Comparison of manual and homogenizer methods for preparation of tick-derived stabilates of *Theileria parva*: equivalence testing using an *in vitro* titration model

V. MBAO[2], N. SPEYBROECK[1], D. BERKVENS[1], T. DOLAN[3], P. DORNY[1], M. MADDER[1], M. MULUMBA[4], L. DUCHATEAU[5], J. BRANDT[1] *and* T. MARCOTTY[1]*

[1] *Department of Animal Health, Institute of Tropical Medicine, Nationalestraat 155, 2000 Antwerp, Belgium*
[2] *Department of Veterinary and Livestock Development, Ministry of Agriculture and Cooperatives, P.O. Box 670050, Mazabuka, Zambia*
[3] *Livestock Services Limited, P.O. Box 24437, 00502 Karen, Nairobi, Kenya*
[4] *Centre for Ticks and Tick Borne Diseases, P/Bag A-130, Lilongwe, Malawi*
[5] *Department of Physiology, Biochemistry and Biometrics, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, B-9820, Merelbeke, Belgium*

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SUMMARY

Theileria parva sporozoite stabilates are used in the infection and treatment method of immunization, a widely accepted control option for East Coast fever in cattle. *T. parva* sporozoites are extracted from infected adult *Rhipicephalus appendiculatus* ticks either manually, using a pestle and a mortar, or by use of an electric homogenizer. A comparison of the two methods as a function of stabilate infectivity has never been documented. This study was designed to provide a quantitative comparison of stabilates produced by the two methods. The approach was to prepare batches of stabilate by both methods and then subject them to *in vitro* titration. Equivalence testing was then performed on the average effective doses (ED). The ratio of infective sporozoites yielded by the two methods was found to be 1·14 in favour of the manually ground stabilate with an upper limit of the 95% confidence interval equal to 1·3. We conclude that the choice of method rests more on costs, available infrastructure and standardization than on which method produces a richer sporozoite stabilate.

Key words: *Theileria parva*, immunization, sporozoite, tick-derived stabilate, homogenization, equivalence testing.

INTRODUCTION

Theileria parva is a protozoan parasite that is transmitted to cattle by the 3-host tick *Rhipicephalus appendiculatus* and causes East Coast fever (ECF). East Coast fever is a disease of major economic importance in Eastern, Central and Southern Africa (Young, Groocock & Kariuki, 1988). Its control is achieved mainly by vector control but also by livestock movement control and immunization. The infection-and-treatment method of immunization (Radley *et al.* 1975) is the only available means of conferring immunity to cattle against homologous challenge (Uilenberg, 1999). The process involves simultaneous inoculation of tick-derived sporozoites and a long-acting tetracycline. Production protocols for *T. parva* sporozoite stabilates have been documented for both manual-extraction by mortar and pestle (Cunningham *et al.* 1973*a*, *b*; Purnell *et al.* 1973) and for tissue homogenizers (Kimbita, Silayo & Dolan, 2001, 2004), the latter being recommended by OIE (2000).

Although the use of homogenizers is considered the standard method for sporozoite extraction, no comprehensive study comparing the yield of sporozoites by each method has been published. Homogenization has advantages in the production of large volume stabilates because large numbers of ticks can be processed fairly quickly and the stabilate diluted, cryoprotected, aliquoted and stored in a much shorter time following their removal from animals. The determination of actual numbers of live sporozoites in stabilates has been a challenge for routine production but the introduction of an *in vitro* titration technique (Marcotty *et al.* 2004) in which an effective dose (ED) is determined and taken as the unit of sporozoite concentration has improved quality assessment in the production process.

The present study describes attempts to quantify the difference between the two methods in terms of sporozoite yield. It was hypothesized that manually ground stabilates, taken as the standard in this study, would produce a higher sporozoite yield than machine-homogenized stabilates, and we set out to quantify this difference by equivalence testing.

* Corresponding author: Department of Animal Health, Institute of Tropical Medicine, Nationalestraat 155, 2000 Antwerp, Belgium. Tel: +32 3247 62 63. Fax: +32 3 247 62 68. E-mail: tmarcotty@itg.be

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Equivalence testing goes a step further than significance testing as it can quantify a predicted maximum difference between 2 parameters that are assigned the null hypothesis of non-equivalence. In our study, the multiplicative model (Diletti, Hauschke & Steinijans, 1991) was used to focus on the ratio of the sporozoite yields of the manual to the homogenizer methods.

