MAXIMIZING *THEILERIA PARVA* SPOROZOITE MATURATION IN ADULT MALE AND FEMALE *RHIPICEPHALUS APPENDICULATUS* BY VARYING TICK AGE AND FEEDING CONDITIONS: *IN VITRO* STUDIES.

E. N. Kimbita and R. S. Silayo. Department of Veterinary Microbiology and Parasitology, Faculty of Veterinary Medicine, Sokoine University of Agriculture P.O. Box 3019, CHUO KIKUU, MOROGORO - TANZANIA.

(Accepted January 9, 1998)

SUMMARY

In vitro infectivity assays were carried out to determine the optimal conditions for stimulating *Theileria parva* sporozoite development in male and female *Rhipicephalus appendiculatus.* The conditions investigated were the interval between nymphal repletion and feeding of adults; feeding on rabbits for four days; incubation (at 37°C, 85% relative humidity) for 2 days followed by feeding on rabbits for 3 days or incubation for 6 days. The ticks were dissected and their salivary glands homogenized to provide sporozoite suspensions for *in vitro* titration assays. Feeding of ticks on rabbits for 4 days produced significantly more sporozoites in females than males (P= 0.010). However, male ticks incubated prior to feeding produced significantly more sporozoites than males fed on rabbits for 4 days (P=0.023). When the ticks were incubated prior to feeding there was no significant difference between the males and females (P= 0.125). Similarly, there was no significant difference between female ticks fed without incubation and those incubated prior to feeding (P= 0.625). Whether the ticks were incubated prior to feeding or fed without incubation, maximal titre were observed at 35 days after repletion as nymphs and a significant decline noted at day 42 with the exception of females (P= 0.021). Incubation for 6 days without feeding produced very low titre, and infective sporozoites were only detected when the ticks were tested 35 days after repletion. At 28 and 42 days, yields were low and the sporozoites were not infective to lymphocytes.

INTRODUCTION

Theileria parva causes fatal diseases of cattle variously known as East Coast fever (ECF), Corridor disease or January disease in East,

Central and Southern Africa respectively. About 25 million cattle are at risk to the disease, of which more than one million may die annually (Mukhebi *et al.*, 1992). *T. parva* is transmitted by a three host tick, *R. appendiculatus* in which the parasite develops through gametogony in the gut and sporogony in the salivary glands. The ticks are found in a wide range of climatic conditions which may have important effects on their life cycle as well as the development and transmission of *T. parva*. Previously, completion of sporogony in *T. parva* was thought to be dependent on the feeding of the tick on a mammalian host (Purnell *et al.*, 1973). However, unfed infected *R. appendiculatus* incubated at 37°C for 6 days have been shown to produce sporozoites infective for cattle (Young *et al*, 1979). It has also been shown that exposure of infected ticks to high ambient temperatures in the field can induce development of infective sporozoites (Young *et al.*, 1987). However, incubated ticks produced few sporozoites compared to those fed on rabbits, and the age of ticks was important if sporozoites were to be produced (Young *et al.*, 1984). Completion of sporogony in unfed ticks could have some practical implications in terms of transmission of *T. parva* in the field. Additional possibility exists for maximizing sporozoite development by varying incubation and feeding conditions of *T. parva*-infected *R. appendiculatus* for stabilate preparation. This paper reports of a new and improved method of stimulating sporogony of in *R. appendiculatus* and also investigates the effects of the age of ticks on production of infective sporozoites.

MATERIALS AND METHODS

Experimental animals.

A sero-negative (To *T. parva, Anaplasma marginale,* and *Babesia bigemina*) Ayrshire (*Bos taurus*) steer, G113, reared indoors under tick-free conditions was used as a blood donor for preparation of peripheral blood lymphocytes. Four Boran (*Bos indicus*) steers BH195, BJ30, BJ182 and BK152 were infected by inoculation of 1 ml of thawed and diluted (1:20) *T. parva* (Muguga 3087) stabilate subcutaneously below and in front the right ear, for transmission of infection to ticks. Prior to infection the animals were screened and found negative for antibodies against *T. parva, B. bigemina* and *A. marginale*. The experimental cattle were maintained indoors on hay and pelleted concentrates (Unga Feeds Co.,Nairobi, Kenya) and drinking water was provided *ad libitum*. Once a week, the

animals were sprayed with dioxathion (Delnav DFF, Wellcome Kenya Ltd, Nairobi, Kenya) except one week prior to and during the feeding of ticks. New Zealand white (NZW) or NZW cross-bred rabbits reared in an insect-proof facility, were used for feeding of ticks.

