Theileria lestoquardi – maturation and quantification in *Hyalomma anatolicum anatolicum* ticks

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SUMMARY

The maturation and quantification of *Theileria lestoquardi* (*T. hirci*) parasites in unfed and partially fed adult *Hyalomma anatolicum anatolicum* ticks was studied using (1) methyl green pyronin (MGP) staining of salivary glands, (2) *in vitro* infection of peripheral blood mononuclear cells (PBM) with parasites harvested from infected ticks and (3) a semi-quantitative polymerase chain reaction (PCR). With MGP staining the greatest infection rate was seen in unfed ticks. Feeding resulted in a gradual reduction in the number of infected acini with a concomitant increase in the maturity of the parasites. *In vitro* infection of sheep PBM with titrated ground-up tick supernate (GUTS) demonstrated that infectivity peaked between 2 and 4 days of tick feeding whereas GUTS prepared from unfed ticks was not infective. The polymerase chain reaction (PCR) was both sensitive and specific, detecting *T. lestoquardi* DNA in unfed and partially fed ticks, with a maximum sensitivity of 0·022 infected acinus/tick in 2-day fed ticks, though it gave no indication of the infectivity of the parasite.

Key words: *Theileria lestoquardi*, *Hyalomma anatolicum anatolicum*, infection rates, *in vitro* titration, PCR.

INTRODUCTION

Theileria lestoquardi (synonym *T. hirci*) (Morel & Uilenberg, 1981) causes pathogenic theileriosis in sheep (Hooshmand-Rad & Hawa, 1973*a*) and goats (Banerji *et al*. 1990). The life-cycle of this parasite is identical to that of *T. annulata* and *T. parva* which cause morbidity and mortality in cattle. Both *T. annulata* and *T. lestoquardi* are transmitted transtadially by at least 1 common vector tick, *Hyalomma anatolicum anatolicum* (Hooshmand-Rad & Hawa, 1973*b*). The disease caused by *T. lestoquardi* occurs in many of the areas where this vector is found: Egypt (Littlewood, 1915), Algeria (Lestoquard, 1924), Serbia (Dschunkowsky & Urodschevich, 1924), Turkey (Baumann, 1939), Iraq (Hooshmand-Rad & Hawa 1973*a*), Iran (Hooshmand-Rad, 1985), India (Banerji *et al*. 1990) Sudan (Tageldin *et al*. 1992), and China (Jianxun & Hong, 1997). In areas where it is a threat to small ruminants, vaccination is recommended and applied in its control (Hooshmand-Rad, 1985). Acaricidal treatment to control ticks on sheep in endemic areas also has an effect on the disease (Jianxun & Hong, 1997).

Many characteristics of *T. lestoquardi* and the disease it causes have not been extensively studied. The work described here, therefore, aims to determine the optimum feeding time of ticks on either a sheep or rabbit necessary for the production of large numbers of infective *T. lestoquardi* sporozoites. The partial feeding of infected adult ticks on mammalian blood is necessary to provide the biological stimulus for the development of infective *Theileria* sporozoites (Theiler & du Toit, 1928).

Three methods were used to assess the quantity of parasite material: microscopical analysis of methyl green pyronin (MGP) stained salivary glands of the infected ticks to determine the number and maturity of infected acini (Walker *et al*. 1979), an *in vitro* infectivity assay, based on the method for infecting bovine peripheral blood mononuclear cells (PBM) with *T. annulata* sporozoites (Brown, 1983) and a semi-quantitative polymerase chain reaction (PCR) (Kirvar *et al*. 1998).

MATERIALS AND METHODS

Experimental design

Adult *H. a. anatolicum* ticks heavily infected with *T. lestoquardi* that had been quiescent at 18 °C for 3 months after moult were fed for between 1 and 6 days on either a sheep or a rabbit. Unfed adult ticks from the same batch were used as the day 0 control. Every day 40 feeding ticks were removed from each host animal. The salivary glands were dissected from 20 of them and stained with methyl green pyronin; the remaining ticks were ground in medium to assess the infectivity of the suspension for sheep PBM *in vitro* and to extract DNA for the PCR.

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Parasite used

The stock of *T. lestoquardi* used in this study named Lahr, originated from Iran and was kindly provided by Dr Parviz Hooshmand-Rad, Razi Institute, Teheran. The parasite was shown to be *T. lestoquardi* and not *T. annulata* by the ability of its sporozoites to infect sheep PBM but not cattle PBM (unpublished observations).

Ticks used

Clean *H. a. anatolicum* nymphs were fed on a sheep with a *T. lestoquardi* parasitaemia between 2·5 and 3·5 %. Nymphs were allowed to moult for 28 days at 28 °C and 85 % relative humidity (RH). The resultant adults were held at 18 °C, 85 % RH for 3 months. A preliminary examination of these adults showed them to be heavily infected and a homogenous batch was selected for use in this experiment.

Tick feeding on sheep and rabbit

A total of 280 adult infected *H. a. anatolicum* ticks (equal numbers of male and female) were placed in a body bag on the back of a clean sheep and allowed to feed for up to 6 days. Every day, starting from day 1, 20 male and 20 female feeding ticks were removed carefully from the animal and processed as described below. The same numbers were fed on the ears of a clean rabbit (Bailey, 1960) and removed as for the sheep. Any ticks not attached on day 1 were removed and not included in the study.