MATERIALS AND METHODS

Preparation of Theileria parva *sporozoite stabilates*

The *T. parva* Katete stock that had been isolated at Kalapula village in Katete, Zambia in 1984 (Marcotty *et al*. 2001) was used. *T. parva* sporozoites were extracted from infected adult *R. appendiculatus* ticks following their infection as nymphs as described by FAO (1984). Briefly, susceptible Friesian cattle were inoculated with *T. parva* Katete subcutaneously next to the pre-parotid lymph node. When infection developed, *R. appendiculatus* nymphs, collected originally from the vegetation near Wafa village (13°35′ S, 32°30′ E, 980 m) in Eastern Zambia were applied to their ears. The engorged ticks were collected and allowed to moult in an incubator at 22 °C and 80–90% relative humidity. Six weeks after moulting, they were fed on rabbits for 4 days to induce sporogony of the parasite (Kimbita & Silayo, 1997). Following removal from the rabbits, 800 ticks were split randomly into 4 groups of 200. Two groups were homogenized using an Omni-mixer Homogeniser® (Omni International, USA, model 17106) following the OIE protocol (OIE, 2000) and labelled H1 and H2. The other 2 groups were ground manually using a mortar and a pestle (M1 and M2). Sporozoites were extracted in 20 ml of cooled (4 °C) Minimum Essential Medium (with Hank's salts, 35 g/l bovine serum albumin and antibiotics).

For homogenization, a large aperture head (shaft Ø 20 mm, rotor Ø 15 mm) was used at low speed (mark 3) for 2 min followed by 3 min of a small aperture head (shaft Ø 10 mm, rotor Ø 7·5 mm) at the same speed. The receptor (Nalgene® wide-mouth plastic bottle, 60 ml) containing the ticks was kept in an ice bath throughout.

The groups for manual extraction were ground separately for 15 min with a pestle in a mortar containing glass fragments. Extraction was performed by 2 persons in turn to assure continuous and intense crushing. A sample was examined under a stereoscopic microscope to check the quality of tick disintegration.

The resulting tick material was made up to 25 ml in medium obtained from rinsing the tools and receptor used for extraction. The suspensions were then centrifuged separately at 50 ***g*** for 5 min in 10 ml centrifuge tubes in a cooled (4 °C) centrifuge. Supernatant fractions were harvested using sterile Pasteur pipettes, leaving the large tick debris behind, and transferred to a beaker to which an equal volume of cold glycerol (150 g/l) MEM/BSA was added drop-wise: first 5 min 1 drop every 2 sec and thereafter 1 drop/sec. The stabilates were stirred continuously in an ice bath throughout this process. Four batches of 50 ml stabilate each, giving a 4 tick-equivalent (t.e.) per ml concentration were produced. They were aliquoted into appropriately labelled 1·5 ml cryogenic vials (Nalgene®). The tubes were placed in an ultra-freezer (−80 °C) for 24 h and then transferred to permanent storage in liquid nitrogen.

Table 1. Number of wells used to titrate stabilates (M, manual; H, homogenized.)

	Stabilate			
Session	M1	M2	H1	H2
1	48		48	
2	71		72	
3		72		72
4	93			91
5		88	95	
6		48		48
Totals	212	208	215	211

In vitro *titrations*

The *T. parva* stabilates were titrated *in vitro* as described by Marcotty *et al*. (2004), with some 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/ml in culture medium (RPMI-1640 with 25 mM HEPES, 15% FCS, gentamycin, 2-mercaptoethanol, L-glutamine). The following day, test *T. parva* stabilates were thawed and diluted serially in 96-well flat-bottom microplates across columns (6 dilutions by 1·5). Then 50 μl of PBMC were 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 in air, centrifuged (210 ***g*** for 10 min) and the excess medium was decanted. Then 150 μl of fresh culture medium were added to each well and the plates re-incubated for 10 days under the same conditions.

On day 10, cyto-centrifuged samples were prepared and stained with Giemsa's stain. Wells positive or negative for schizonts were scored 1 or 0 respectively. A total of 6 sessions were set up (Table 1).

Statistical analysis

The binary results were analysed using a random effect logistic regression in Stata8/SE (StataCorp,

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2003). The proportion of positive wells was the response variable and the logarithm of the stabilate concentration and the method of extraction were used as explanatory variables. The test session was taken as a random effect given the importance of the clustering effect within test sessions (Marcotty *et al*. 2004).