Media.
The growth medium used in these studies was Leibovitz-15 (L-15) (Sigma Chemicals Company, St Louis, MO, USA) supplemented with 15% foetal bovine serum (FBS)(Hyclone Laboratories, Utah, USA), 10% tryptose phosphate broth (Gibco Laboratories, Grand Islands, NY, USA), 50 mg/ml gentamicin (Flow laboratories, Irvine, UK), 100 I.U/ml penicillin and 100 mg/ml streptomycin (Sigma). Eagle's Minimum essential medium (MEM) with Earle's salts (Sigma) and 3.4% bovine serum albumin (BSA) (Sigma) with the addition of 50 units nystatin/ml (Gibco) was used for dissection of ticks. Eagle's MEM with Earle's salts (Sigma) and 3.4% bovine serum albumin (BSA) (Sigma) and 7.5% glycerol (Sigma) was used to dilute sporozoite stabilates.

Ticks and parasite stabilate.
Uninfected *R. appendiculatus* originally obtained from the East African Veterinary Research Organization (EAVRO) Muguga, were maintained by feeding on rabbits as described by Bailey (1960). *Theileria parva* (Muguga 3087) stabilate was used in these studies.

Peripheral blood lymphocytes.
Blood was withdrawn aseptically from the jugular vein of steer G113 and the lymphocytes were separated according to the method of Brown (1979).

Preparation of sporozoites suspensions.
Adult infected ticks were stimulated by either of three methods described below to induce maturation of sporozoites. The ticks were removed from the rabbits' ears and washed with soap and water. They were then dissected aseptically as described by Purnell and Joyner (1968).

The effects of different methods of stimulation of sporogony on the yield of sporozoites.
Nymphs of *R. appendiculatus* were fed on two steers BH195 and BJ30 which had been infected with *T. parva* (Muguga 3087) and were harvested 17 days after infection when the piroplasm parasitemia was rising. The nymphs were allowed to moult and 42 days after repletion as nymphs, using a stratified random sampling based on sex, 300 adult ticks were divided into three groups A, B and C. Ticks in group A were applied

in a bag to the right ear of a rabbit and allowed to feed for 4 days before they were removed and washed with soap and water. Ticks in group B were incubated aerobically at 37°C and a relative humidity of 85% for 2 days before being fed on rabbits for 3 days. Ticks in group C were incubated at 37°C and a relative humidity of 85% for 6 days as described by Young *et al.* (1984). Twenty male and 20 female ticks from each group were dissected aseptically and salivary glands from male ticks collected separately from those of female ticks, in growth medium. The salivary glands were homogenized in 100 ml growth medium and centrifuged at 50 'g' for 5 minutes. Ninety ml of the supernatant were collected and made up to 100 ml using growth medium. The supernatant was titrated by the method of Spooner (1990) and incubated with lymphocytes as described by Brown *et al* (1973). The cultures were maintained and infectivity of the sporozoites was evaluated as described elsewhere (Kimbita and Silayo, 1997).

Stimulation of sporogony was in addition carried out at 28, 35 and 42 days post nymphal repletion. Stimulation conditions and evaluation of infectivity were as indicated above.

Statistics.

Title of infectivity were compared using the Analysis of Variance. The level of significance was $P<0.05$.

RESULTS

The effects of different methods of stimulation of ticks on the yield of sporozoites.

The effects of different methods of stimulation on sporozoite yield as assayed by the *in vitro* titration of infectivity are shown on Fig. 1. Female ticks fed on rabbit for 4 days produced significantly ($P= 0.010$) more infective sporozoites than their male counterparts. Sporozoites from male ticks, incubated prior to feeding had a significantly higher title ($P= 0.023$) than those of males fed only on rabbits for 4 days. No significant difference in the sporozoite infectivity was observed between male and female ticks ($p=0.642$) when they were incubated for 2 days at 37°C followed by feeding on rabbits. Similarly there were no significant differences between females incubated prior to feeding and those fed without incubation ($P= 0.625$).