Processing of ticks

Dissected salivary glands were removed from 10 male and 10 female ticks, teased out on a microscope slide and stained with methyl green pyronin, as described previously for the detection of *T. annulata* in *H. a. anatolicum* ticks (Walker *et al.* 1979). The number of infected acini/tick was counted at ×100 magnification. An estimation of the proportion of immature, partially mature and mature parasites in the acini (pink/red represents immature parasites, red/purple represents partially mature parasites and purple/green represents mature parasites) was made.

Assessment of *T. lestoquardi* sporozoite numbers by infection of sheep PBM *in vitro* was based on a method described previously for the infection of bovine PBM with *T. annulata* (Brown, 1983). Briefly, 10 male and 10 female ticks were surface sterilized and ground up in 5 ml of MEM/3·5 % Bovine Plasma Albumin (BPA) containing 200 units/ml penicillin, 200 μg/ml streptomycin and 100 units/ml nystatin. The suspension was centrifuged at 50 ***g*** for 5 min. The supernatant, representing material obtained from 20 ticks was considered to have a concentration of 4 tick equivalents (t.e.)/ml. A 0·5 ml volume of this ground up tick supernate (fresh GUTS) was used for *in vitro* infectivity testing. Similarly, a 0·5 ml volume was frozen at −20 °C and later used to extract DNA for PCR.

For the *in vitro* infectivity test, fresh PBM were separated from 10 ml of lithium heparinized blood of a naive sheep using Ficoll Paque and washed (Brown, 1983). The cells were resuspended in complete medium (RPMI 1640/20 % foetal calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin and 5×10^{-5} M 2-mercaptoethanol), counted and adjusted to 2×10^{6}/ml. Fresh GUTS dilutions of 1, 0·2, 2×10^{-2}, 2×10^{-3}, 2×10^{-4} and 2×10^{-5} t.e./ml were prepared in complete medium. Equal volumes (1 ml each) of the PBM suspension and the GUTS dilutions were added to a single well of a 24-well tissue culture plate. The resulting final concentrations were 10^{6} sheep PBM/ml and 0·5, 0·1, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} t.e./ml GUTS. Plates were kept in a humidified 5 % CO_2 incubator for 28 days. The cultures were maintained by removing 1 ml of medium and adding the same volume of fresh medium after 4 days and then twice weekly. Cytospin samples taken from each well after 7, 14, 21 and 28 days were stained with Giemsa and 200 cells counted to estimate the percentage infected with macroschizonts. Where no macroschizont infected cells were found in a count of 200 cells, the remainder of the cytospin sample was scanned (generally 2000–5000 cells) for positive infection and, if found, noted as +.

The number of sporozoites present in the original tick sample was considered to be directly proportional to the greatest dilution of tick material that transformed the sheep PBM to cultures in which macroschizonts were observed. With this method a useful comparison of numbers of infective sporozoites could be made between ticks feeding for different periods on either a sheep or rabbit.

Extraction of DNA from ticks and PCR was performed using a previously frozen 0·5 ml volume of fresh GUTS equivalent to 2 ticks which was thawed and centrifuged at 16000 ***g*** in a microfuge. The pellet was rinsed once in sterile PBS and centrifuged once more. The washings were discarded and 180 μl of lysis buffer from the QIAamp Tissue Kit for DNA extraction (Qiagen Ltd, Germany) added to the pellet. The DNA was then extracted according to the manufacturer's instructions. The purified DNA in a 400 μl volume of AE buffer was stored at −20 °C and used when required.

Uninfected tick DNA, required for a negative control in the PCR and as a diluent for the infected tick material, was obtained by grinding 100 unfed adult *H. a. anatolicum* ticks in liquid nitrogen using a sterile pestle and mortar which had been kept at −70 °C for 1 day. Following evaporation of the

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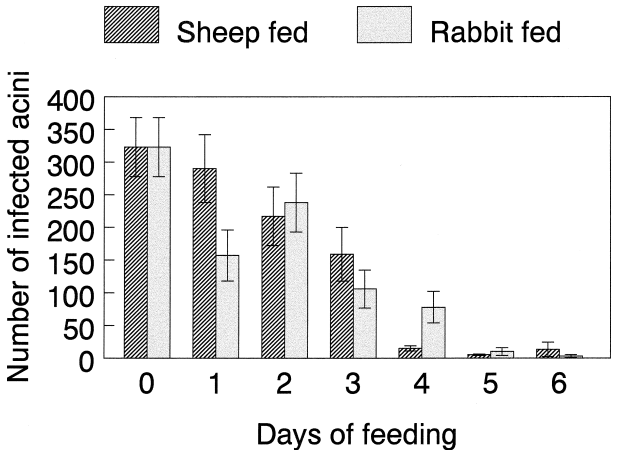

Fig. 1. Histogram showing the mean number of acini infected with *Theileria lestoquardi* in 10 male and 10 female *Hyalomma anatolicum anatolicum* ticks fed on either a sheep or a rabbit. Bars represent standard error of the mean.

liquid nitrogen, the ground-up tick powder was aliquotted into 1·5 ml Eppendorf tubes so that each tube received the equivalent of 10 ticks (70 mg material). Lysis buffer and proteinase K were added to the crushed ticks and the QIAmp Tissue Kit (Qiagen Ltd) protocol followed. The purified DNA was pooled and kept at −20 °C until use.

