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# Immunisation against East Coast fever by the infection and treatment method: evaluation of the use of ice baths for field delivery and appraisal of an acid formulation of long-acting tetracycline

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**Abstract**

Immunisation by the infection and treatment method using the Katete strain is currently the most efficient prophylactic technique to control East Coast fever (ECF) in the endemic areas of the Eastern Province of Zambia. The maintenance of the cold chain in liquid nitrogen up to the time of inoculation and the cost of the reference long-acting oxytetracycline (Terramycin LA®, Pfizer) are the main drawbacks of the method. The work presented in this paper aims at reducing the cost of immunisation against ECF by using an ice bath for the field delivery and a cheaper long-acting oxytetracycline formulation as chemotherapeutic agent. In experimental conditions, the results from 40 calves immunised after various periods of storage on ice ranging from 4 to 32 h indicate that deferred immunisation performed with a stabilate kept on ice for up to 6 h after thawing has an efficiency of 90%. Moreover, sporozoites kept on ice were still surviving 32 h after thawing. In a field trial, 91 calves were inoculated with a stabilate kept for 3.5–5.5 h after thawing and dilution whereas 86 calves were immunised using the standard method. Clinical and parasitological reactions to immunisation were monitored as well as the seroconversion. In the field trial, the deferred immunisation was more efficient than the standard method. The acid formulation of oxytetracycline that was tested was found as suitable as the reference alkaline formulation for the

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chemotherapeutic control of the Katete strain in ECF immunisation. One indoor trial was carried out on 10 animals and a field trial involved 93 calves. 

*Keywords:* *Theileria parva*; Immunisation; Cold chain; Oxytetracycline; Zambia

## 1. Introduction

East Coast fever (ECF) is a tick borne disease of cattle caused by the protozoan *Theileria parva*. This agent is responsible for a severe lymphoproliferative disease and a high mortality rate reaching 50% in endemic areas of eastern Zambia (Billiouw et al., 1999). In case of recovery, cattle develop a strong immunity against subsequent homologous challenges. ECF is mainly transmitted by the ixodid tick *Rhipicephalus appendiculatus*. It is the main health constraint for raising indigenous cattle in eastern Zambia (Nambota et al., 1994).

ECF may be controlled through tick control (regular acaricide spray, dip, spot-on or pour-on), curative treatment (parvaquone or buparvaquone) or immunisation. So far the only effective method of immunisation applicable in the field is the infection and treatment (I&T) method described by Radley et al. (1975). It consists in the inoculation of live (cryopreserved) and virulent *T. parva* sporozoites to naive cattle together with a long-acting tetracycline. Since 1987, I&T with a local *T. parva* strain, i.e. the Katete strain, has been extensively applied in calves in Eastern Zambia (about 142,000 immunisations between 1987 and 1999). Maintenance of the cold chain up to final delivery in the field, usually by transporting stabilates in liquid nitrogen, is a serious constraint implying the use of four wheel drive vehicles. The total cost of I&T, including transport, had been estimated around US$ 9.5 for a 80 kg bodyweight calf (D'Haese et al., 1999). If the vaccine could be transported to the field on a motorbike, the cost would be reduced by about 10%. So far, Terramycin LA® (Pfizer) is the only formulation recommended for I&T. Its usage brings the cost of the long-acting oxytetracycline to about 10% of the total immunisation cost. In Zambia, some other alkaline or acid long-acting oxytetracycline formulations are available. In 1998, the cost of acid formulations like Oxyject LA® (Dopharma) reached about 35% of the price of Terramycin LA® (alkaline formulation). The aim of the present work is to reduce the cost of ECF immunisation without affecting its efficiency and to ease the handling of the vaccine in the field. In this respect, the use of cheaper long-acting oxytetracycline formulations and alternative methods for the handling and delivery of the vaccine in the field were evaluated.