It was further observed that sporozoites harvested from ticks which were stimulated by incubation at 37°C for 6 days were not infective to bovine lymphocytes. This was also evident when ticks were incubated for 10 days at 37°C.

The effects of different times after

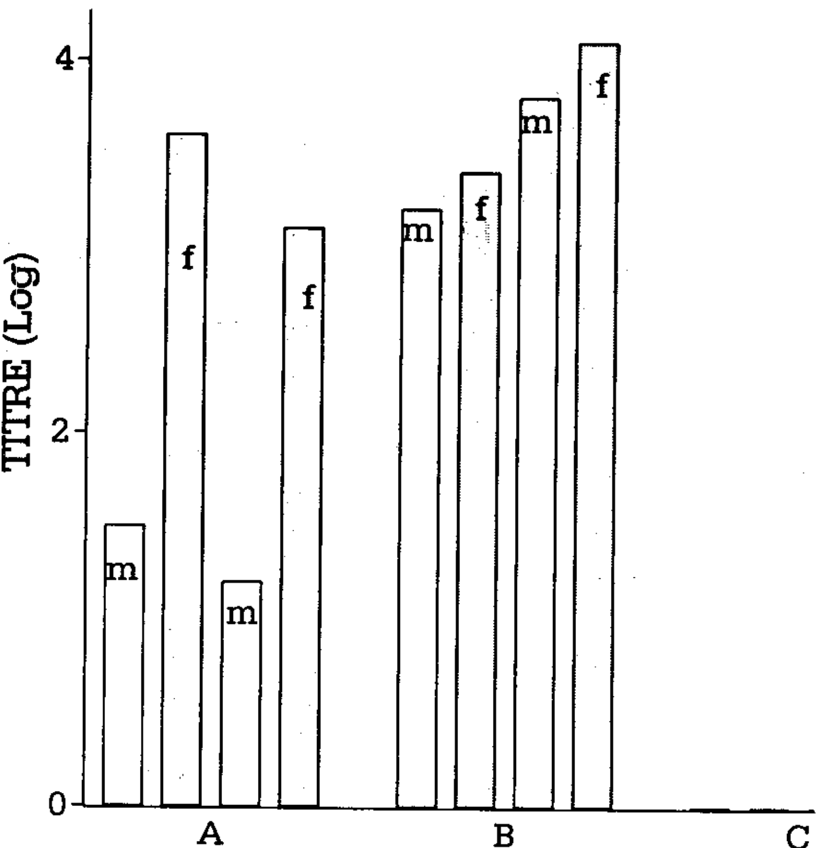

Figure 1. The effect of incubation on sporogony of *Theileria parva* in *Rhipicephalus appendiculatus* ticks as assayed *in vitro*. Ticks stimulated by method A were fed on rabbits for 4 days whereas in method B ticks were incubated at 37°C and 85% relative humidity for 2 days and then fed on rabbits for 3 days. In method C ticks were incubated at 37°C and 85% relative humidity for 6 days without feeding on rabbits. M and F indicate male female ticks respectively.