Infected tick (containing parasite) DNA was diluted with uninfected tick DNA. The dilutions made corresponded to 0·025, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and, in some cases, 10^{-6} t.e./50 μl reaction volume (named A, B, C, D, E and F respectively) when the volumes used for the PCR were taken into account.

The primers used to detect *T. lestoquardi* have not been previously published. These were 5′ GTGCCGCAAGTGAGTCA 3′ and 5′ GGACTGATGAGAAGACGATGAG 3′ from the gene coding for the 30 kDa *T. lestoquardi* merozoite surface antigen. These primers amplify a 785 bp product with *T. lestoquardi* DNA but not *T. annulata* DNA (Kirvar *et al*. 1998). The other reagents for the PCR were 0·2 mM each dNTP, 0·1 mM each primer, 20 mM Tris–HCl, pH 8·55*, 16 mM $(NH_4)_2SO_4$*, 2·5 mM $MgCl_2$*, 150 μg/ml BSA*, (*contained in the ×10 reaction buffer supplied with *Taq* Polymerase), *Taq* Polymerase (Thermometric Ltd), 0·025 U/μl and 5 μl test or control DNA sample in a final volume of 50 μl. Thin-walled 200 μl tubes were used in an Omn-E PCR thermal cycler (Hybaid) using the heated lid. Cycling conditions were 94 °C for 3 min followed by 40 cycles at 94 °C for 1 min, 65 °C for 1 min and 72 °C for 1 min. A final extension step of 72 °C for 5 min was also used. In addition to the test DNA samples, a positive control (purified DNA from *T. lestoquardi* infected tick salivary glands), a negative sample control (containing tick DNA only) and a no DNA negative control (5 μl of Milli-Q water substituted for DNA) were included in the PCR amplification. The PCR products (15 μl) were separated on a 1·5 % agarose gel containing 0·5 μg/ml ethidium bromide according to standard methods (Sambrook, Fritsch & Maniatis, 1989). The gels were visualized using an ultra-violet transilluminator and photographed using a Polaroid MP4 camera with type 55 film and a Kodak Wratten 23S filter.

In order to demonstrate that the PCR product bands were specific to the 30 kDa gene, a 17-mer sequence (5′GTAGACTTTCATACTC3′) from within the gene was labelled with digoxigenin (Boehringer Mannheim) and used as a probe following Southern blotting of the gel. The procedure followed is described in the Hybridization Protocols booklet supplied by Boehringer Mannheim. The stained blots were photographed as above using a Kodak Wratten 3S filter.

RESULTS

Salivary gland infection rates

The mean number of infected acini/unfed and fed tick examined is represented in Fig. 1. The unfed ticks that were used as a baseline for the comparison of the fed ticks showed the highest rate of infection. In this batch of ticks, infected acini stained pink/red indicating the immaturity of the parasites. As the ticks began to feed, a relatively high infection rate was maintained for the first 2 days of feeding with a noticeable trend toward maturity of the parasites, as indicated by the purple/green rather than the pink colour of the infected acini. In addition to the change in colour, the density of staining also decreased during this time. Grades of maturity of the infected acini from day 0 to day 6, estimated on the basis of their colour is depicted in Fig. 2. Generally, infected acini were larger than uninfected acini and the infected acinar cell nuclei were hypertrophied and stained green. There was a gradual reduction in the number of infected acini during the feeding period so that by day 5, any remaining infected acini were difficult to see, particularly as the acini had enlarged and their structure changed. On days 5 and 6, methyl green pyronin-stained salivary glands showed little evidence of previous infection with parasites indicating that most of the sporozoites had been released from the ticks into the host. The decreasing trend in infection rates was similar for both sheep and rabbit fed ticks. There was a large variation in the numbers of infected acini/tick on the days of inspection, but most of this was due to degenerated glands where it was impossible to count numbers of infected acini accurately. This accounts for the sudden dip in values of infection rates in the day 1 rabbit-fed ticks. In most cases female ticks were more heavily infected than the male ticks. Photographs of *T. lestoquardi* in the salivary glands of *H. a. anatolicum* at various stages of maturity are represented in Fig. 3.