## 2. Materials and methods

Immunisation against ECF by the infection and treatment method consists of two injections. A *T. parva* sporozoite stabilate is inoculated subcutaneously over the pre-parotidian lymph node whereas a long-acting oxytetracycline is injected intramuscularly (Radley et al., 1975).

### 2.1. Cattle

Fresian calves were used for indoor trials. They were purchased from commercial farms in Chipata and Lusaka where strict tick control was applied. The calves (60–100 kg bodyweight) were free of ticks, seronegative for ECF and free of any blood parasite when they were brought in. They remained under strict tick control for the whole period of the trials. *T. parva* seronegative local Angoni calves (small Zebu type) were used for the field trials. They grazed in communal pastures where ECF is endemic. They were immunised in November or December, i.e. at the beginning of the main adult *R. appendiculatus* activity period (Billiouw et al., 1999). The bodyweights of both experimental and field cattle were measured using the barymetric method (Rondo®).

### 2.2. T. parva sporozoite stabilate

The stabilate used was produced in Chipata, Zambia. A local strain named Katete, was isolated from cattle in Katete in 1983 by D. Geysen (BADC project, Chipata, personal communication). The medium used is Eagle's minimum essential medium (MEM), 25 mM HEPES (Life Technologies #11012-010) with 3.5% BSA (Acros #24.040.1000), 7.5% w/v glycerol (Merck #1.04094.1000) and antibiotics. The stabilate is stored in liquid nitrogen and thawed before use in water at 38–40°C. It is then diluted at the 1/100 standard dilution (equivalent to the infective material collected from 0.1 tick) for immunisation while the neat stabilate (1/1 concentration) is used for the lethal challenges. For the deferred immunisation, the stabilate is also thawed in 38–40°C water and diluted 10-fold. It is then plunged and kept in an ice bath (±2°C) until use.

### 2.3. Oxytetracycline

Long-acting oxytetracycline (20 mg/kg bodyweight) was used to control the clinical reactions during immunisation. The Pfizer's, Terramycin LA®, was used as a reference. It is an alkaline oxytetracycline dihydrate formulation. In these trials, the use of the cheapest long-acting oxytetracycline available in Zambia, (Oxyject LA® — Dopharma, an acid formulation) was assessed at the same dosage.

### 2.4. Clinical, parasitological and serological monitoring

During indoor trials, the experimental animals were monitored for development of fever, *T. parva* schizonts and *T. parva* antibodies. The rectal temperature of the calves was recorded daily. Fever was defined as a rectal temperature ≥40°C. When lymph nodes were found to be enlarged, lymph biopsies were taken and stained with May–Grünwald for schizont detection. Schizont scoring was calculated as the proportion of infected lymphocytes [macroschizont index (MSI) = number of schizonts observed/number of lymphocytes examined]. A dysimmunisation is defined as an immunisation producing severe side effects, i.e. fever and presence of schizonts in lymph node biopsy. Indirect immunofluorescence antibody test (IFAT) on *T. parva* schizont antigen (Burridge and Kimber, 1972) was carried out weekly

to monitor the specific antibody titres. Fluorescence at 1/40 sera dilution was considered as positive. To test the infectivity of the stabilate, the immunised animals and a negative control were challenged 4–8 weeks after immunisation with a lethal dose of the stabilate, in the absence of tetracycline. An animal was said to be resistant to the lethal challenge if the clinical reaction was absent or moderate according to the classification of Morzaria et al. (1987) (duration of pyrexia and macroschizonts for less than 8 days, no mortality due to ECF).

In the field trials, the animals were regularly examined. Rectal temperature was recorded and lymph node biopsies were collected from sick animals only. Blood samples were taken at the time of immunisation and 28–42 days post-immunisation to assess the seroconversion. The calves that were found seropositive at the time of immunisation were discarded. Again, a 1/40 cut-off titre was used.