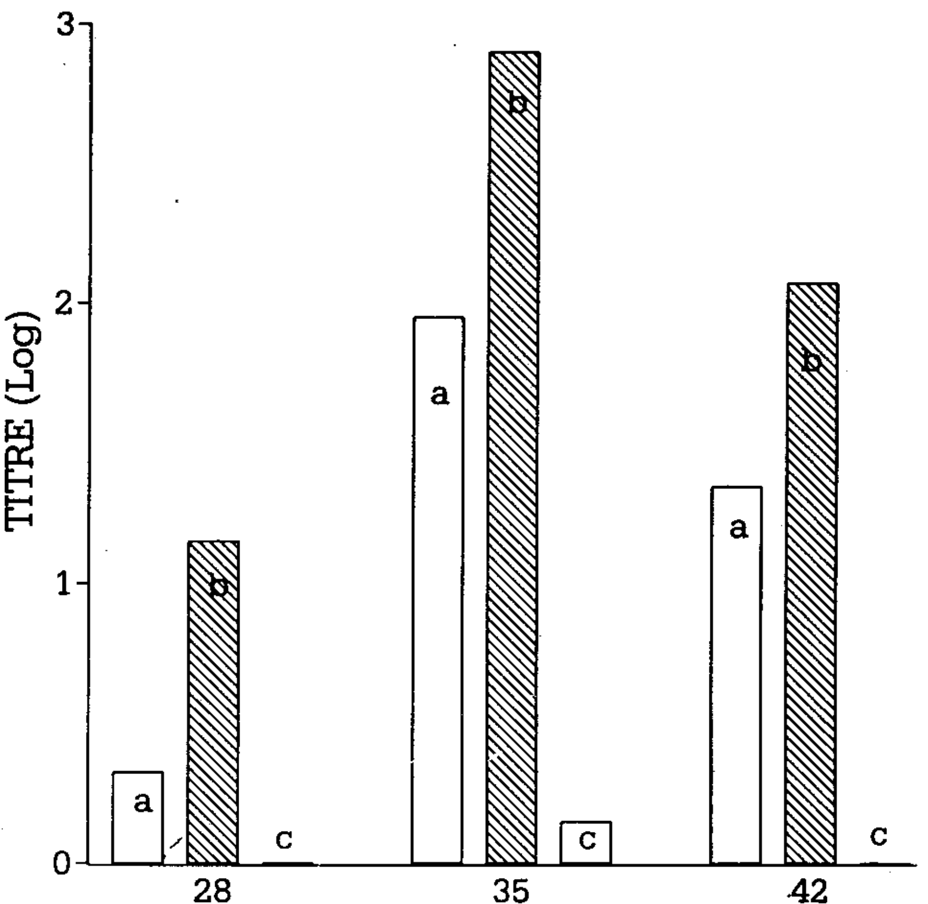

Figure 2. The mean titre of *Theileria parva* sporozoite infectivity in *Rhipicephalus appendiculatus* at different times after repletion as assayed *in vitro*. In method A ticks were fed on rabbits for 4 days while in method B they were incubated at 37°C and 85% relative humidity for 2 days and then fed on rabbits for 3 days. In method C ticks were incubated at 37°C and 85% relative humidity for 6 days.

not infective to bovine lymphocytes. This was also evident when ticks were incubated for 10 days at 37°C. The effects of different times after repletion on the yield of sporozoites as assayed *in vitro* are shown in Fig. 2. There was a significant (P= 0.021) increase in the titre of sporozoite infectivity at 35 days after tick repletion compared to 28 days post repletion. A decline in the titre of sporozoite infectivity was observed when ticks were tested 42 days after repletion with the exception of female ticks fed on BK152 and incubated prior to feeding. The sex related differences and effect of incubation reported above were also observed when ticks were tested 28 and 35 days post-repletion. Ticks incubated without feeding produced significantly (P=0.04) low titre and infective sporozoites were only detected when they were tested 35 days after repletion. However, there was no significant difference (P=0.175) between male and females incubated without feeding.

DISCUSSION

The results of this study have shown that the development of infective sporozoites was dependent on incubation conditions, presence of host and the times after nymphal tick repletion. Maximal yield of infective sporozoites were evident when ticks were used 35 days after repletion with the exception of females incubated at 37°C for two days prior to feeding on rabbits. These ticks maintained high levels even at 42 days. These results are in agreement with those of Young *et al.* (1984) that the time after repletion was important if infective sporozoites were to be produced. In their study, Young *et al.* (1984) found that ticks produced infective sporozoites if incubated 27-41 days after repletion. The possible explanation for low titre of infective sporozoites in young ticks may be that they may not have enough kinetes in salivary glands whereas at 35 days they have enough kinetes infecting the glands. At 42 days it is possible that the parasite may have exhausted some essential nutrients for completion of sporogony.

Feeding of ticks was important and this is supported by the observation that incubation alone could not stimulate significant numbers of infective sporozoites. It was further evident that incubation prior to feeding resulted in increase in infective sporozoites only in male but not in female ticks. The finding that incubation at 37°C resulted in the transmission of infection to bovine

lymphocytes is indicative of the possibility that under field conditions when ticks are exposed to high environmental temperatures such as those of semi-arid areas can still transmit *T. parva* infection as also observed by Young *et al.* (1979, 1984).