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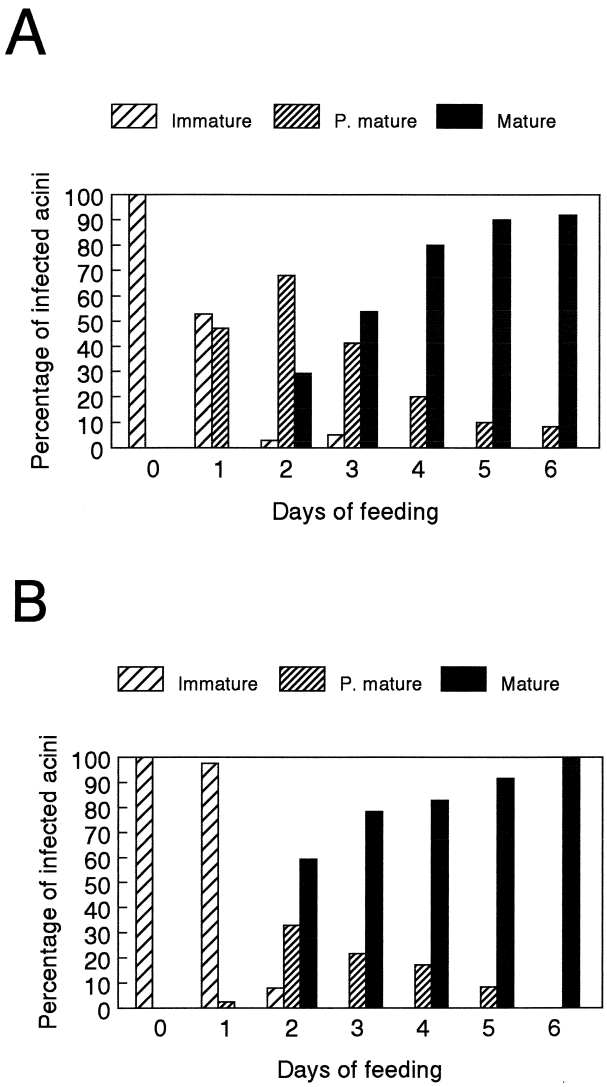

Fig. 2. Histogram showing the grades of maturity of acini infected with *Theileria lestoquardi* for unstimulated ticks and ticks fed on a sheep (A) or on a rabbit (B) for a period of 6 days. Results are expressed as a percentage of the total number of infected acini. P = Partially mature.

In vitro *infection of sheep PBMs*

Unfed ticks were uninfective for sheep PBM despite the large parasite load seen by salivary gland staining. Giemsa-stained cytospin smears of GUTS obtained from unfed ticks showed that the parasite was present as large 'plates' of irregular cytoplasmic and nuclear material. This is assumed to be the sporoblast stage of the parasite. By day 1 of feeding, sporoblasts were beginning to mature to sporozoites as seen by the infection of PBM. Ticks partially fed for 2 days on sheep generally produced the highest infection counts and the greatest dilution endpoint (Table 1 and Fig. 4). Following 7 days in culture, 10^{-5} t.e./ml of 2 day sheep fed tick GUTS produced infection, corresponding to 0·002 infected acini (Table 2). Since no greater dilution was tested, it is not possible to categorically state that this value represents the endpoint of infection for this sample although