### 2.5. Experimental design

In total, six experiments were carried out. The objective of the first one being to define the parameters of a standard immunisation, the next three trials, referred to as deferred immunisations, aimed at assessing the survival of *T. parva* sporozoite stabilates kept on melting ice and their efficiency as immunising agents. Two indoor experiments were conducted to study the survival of stabilate on ice. A field trial was then carried out on a larger number of animals. The objective of the last two experiments, one under experimental conditions and one in the field, was to assess the use of Oxyject LA® (Dopharma) as chemoprophylactic agent for immunisation.

#### 2.5.1. Experiment 1: standard immunisation (indoor)

Ten calves were immunised immediately after thawing the stabilate. These animals and one negative control were challenged with an homologous stock 42 days later.

#### 2.5.2. Experiment 2: indoor deferred immunisation trial (4–8 h)

Twenty animals were split into five groups of four calves each. The groups were immunised 4, 5, 6, 7 or 8 h after thawing the stabilate, period during which the stabilate was maintained in melting ice. The same stabilate was used in all groups. Additionally, two animals were immunised immediately after thawing as positive controls. Terramycin LA® was used as chemoprophylactic agent. All animals including two naïve calves were challenged on day 41 post-immunisation with a lethal homologous dose.

#### 2.5.3. Experiment 3: indoor deferred immunisation trial (8–32 h)

The set up was very similar to the previous experiment, including the use of two positive and two negative controls. Again, Terramycin LA® was used. Five groups of four animals were immunised 8, 12, 16, 24 or 32 h after thawing the stabilate. The animals were challenged 42 days later.

#### 2.5.4. Experiment 4: field deferred immunisation trial

This trial comprised 177 seronegative Angoni calves. Ninety-one of them, randomly selected, were immunised using the deferred immunisation method (3.5–5.5 h in ice bath

prior to immunisation) and the other 86 were immunised by the standard method (i.e. immunisation within 45 min after thawing and stabilates kept at ambient temperature). Two vaccinators immunised alternatively by the standard or by the deferred immunisation method. The calves were bled 35–38 days later and their specific antibody titres were determined.

#### 2.5.5. Experiment 5: indoor Oxyject LA® trial

Ten calves were immunised using Oxyject LA® instead of Terramycin LA® as chemoprophylactic agent. The stabilate was inoculated shortly after thawing. The animals were challenged 43 days post-immunisation. Controls comprised one calf given the immunising stabilate without tetracycline and another given the lethal challenge.

#### 2.5.6. Experiment 6: Oxyject LA® field trial

Ninety-three seronegative Angoni calves were immunised by the deferred immunisation method (i.e. stabilates kept in melting ice for 2–4 h) and Oxyject LA® (Dopharma) was the chemoprophylactic agent used. The calves were bled for serology 42 days after immunisation.

### 2.6. Statistical analysis

Binomial data were summarised by means of proportions and analysed using either contingency tables or logit models as indicated. All hypothesis testing was done using the Chi-square statistic except for contingency tables with expected cell values less than 5 where the Fisher's exact test was used.

## 3. Results

### 3.1. Indoor trials

Two animals died from causes unrelated to ECF, one 30 days after immunisation (verminosis) and the other 13 days after lethal challenge (pink eye complications). They were not recorded as cases of dysimmunisation and both of them were excluded from the results of the lethal challenge. Standard immunisation with Terramycin LA® (experiment 1) produced little side effects. Mild swelling of the pre-parotidian lymph node was noticed, fever was moderate or absent and very few schizonts were observed in lymph node biopsies. The side effects observed after the deferred immunisation (experiments 2 and 3) were even milder (no fever and no schizonts at all were observed) while they were more severe when Oxyject LA® was used instead of Terramycin LA® (experiment 5). The number of animals with fever or schizonts was significantly higher in the group that was immunised with Oxyject LA® (experiment 5) than in the deferred immunisation group (combined data of experiments 2 and 3) ($P < 0.05$) (Table 1). However, no dysimmunisations were observed in any of the groups (Table 1). All animals belonging to the standard and Oxyject LA® immunisation groups seroconverted and survived the homologous lethal challenge