The model can be written as follows:

$$\log\left(\frac{\pi}{1-\pi}\right) = \alpha + \beta_{\ln(t.e.)} \times \ln(t.e.) + \beta_{method} \times method + \nu + \varepsilon$$

where π is the proportion of positive wells, α a constant, $ln(t.e.)$ the natural logarithm of the sporozoite dose in tick-equivalents, $\beta_{ln(t.e.)}$ the coefficient of the stabilate dose, *method* the method used (0 for homogenizer and 1 for hand-extraction), β_{method} the coefficient of the method, ν the cross-sectional random effect of the session and ε the residual error.

The coefficients of the model were used to estimate ED_{50}, the dose that is effective in 50% of the cases, for the two methods:

$$\ln(ED_{50}) = -\frac{\alpha + \beta_{method} \times method}{\beta_{\ln(t.e.)}}.$$

A comparison of the sporozoite yield by the multiplicative model of the ratio $ED_{hand}/ED_{machine}$ was then made by non-linear combinations of estimators:

$$\ln(ED_{hand}) - \ln(ED_{machine}) = -\frac{\beta_{method}}{\beta_{\ln(t.e.)}}$$

$$\Rightarrow \frac{ED_{hand}}{ED_{machine}} = \exp\left(-\frac{\beta_{method}}{\beta_{\ln(t.e.)}}\right).$$

In the first step, data were regressed independently for the 4 stabilates (2 methods × 2 replications). In the second step, the data were pooled and average estimates generated to obtain a global model per method. The maximum expected ratio was calculated using the delta method (Oehlert, 1992).

RESULTS

The regression model showed a highly significant effect of the natural logarithm of the dose expressed in tick-equivalents, $ln(t.e.)$, on the predicted proportion of positive wells (odds ratio = 16, $P < 0{\cdot}001$). Fig. 1 plots the predicted proportion of positive wells against the stabilate dose for each of the 4 stabilates i.e. 2 that were manually extracted (m) and 2 obtained by homogenization (h). Whereas the curves of the manually produced stabilates overlap, the difference between the ED_{50} of the 2 homogenized groups was 0·01 t.e. and their ratio 1·9 (95% CI: 1·5–2·5).

In the global model (log likelihood = −334, $\chi^2 < 0{\cdot}001$), the difference between the two methods was marginally non-significant (odds ratio = 1·45, $P = 0{\cdot}058$). Fig. 2 shows the estimates and confidence intervals of logistic regression model of the pooled data. The ED_{50} of the two methods are 0·13 t.e. and 0·15 t.e. for the manually ground and the homogenized stabilates respectively.

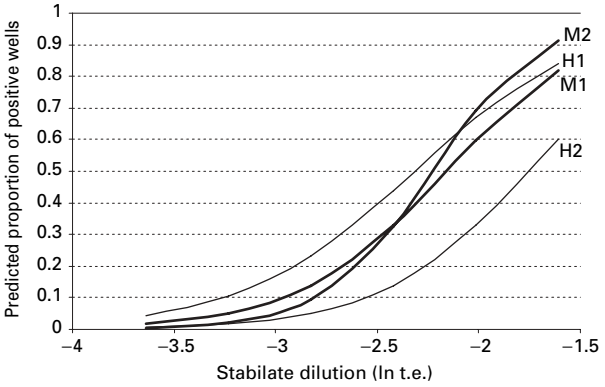

Fig. 1. Variability within and across methods (Manual, thick lines; Homogenizer, thin lines).

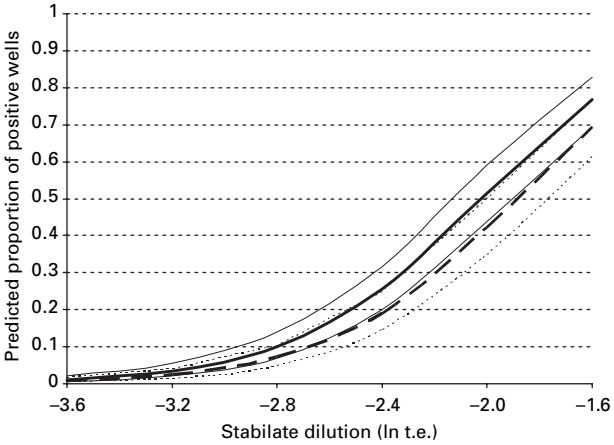

Fig. 2. Titration curves of manually and homogenizer extracted stabilates: estimates (Manual, bold solid line; Homogenizer, bold dashed line) and 95% confidence intervals (thin lines). Upper limit for the Homogenizer curve is partly obstructed by the average of Manual curve.