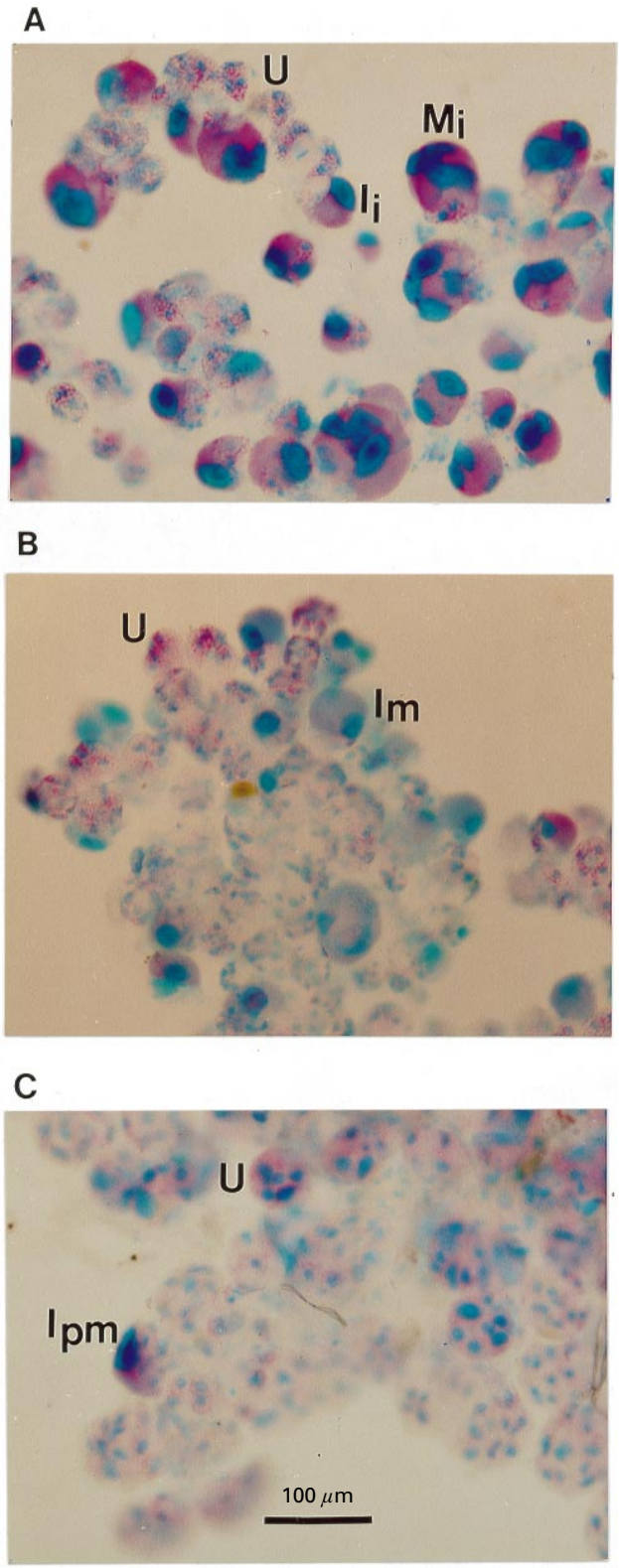

Fig. 3. Parasite masses in the salivary glands of adult *Hyalomma anatolicum anatolicum* ticks. *Theileria lestoquardi* in (A) unfed ticks; (B) 2-day fed ticks and (C) 4-day fed ticks. Methyl green pyronin staining. (U = uninfected acinus; I = infected acinus; M = multiple infection in 1 acinus; subscripts i, pm and m indicate the degree of maturation of the parasite; immature, partially mature and mature, respectively). Note the almost complete loss of *Theileria* from this 4-day fed tick.

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Table 1. Percentage of cells containing macroschizonts in culture wells (A) 7 days and (B) 14 days after *in vitro* infection of sheep PBM with ground-up tick supernate (GUTS) derived from unfed and 1–6 days sheep or rabbits fed *Hyalomma anatolicum anatolicum* ticks infected with *Theileria lestoquardi*

	GUTS density (t.e./ml)	Unfed tick GUTS	Sheep fed tick GUTS (days of feeding)						Rabbit fed tick GUTS (days of feeding)					
		0	1	2	3	4	5	6	1	2	3	4	5	6
A	0·5	0	86·5	92	6·5	Dead	Dead	Dead	47	84	75·5	64	Dead	Dead
	0·1	0	18	65	3·5	3·5	+	1·5	2	63	70	40	4·5	+
	10^{-2}	0	1	10	0·5	+	0	0	+	3·5	3	2	+	0
	10^{-3}	0	0	0·5	0	+	0	0	0	2·5	+	0	0	0
	10^{-4}	0	0	+	0	0	0	0	0	0	+	0	0	0
	10^{-5}	0	0	+	0	0	0	0	0	0	0	0	0	0
B	10^{-2}	0	33	82	28·5	18	0·5	7·5	18	75·5	86·5	57	18	4
	10^{-3}	0	1	21	19	6	0	0	0	35	43·5	3	N.C.	0
	10^{-4}	0	0	7·5	0	+	0	0	0	3·5	6	4	0	0
	10^{-5}	0	0	0·5	0	0	0	0	0	0	0	0	0	0

N.C., No count.
+, One schizont infected cell/2000–5000 cells.

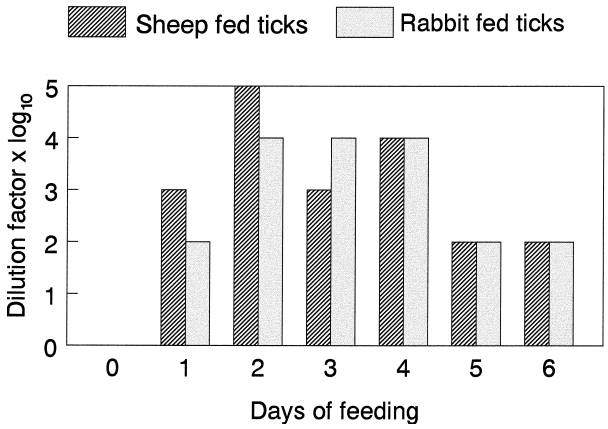

Fig. 4. GUTS dilution endpoint of infection (represented on a positive scale of 10-fold dilution factor) by sporozoites from fresh GUTS of ticks from various duration feeds on either a sheep or rabbit.

judging by the low number of schizont infected cells generated, it is unlikely to have titrated much further. Large numbers of sporozoites were still obtained on day 4 of the sheep fed ticks and on days 2, 3 and 4 of the rabbit fed ticks, though the numbers were approximately 10-fold less than those harvested from the 2 day sheep-fed ticks. Less infective parasite material was produced on days 1, 5 and 6 of feeding on either animal. The value obtained for 3 day sheep fed ticks appears anomalous and may be attributed to the peculiarity of culture conditions as the trend in macroschizont-infected cell percentages would indicate that the 10^{-4} t.e./ml concentration should also be positive. Despite this, the greatest number of sporozoites were produced at 2 days of feeding on a sheep and 2–4 days of feeding on a rabbit.

Infectivity assessment was difficult or impossible in some 0·5 t.e./ml density wells due to cell death. This may have been due to the toxic effect of the tick material as this inhibition of evident infection and transformation increased with time of feeding. Day 14 percentage infections (see Table 1) omit the 0·5 t.e./ml wells, many of which contained only dead cells, and the 0·1 t.e./ml wells, most of which (except unfed ticks) had very high infections.