Table 1
Indoor immunisation trials parameters[a]

| | Number of calves | Number of cattle with fever | Mean period to fever (days) | Mean duration of fever (days) | Number of cattle with schizonts | Mean period to schizont observation (days) | Mean max. MSI (%) | Mortality (number of deaths) |
|---|---|---|---|---|---|---|---|---|
| Standard immunisation (experiment 1) | 14[b] | 2 | 18.5 ± 2.1 | 3.5 ± 0.7 | 1 | 14 | 0.5 | 0 |
| Deferred immunisation (experiments 2 and 3) | 40[b] | 0 a | | | 0 c | | | 1[c] |
| Oxyject LA® immunisation (experiment 5) | 10 | 2 b | 15.5 ± 2.1 | 1.5 ± 0.7 | 3 d | 15.8 ± 0.7 | 0.6 ± 0.5 | 0 |

[a] Different letters in a same column indicate a statistically significant difference (Fisher's exact test, $P < 0.05$).
[b] The positive controls of the deferred immunisations are included in the standard immunisation group.
[c] Died of verminosis 30 days post-immunisation.

Table 2
Indoor immunisation trials: lethal challenge parameters

| | Number of calves challenged | Number of cattle with fever | Mean period to fever (days) | Mean duration of fever (days) | Number of cattle with schizonts | Mean period to schizont observation | Mean max. MSI (%) | Mortality (number of deaths) |
|---|---|---|---|---|---|---|---|---|
| Experiment 1 | | | | | | | | |
| Standard immunisation | 10 | 1 | 8 | 1 | 0 | | | 0 |
| Experiment 2 | | | | | | | | |
| Deferred immunisation 0 h | 2 | 1 | 17 | 2 | 0 | | | 0 |
| Deferred immunisation 4 h | 4 | 0 | | | 0 | | | 0 |
| Deferred immunisation 5 h | 4 | 1 | 9 | 7 | 1 | 7 | 20 | 1 |
| Deferred immunisation 6 h | 4 | 0 | | | 0 | | | 0 |
| Deferred immunisation 7 h | 4 | 1 | 15 | 1 | 1 | 7 | 5 | 0 |
| Deferred immunisation 8 h | 4 | 0 | | | 0 | | | 1[a] |
| Experiment 3 | | | | | | | | |
| Deferred immunisation 0 h | 2 | 1 | 9 | 1 | 0 | | | 0 |
| Deferred immunisation 8 h | 3[b] | 1 | 8 | 17 | 1 | 8 | 25 | 1 |
| Deferred immunisation 12 h | 4 | 2 | 8.5 ± 0.7 | 11 ± 5.7 | 2 | 8 ± 1.4 | 13 ± 17 | 1 |
| Deferred immunisation 16 h | 4 | 3 | 8.7 ±1.2 | 13.7 ± 3.8 | 3 | 8 ± 1.7 | 10.3 ± 12.9 | 3 |
| Deferred immunisation 24 h | 4 | 1 | 8 | 9 | 1 | 7 | 25 | 1 |
| Deferred immunisation 32 h | 4 | 2 | 7 ± 1.4 | 12 ± 5.7 | 2 | 8.5 ± 2.1 | 15 ± 14.1 | 2 |
| Experiment 4 | | | | | | | | |
| OxyjectLA® immunisation | 10 | 1 | 17 | 1 | 0 | | | 0 |
| Experiments 1, 2, 3 and 5 | | | | | | | | |
| Negative controls | 6 | 6 | 8.2 ± 1.2 | 13.5 ± 8.9 | 6 | 10.7 ± 4.1 | 16.8 ± 19.3 | 6 |

[a] Died of pink eye complication 13 days after the challenge.
[b] One animal died of worm infestation 30 days post-immunisation, before the challenge.