Sex differences in the production of infective sporozoites which was evident in ticks fed to rabbits without incubation is in support of presence of few type II acini in males (Irvin *et al.* 1981). However, the observation that incubation prior to feeding of ticks resulted in comparable titre in both sexes suggests that male and female ticks have the same potential of producing infective sporozoites. This finding is in agreement with histological studies which suggested the same carrying capacity of parasites by both sexes of ticks (Purnell and Joyner 1968). Since male *R. appendiculatus* moult two days later than female ticks and that the appearance of *T. parva* kinetes in the haemolymph coincides with moulting, it is then possible that the delay in the moulting of male ticks was responsible for delayed development of the parasites. Thus, incubation of males prior to feeding on rabbits allows the continued development of the parasites which is completed upon feeding on rabbits. Therefore, the initial incubation of ticks may synchronize the development of the parasites in the tick, thereby increasing the output of infective sporozoites. In the field, male ticks do not engorge but feed for short periods after which they go in search of females for mating. This feeding behavior is thus not conducive to rapid completion of parasite development under natural conditions. It is worth noting that the practical use of this finding is that incubation of ticks provides an opportunity to increase the contribution of males in the overall output of infective sporozoites in the preparation of stabilates.

Therefore, the results indicate that in order to improve the infectivity of sporozoite stabilates there is a need to maximize the component of infectivity attributed to male ticks and this can be achieved by using ticks 35 days after repletion and for which sporogony has been stimulated by incubating at 37°C prior to feeding on rabbits.

ACKNOWLEDGEMENTS.

We thank the Director, ILRAD for supporting one of us (ENK) through a research fellowship. Dr. T T. Dolan is thanked for supervising the research. We thank Mr. S. Mwaura for dissecting the ticks and staining the salivary glands.

REFERENCES

Bailey, K. P. (1960). Notes on the rearing of *Rhipicephalus appendiculatus* and their infection with *Theileria parva* for experimental transmission. *Bull. Epizoot. Dis. Afr.* 8: 33-43.

Brown, C. G. D. (1979). Propagation of *Theileria*. In: *Practical Tissue Culture Applications.* (Edited by K. Maramorosch and H. Hirumi). Academic Press, New York, pp 223-254.

Brown, C. G. D., Stagg, D. A., Purnell, R. E., Kanhai, G. K. and Payne, R. C. 1973. Infection and transformation of bovine lymphoid cells *in vitro* by infective particles of *Theileria parva*. *Nature* 245: 101-103.

Irvin, A. D., Boarer, C. D. H., Dobbelaere, D. A. E., Mahan, S. M., Masake, R. and Ochama, J. G. R. (1981). Monitoring *Theileria parva* infection in adult *Rhipicephalus appendiculatus* ticks. *Parasitol.* 82: 137-147.

Kimbita, E. N. and Silayo, R. S. (1997). Use of an *in vitro* infectivity assay in comparison with histological techniques in the study of *Theileria parva* sporozoite maturation. *Vet. Parasitol.* 70: 83-97.

Mukhebi, A.W., Perry, B.D. and Kruska, R. (1992). Estimated economics of theileriosis control in Afr. *Prev. Vet. Med.* 12:73-85.

Purnell, R. E., Brown, C. G. D., Cunningham, M. P., Burridge, M. J., Kirimi, I. M. and Ledger, M. A. (1973). East Coast fever: Correlation between morphology and infectivity of *Theileria parva* developing in its tick vector. *Parasitol.* 66:539-544.

Purnell, R. E. and Joyner, L. P. (1968). The development of *T. parva* in the salivary glands of the tick *R. appendiculatus*. *Parasitol.* 58:725-732.

Spooner, P. R. (1990). The effects of oxytetracycline on *T. parva in vitro*. *Parasitol.* 100:11-17.

Young, A. S., Leitch, B. L. and Mutugi, J. J. (1984). Some factors controlling the stimulation of sporogony of *T. parva* in its tick vector *Rhipicephalus appendiculatus*. *Int. J. Parasitol.* 14:97-102.

Young, A. S., Leitch, B. L., Morzaria, S. P., Irvin, A. D., Omwoyo, P. L., and De Castro, J. J. (1987). Development and survival of *Theileria parva parva* in *Rhipicephalus appendiculatus* exposed in the Trans-Mara, Kenya. *Parasitology* 94: 433-441.

Young, A. S., Leitch, B. L. and Omwoyo, P. L. , (1979). Induction of infective stages of *T. parva* by exposure of host ticks to high temperatures. *Vet. Rec.* 105:531-533.