For most tick batch samples, 14 days in culture was necessary for the lower concentrations of GUTS material to produce macroschizont-infected cells which could be detected by microscopy (i.e. 1 in 5000 cells). Culture for a further 2 weeks did not increase the sensitivity of detection.

PCR

For each day of feeding, including the unfed ticks, a PCR product was obtained at the greatest concentration of DNA tested (0·025 t.e.s of DNA). Clear differences were obtained in the intensity of the PCR product and the dilution of DNA detected. Two day sheep fed ticks appeared to contain the greatest amount of *T. lestoquardi* DNA as the dilution representing 10^{-4} t.e.s gave a positive signal. The PCR for the sheep-fed ticks and the Southern blot can be seen in Fig. 5. The PCR results obtained for the rabbit-fed ticks showed the same trends (results not shown).

The sensitivity of this PCR in terms of number of acini detected was estimated by assuming the tick batches used for salivary gland staining and the PCR contained equivalent numbers of infected acini. It was then possible to compare the limit of dilution obtained in PCR with the number of infected acini. For example, on day 2 of sheep fed ticks (when 220 infected acini/tick were detected by salivary gland staining), the DNA from 10^{-4} t.e. could be detected. When dilutions are taken into account, this is

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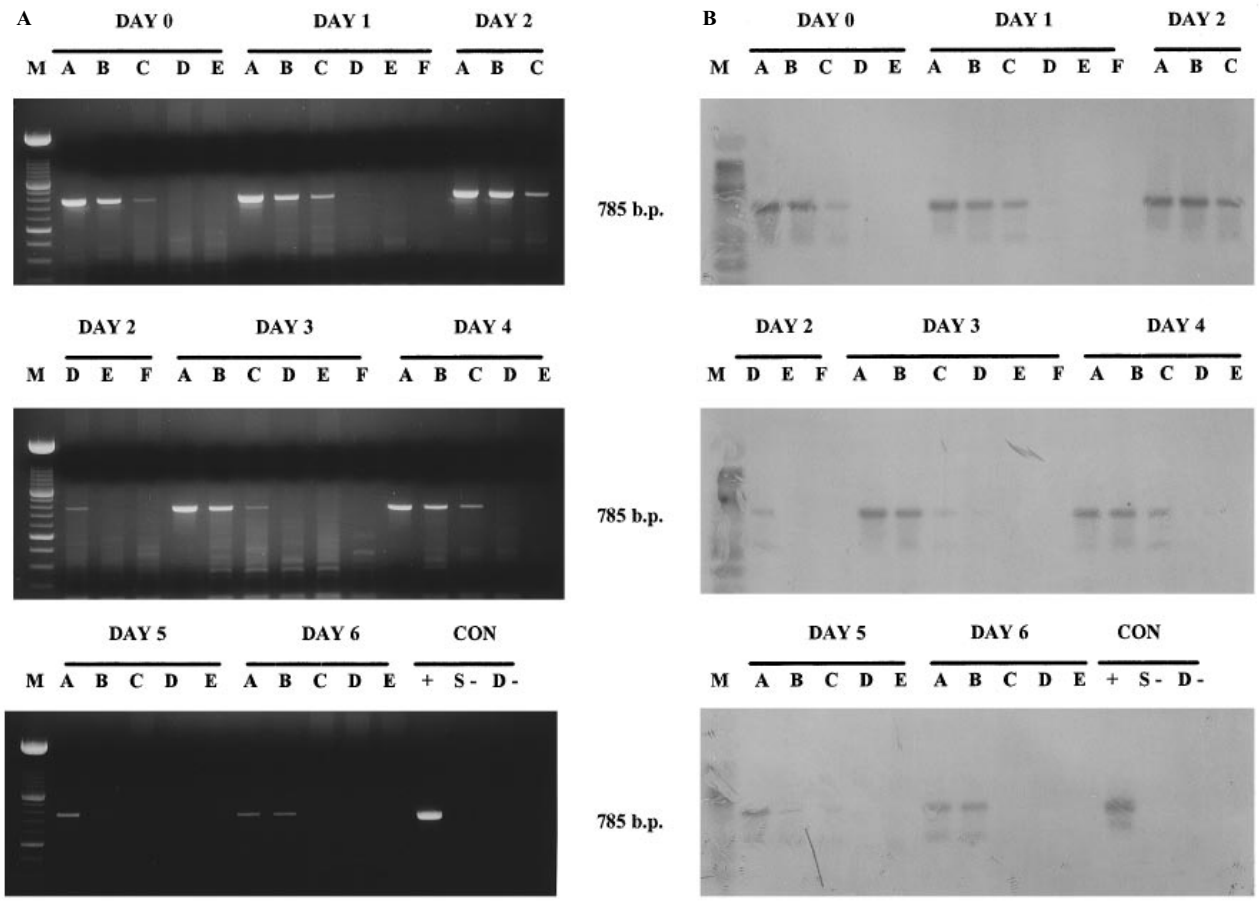