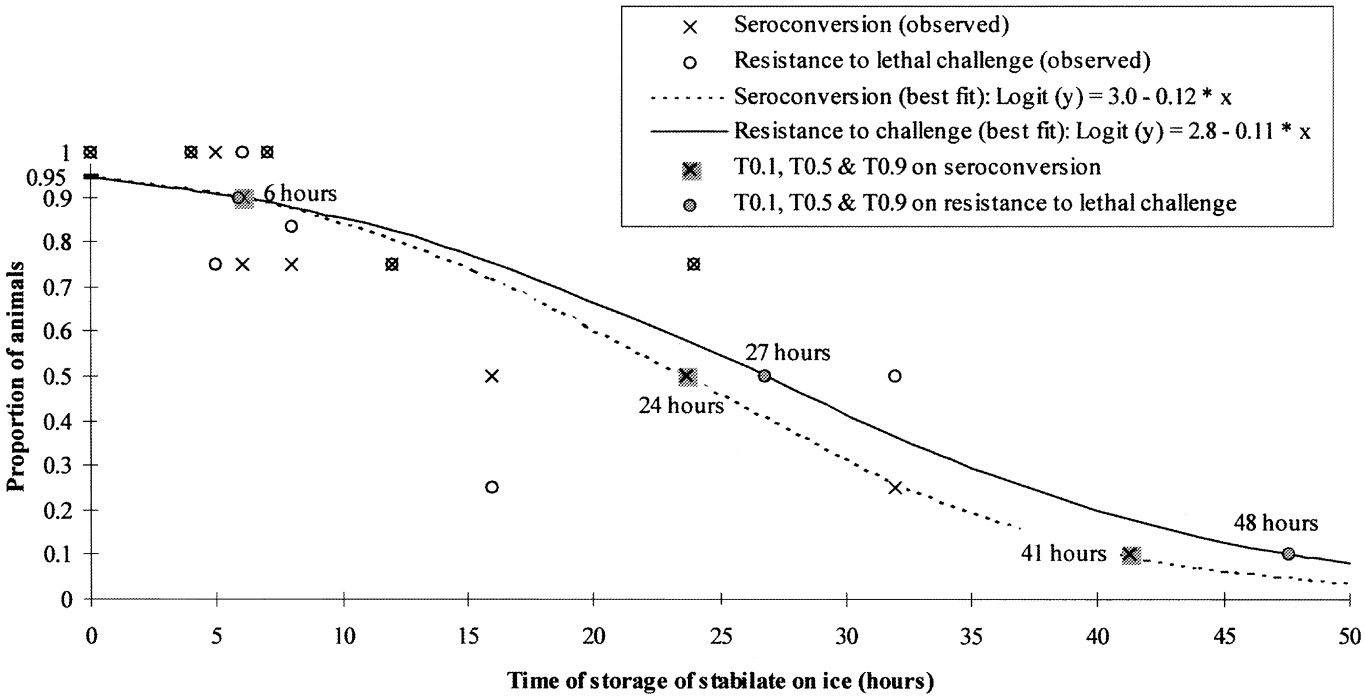


Fig. 1. Indoor deferred immunisation trials (experiments 2 and 3).

Table 3
Field trials: dysimmunisation and seroconversion ratios[a]

| | Number of calves | Number of dysimmunisations | Number of deaths before blood sampling | Number of calves sampled for IFAT | Number of calves with serconversion |
|---|---|---|---|---|---|
| Standard immunisation (experiment 4) | 86 | 2 (2.3%) | 3 | 77 | 54 (70%) a |
| Deferred immunisation with Terramycin LA® (experiment 4) | 91 | 0 | 3 | 86 | 74 (86%) b |
| Deferred immunisation with Oxyject LA® (experiment 6) | 93 | 1 (1.1%) | 1 | 91 | 75 (82%) |

[a] Different letters in a same column indicate a statistically significant difference (Chi-square test, $P < 0.05$).

whereas there was a significant effect of the time of storage of the stabilate on ice on the seroconversion and resistance to lethal challenge ratios ($P < 0.05$) (Table 2 and Fig. 1). The best fit curves and the time of storage on ice producing a 10, 50 or 90% ($T_{0.1}$, $T_{0.5}$ or $T_{0.9}$) seroconversion or resistance to homologous challenge were calculated using a logit model (Fig. 1).

