 1 h on ice did not reduce the quality or infectivity of stabilates significantly. This is important as the process of preparing stabilates, particularly centrifuging and aliquoting, takes time before the stabilate is ready for freezing. In addition, including "batch" as a random effect not only takes into account the tick batches but the different methods of grinding (Omni-mixer Homogeniser®, Ultraturax® and manual) used in these experiments.

In conclusion, the study confirms the findings of Kimbita *et al.* (2004) that RPMI 1640 is as effective as MEM in supporting sporozoite infectivity. Therefore, it is recommended that RPMI 1640 that is properly supplemented can be used as an alternatively cheaper freezing medium in *T. parva* stabilate production where MEM is either too costly or not available. We also showed that there is an estimated loss in sporozoite infectivity of 35% when stabilates are refrozen. This loss needs to be adjusted for in both research and field use stabilates.

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