Fig. 5. (A) Ethidium bromide-stained agarose gel showing the results of the PCR detecting *Theileria lestoquardi* in adult *Hyalomma anatolicum anatolicum*. Ticks were either unfed (day 0) or fed on a sheep for 1–6 days. Dilutions A–F represent 0·25, 0·1, 0·01, 10^{-3}, 10^{-4} and 10^{-5} t.e./sample. M = base pair marker (XIV, Boehringher Mannheim); CON = controls; +, *T. lestoquardi* and tick DNA (positive sample control); S-, uninfected tick DNA (negative sample control); D-no DNA control. (B) Southern blot of the gel using a digoxigenin labelled 17-mer oligonucleotide sequence from the 30 kDa *T. lestoquardi* merozoite surface gene as a probe. The Boehringer Mannheim XIV base pair marker was also labelled with digoxigenin and used as a probe for itself.

equivalent to 0·022 infected acini/tick, which is more sensitive than currently used histological methods (Table 2).

DISCUSSION

Although much work has been done in the past on the maturation and quantitation of *T. parva* (Purnell & Joyner, 1968; Irvin *et al*. 1981) and *T. annulata* (Samish & Pipano, 1978; Singh *et al*. 1979; Walker & McKellar, 1983) there has been no published information on *T. lestoquardi* maturation.

This study aimed to determine the optimum time required for the maturation of *T. lestoquardi* sporozoites in *H. a. anatolicum* ticks and to test whether the partial feeding of infected ticks on the natural host of this parasite (i.e. sheep) improved over partial feeding on a rabbit. Three methods of detection were used in an attempt to quantify the parasites produced in this manner.

All 3 methods used in this study had advantages and disadvantages. With methyl green pyronin staining of salivary glands, infection could be seen clearly in ticks that were healthy; however, some glands had started to degenerate and in these situations it was difficult to interpret results. In the healthy ticks it was possible to estimate the maturity of the parasite by the colour of the staining. This method is clearly a useful technique which detects the number of infected acini and to a degree the level of maturity of the parasite. However, it is less reliable as tick feeding advances when uninfected acini enlarge and stain similarly to infected acini. This study showed that most of the *T. lestoquardi* infection disappeared from the glands 5 days after feeding. Some ticks did retain smaller numbers of infected acini but this may have been due to slower feeding of that particular tick (which could also be gauged by the weight of the tick), failure of some parasite masses to mature and hence remain in the gland or due to a natural state whereby ticks remain infective for long periods. Samish & Pipano (1978) showed that adult *H. detritum* ticks infected with *T. annulata* were still infective after feeding on a rabbit for 20 days. In

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Table 2. Comparison of the sensitivity of detection of *Theileria lestoquardi* in *Hyalomma anatolicum anatolicum* ticks using histology, *in vitro* infection and PCR

(The ticks were either unstimulated (day 0) or fed on a sheep for 1–6 days. The figures in bold in parentheses in the last 2 columns indicate the sensitivity of detection in terms of infected acini.)

Day of feeding	Mean no. of infected acini/tick	*In vitro* titration of sheep PBM (t.e.s detected)	PCR titration (t.e.s detected)
0	320	0 **(0)**	10^{-3} **(0·32)**
1	280	10^{-2} **(2·8)**	10^{-3} **(0·28)**
2	220	10^{-5} **(0·002)**	10^{-4} **(0·022)**
3	160	10^{-3} **(0·16)**	10^{-3} **(0·16)**
4	20	10^{-4} **(0·002)**	10^{-3} **(0·02)**
5	10	10^{-2} **(0·1)**	$2{\cdot}5 \times 10^{-2}$ **(0·25)**
6	20	10^{-2} **(0·2)**	10^{-2} **(0·2)**

addition, the fact that male ticks can remain on their host, taking small, repeated blood meals for several weeks indicates that there is a broad time-scale in which they will continue to harbour and supply small doses of sporozoites. The colours of the infected acini in an individual tick often showed them to be at different stages of maturity, a condition also reported by Purnell & Joyner (1968) and Kimbita & Silayo (1997) for *T. parva* in *R. appendiculatus* ticks. In some ticks that had fed for 4–5 days, there was evidence of partially mature acini whereas it would be expected that the parasites would have matured by this time.

If infected ticks had been collected from an area where both *T. annulata* and *T. lestoquardi* existed, as suggested by Walker *et al*. (1983), it would be very difficult to distinguish between these parasite species using only this method of staining, despite the unpublished observations that *T. annulata*-infected acini tend to stain more densely and are more enlarged than is the case with *T. lestoquardi*. In field infections where a large proportion of infected ticks only possess between 1 and 10 acini infected with *Theileria* (Sangwan, Chhabra & Samantaray, 1986) a distinction on the basis of staining would make speciation of the parasite extremely difficult and unreliable.