### 3.2. Field trials

The results indicate that the standard immunisation produced the highest number of dysimmunisation [2 on 86 calves (2.3%)] and the lowest seroconversion [54 on 77 calves (70%)]. The immunisation parameters in the two deferred immunisation groups, with Terramycin LA® or Oxyject LA® are similar although one case of dysimmunisation was recorded in the group immunised with Oxyject LA® (Table 3). A rather high number of deaths was recorded, without significant differences between the groups. The cause of the deaths could not be identified.

## 4. Discussion

The results of the indoor trials on deferred immunisation indicate that the *T. parva* Katete stabilate is still infective up to 32 h after thawing when kept on ice. However, its viability is seriously reduced. Musisi and his colleagues (1996) observed that the Muguga trivalent vaccine was still viable up to 15 h after thawing when stored at 4°C. According to the results of the present work, a viability of about 75% is observed after such a period of storage on ice. The effect of the time that stabilates are left on ice on the infection parameters is similar to that which is observed after dilution (Radley, 1981) or irradiation (Cunningham et al., 1973) of sporozoite suspensions, where important individual variability was observed. The loss of viability could be simply due to the effect of the exhaustion of the sporozoites or to a modification of the environment, e.g. pH. Yet, the diluent that was used contained a strong buffering system and no change in colour of phenol red was observed. The estimation of the $T_{0.1}$ revealed that 90% of the animals immunised with a stabilate kept for 6 h on ice seroconverted and became resistant to an homologous challenge. It must be pointed out that immunisation performed immediately after thawing has, according to the curve, an efficiency of 95% only. A reduction of efficiency from 95 to 90% due to the storage on ice for 6 h is probably acceptable for field immunisation, especially if it is accompanied by an important reduction of the cost of field immunisation.

In a previous paper (Marcotty et al., 2000), standard immunisation against ECF using the Katete strain under field conditions produced about 81% of seroconversion, 0.8% of dysimmunisation and a protection rate of over 90%. The results of the present field trials showed a higher efficiency of the deferred immunisation. This might be due to some drawbacks of the standard immunisation. First, small quantities of vaccine were thawed at once, for immediate use, while for the deferred immunisation, larger quantities of vaccine were prepared, to be used over a longer period of time. By preparing larger quantities of vaccine at once, the effect of the variability of viability between different vials of infective material is attenuated. Secondly, the effect of time on stabilate kept at ambient temperature

is expected to be much more pronounced than at 2°C, implying again more variability in the course of a standard immunisation session. This could however not be confirmed in this trial.

An apparent reduction of the dysimmunisation rate in the deferred immunisation is certainly an advantage provided that the protection rate remains unchanged. It can be explained by a more homogenous vaccine, as mentioned above, or by a loss of virulence by the sporozoites (reduction of viability or attenuation). Interference on the seroconversion by natural challenges in the field trials was probably insignificant: no case of ECF was recorded between immunisation and sampling, although some calves died. In addition, the field trials were carried out at the end of the warm dry season and just before the main adult *Rhipicephalus appendiculatus* activity peak. Furthermore, in the event of a natural challenge, the absence of clinical cases would mean that the animals were adequately immunised.

Continuous maintenance of a cold chain by liquid nitrogen implies the presence of highly qualified staff in the field for the preparation of the vaccine. On the contrary, if the stabilate is diluted and transported on ice, paraveterinarians using small forms of transport such as motorcycles can easily inoculate it into animals. Such an ECF immunisation distribution network would be dramatically cheaper, in contrast to a set-up that makes use of four wheel drive vehicles.