The ratio ED(*hand*)/ED(*machine*) was estimated as 1·14 (95% CI: 0·99–1·30). The average curves of the two regressions are horizontally equidistant and the dilution factor corresponds to the difference of stabilate potency between the two methods. In other words, machine-ground stabilate was estimated to correspond to a dilution of the manually ground stabilate by 1·14.

DISCUSSION

The manually produced stabilate had a marginally higher yield of sporozoites than the homogenized stabilate, as was hypothesized. Our interest was also to see, not just the significance of the difference but 'how excessive' the difference might be. Previously,

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aspects of *T. parva in vitro* infections have been compared by analysis of variance and statistical significance (Kimbita & Silayo, 1997; Kimbita *et al.* 2001; Wilkie, Kirvar & Brown, 2002; Kimbita *et al.* 2004; Marcotty *et al.* 2004). Here we show the usefulness of equivalence testing; it gives a quantitative measure of how inferior or superior the test parameter (homogenization) is compared to a reference method (manual extraction). The results show that the difference between manual and homogenizer extraction is minimal, in terms of viable sporozoite yield, as the ratio of the ED does not exceed 1·3 (95% confidence interval upper limit) and it is concluded that both methods have equivalent efficiency. It is likely that, if the analysis had been based solely on significance testing, more repetitions of *in vitro* comparisons would have narrowed the confidence intervals and yielded a significant difference. It is for this reason that equivalence testing is recommended as it can show that certain factors, in this case extraction method, do not yield very different results.

Manual-extraction presents the advantage of a much reduced risk of overheating and is easier for monitoring thorough extraction. In addition, the equipment is readily available in most laboratories. However, it is labour intensive, more time consuming and carries a higher risk of contaminating the stabilate. This is more so with *T. parva* stocks like Chitongo (Geysen *et al.* 1999), a stock used in southern province of Zambia for which the immunizing dose is 20 times more than that of Katete. For this stock, tens of thousands of ticks are ground per batch of stabilate. These disadvantages are reduced when using a homogenizer.

Homogenized stabilates showed a relatively higher variability in their titration curves than the manually produced batches. This reflected a higher repeatability for the manual method. The observation was contrary to the expectation that homogenization would give a higher repeatability. It is possible that in the manual method, the ticks were more thoroughly ground. This could also explain the lower ED_{50} for the manual method. This observation might indicate that machine homogenization requires further standardization. However, only 2 stabilates per method were produced and this could be a case of random variation rather than a trend. Furthermore, the difference between the two homogenization repetitions (corresponding to a dilution of 1·9 times) is still rather small, considering other sources of variation such as tick infection rates, stabilate production and/or storage.

Therefore, it would be important to study within-method variability based on a greater number of repetitions and also to test different brands of homogenizer and homogenizing heads in different laboratories (reproducibility). The use of a single homogenizing head could greatly increase the undesirable proportion of whole ticks and tick tissues that are still intact (Berkvens, unpublished observations). It is postulated that the larger head disintegrates the ticks while the smaller head opens up the tick salivary gland acini to release more sporozoites, making the use of the two heads essential for good yields. Furthermore, over-heating of the stabilate could result from excessive speed and/or prolonged homogenization due to blunt homogenizing heads. The effects of prolonged homogenization, while keeping the suspensions at low and preferably constant temperature, have not been studied. This could pose a further risk, if indeed, 'over-homogenizing' induced a mechanical stress for the sporozoites. For these reasons, it might be worthwhile to develop 'homogenizing indicators' to evaluate the extraction quality. Examples could include *in vitro* titrations and monitoring the proportion of uncut ticks. Such indicators could be used to calibrate tick densities, time of extraction, speeds, and other factors for optimum stabilate production. These indicators would also be useful in evaluating the repeatability and reproducibility of the two methods.

Finally, it should be noted that the batches of ticks were ground using a homogenizer and container with only 200 ticks in 20 ml of media while OIE (2000) recommended batches of 1000 in 50 ml. This was limited by the small numbers of ticks available and the need to have sufficient volume to cover the homogenizing heads. These deviations from the standards could have resulted in the higher variability observed among the machine ground stabilates. Differences in terms of shapes of machine receptacles, volume and tick density might also have important effects on the extraction quality and on the optimal extraction speed and time.

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