In vitro infection of sheep PBM with GUTS is probably the most relevant parameter representing the infectivity of the parasite material and thus indirectly mature, viable sporozoite numbers. This method is an improvement over the traditional methods used for titration of stabilate doses (Cunningham *et al*. 1973) in that it obviates the need for infecting large numbers of animals *in vivo*. The rise and fall of the infectivity of the GUTS material obtained during the experiment closely matched the pattern that would be expected from knowledge of the maturation of *T. annulata* in the salivary glands of *Hyalomma* spp. (Gill, Bhattacharyulu & Kaur, 1977; Singh *et al*. 1979; Walker & McKellar, 1983). In the case of *T. parva* in the salivary glands of adult *R. appendiculatus*, while showing the same pattern of increase then decrease in infectivity, the peak of maturation and infectivity was 4–5 days (Purnell & Joyner, 1968; Purnell *et al*. 1973; Kimbita & Silayo, 1997).

Two day sheep fed ticks produced approximately 10 times more sporozoites than any other batch of ticks used in this experiment. This represented more infective sporozoites overall from the sheep fed ticks than from the rabbit fed ticks and was the highest yield from 1 harvest. However, as the peak yield from sheep fed ticks appeared to be of shorter duration, timing of maturation may be more critical than with the rabbit feeding which provided reasonable sporozoite yields over a longer period. Whether the ticks fed more rapidly and the parasites matured more synchronously on the sheep than in the rabbits, perhaps due to differences in the temperature of the host or the preference of the parasite for the host to which it has become adapted, is not known but can go some way towards explaining the differences in the timing of maturation of sporozoites observed on both species. It is also possible that the variation in tick infection rates reflected directly on these results. The *in vitro* titration of infectivity method failed to detect *T. lestoquardi* in unfed ticks. Thus it would be unsuitable for parasite detection and quantification in unfed ticks collected from the field. However, this could be overcome by partially feeding the ticks on a rabbit in the laboratory, by heating the ticks to 37 °C at high RH (Samish, 1977) or by the use of either of the other 2 methods described in this paper depending on the level of parasite identification required.

Results of the PCR showed that *T. lestoquardi* could be detected in both unfed ticks and ticks feeding on a sheep and a rabbit. Serial dilutions of DNA extracted from a batch of infected ticks in uninfected tick DNA allowed comparative estimations to be made of the number of parasites present in the original samples. This method of dilution also simulated the different infection rates of ticks that would be present in the field. The described semi-quantitative PCR has been shown to be able to detect the parasites to a level of 0·022 infected acini/tick which is more sensitive than the current criteria used to describe tick infection rates in epidemiological surveys (Buscher & Otim, 1986). *T. lestoquardi* could be detected to a level below 1

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infected acinus/tick in all the ticks, whether unfed or partially fed for between 1 and 6 days. Perhaps more importantly, the PCR can distinguish between *T. lestoquardi* and *T. annulata* in the tick (Kirvar, 1998) without having to perform expensive and time-consuming infections of sheep and cattle to determine the parasite species. The specificity of this method needs to be evaluated further by testing more stocks of *T. lestoquardi* and *T. annulata* and also *Babesia equi* which can be transmitted by *H. a. anatolicum* (Friedhoff, 1988) and is an additional complicating factor in studying the epidemiology of *Theileria* spp. in *Hyalomma* ticks.

The results of the PCR alone must be interpreted with caution when analysing field-collected ticks as this method only detects parasite DNA but gives no indication of the viability of the parasite. In the experimental situation described here, the presence of live parasites was verified by *in vitro* infectivity testing. With ticks collected from the field, either free or feeding, the test would also pick up intact or degraded parasite DNA in the salivary glands that would not be infective for susceptible hosts. Moreover, schizonts and piroplasms or partially degraded DNA arising from them that may be present in the mammalian host and taken up by the tick during feeding could also be detected by PCR. Similar doubts have been expressed for other tick-transmitted parasites (Peter *et al*. 1995; d'Oliveira *et al*. 1997, Watt *et al*. 1997). This reinforces the need to interpret PCR results with knowledge of the relevant vectors and transmission patterns of each parasite species. The possibility of obtaining false positive PCR results could be eliminated by allowing engorged ticks to moult at a relevant temperature and analysing the DNA in the emergent instar. For ticks that have partially-fed and have been removed from an animal, a suitable control would be to check for the presence of parasites in the host's blood. This method also offers the advantage that ticks can be collected in ethanol and processed as time allows (d'Oliveira *et al*. 1997; Watt *et al*. 1997). The PCR might also be used to detect *T. lestoquardi* in nymphal ticks in which salivary gland dissections and other manipulations are cumbersome.

In conclusion we recommend that a median value of 3 days of tick feeding on a rabbit should be carried out to obtain large numbers of *T. lestoquardi* sporozoites. The results of this study showed that there were insufficiently large differences between the numbers of parasites generated by feeding ticks on a sheep or a rabbit to warrant the use of a sheep, particularly as sheep are susceptible to infection by *T. lestoquardi* and rabbits are not. The methods of parasite detection and quantification described in this paper were found to complement each other. The *in vitro* infectivity titration can be used to estimate sporozoite numbers for stabilate production though it cannot be used to detect infection in ticks unstimulated either by feeding or incubation. For field surveys, methyl green pyronin staining of fresh salivary glands is adequate for a rapid assessment and the PCR can be used to detect infection in single whole ticks or large batches of ticks while simultaneously identifying the parasite infecting the ticks to the species level.

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