An other way of reducing the cost of ECF field immunisation is by the use of cheaper brands of long-acting oxytetracycline. Acid formulations are far less expensive than the alkaline ones that have been used so far. The pain induced at the site of inoculation is one of the disadvantages of the acid formulations. However, cattle keepers in the Eastern Province of Zambia have been using acid formulations for a long time without major complaints about this side effect. In fact, the Angoni cattle seems to be fairly tolerant to the irritant effect of the acid formulation when compared to the Friesian breed. The only concern remains the efficiency of the acid formulations to control *T. parva* in the process of immunisation.

Tetracyclines control the multiplication of parasites by inhibiting the synthesis of parasitic proteins (Aiello and Mays, 1998). This delays the development of the parasite, an effect which abates with time as the plasmatic concentration of the oxytetracycline reduces, by then the host's immune system should be capable to control the infection. Yet, long-acting oxytetracycline formulations might suppress excessively the less virulent *T. parva* stabilates, hampering the efficiency of immunisation, as reported by Hove et al. (1995) on immunisation trials using the Boleni strain and an acid long-acting oxytetracycline.

With Terramycin LA®, the plasmatic peak is observed 4 h after a 20 mg/kg intramuscular injection and reaches about 3 μg/ml. The half-life is about 24 h. The critical plasmatic concentration (0.5 μg/ml) is reached 84 h after an intramuscular injection (Davey et al., 1985).

If the pharmacological profiles of both alkaline and acidic formulations are similar, they are expected to have a similar suitability for ECF immunisation. The role of the oxytetracycline being to control the virulence of the inoculated *T. parva*, efficiency can be monitored by the proportion of dysimmunisations and reactions after immunisation. No significant difference was observed neither in experimental conditions nor in the field trial. The high percentage of calves with seroconversion does not only confirm that the virulence of the stabilate used for immunisation was sufficient but it also shows that Oxyject LA® did not control excessively the development of *T. parva* in the host.

These results show that any long-acting tetracycline presenting similar plasmatic profiles may be considered for ECF immunisation by the infection and treatment method. This will broaden the market and allow for competition between the manufacturers, which can only reduce the cost of immunisation to the benefit of the cattle keepers. The fact that the cost of the chemotherapeutic component is markedly reduced makes the cost for immunisation of adult cattle in epidemic situations almost the same as for calf immunisation (Elyn, personal communication). However, according to the manufacturers' recommendations, care should be taken not to use milk for human consumption within 5–7 days of a long-acting oxytetracycline (20%) injection (Terramycin LA® and Oxyject LA®, respectively) and beef within 14–28 days (Oxyject LA® and Terramycin LA®, respectively).

## 5. Conclusion

The deferred immunisation method is still sufficiently efficient up to 6 h after thawing if the *T. parva* sporozoite stabilate is kept on ice. Consequently, the reduced cost of immunisation and the simplification of the field delivery method compensates, to a large extent, the minimal loss in efficiency. *T. parva* sporozoites were found to be alive up to 32 h after thawing and storage on ice. The effect of a long storage time on ice is similar to that observed by dilution or irradiation of stabilates on infection parameters in cattle probably by reducing the number of viable sporozoites. In large scale immunisation programmes, the deferred immunisation presents the advantage of giving more homogenous results when compared with the standard method. The acid formulation of long-acting oxytetracycline was found suitable for the chemotherapeutic control of *T. parva* type Katete in the infection and treatment method of immunisation in Angoni cattle. Its use would reduce the cost of immunisation, allowing for a consideration of immunising adult cattle in epidemic situations. Any long-acting oxytetracycline formulation with a similar plasmatic pattern could probably be considered for use in ECF immunisation. This would in turn allow the use of cheaper and widely available drugs for ECF immunisation.

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