Detection of *Theileria annulata* in cattle and vector ticks by PCR using the Tams1 gene sequences

E. KIRVAR[1]*, T. ILHAN[1], F. KATZER[2], P. HOOSHMAND-RAD[3], E. ZWEYGARTH[4], C. GERSTENBERG[5], P. PHIPPS[6] *and* C. G. D. BROWN[1]

[1] *Centre for Tropical Veterinary Medicine, University of Edinburgh, Easter Bush, Roslin, Midlothian EH25 9RG, UK*
[2] *Department of Veterinary Parasitology, University of Glasgow, Bearsden Road, Glasgow G61 1QH, UK*
[3] *Department of Protozoology and Production of Protozoal Vaccines, Razi State Vaccine and Serum Research Institute, PO Box 11365–1558, Tehran, Iran*
[4] *Protozoology Division, Onderstepoort Veterinary Institute, Private Bag X5, Onderstepoort, 0110, South Africa*
[5] *University of Cambridge Department of Clinical Veterinary Medicine, TBA Equine Fertility Unit, Mertoun Paddocks, Newmarket, Suffolk CB8 9BH, UK*
[6] *Central Veterinary Laboratory, Parasitology Department, New Haw, Addlestone, Surrey KT15 3NB, UK*

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SUMMARY

A Polymerase Chain Reaction (PCR) and Southern blot hybridization for the detection of *Theileria annulata* are described. The PCR used primers amplifying a 785 base-pair fragment of the *T. annulata* gene which encodes the 30 kDa major merozoite surface antigen, Tams1. The sensitivity of the PCR in bovine blood was 1 piroplasm in 1 μl of blood. *T. buffeli*, *T. parva*, *Babesia bigemina*, *B. bovis* and *B. divergens* were not detected. The PCR detected down to 1 infected acinus/tick in resting and partially fed adult *Hyalomma anatolicum anatolicum* ticks and was negative for *T. lestoquardi* and *T. equi*, which are transmitted by this tick but are not infective to cattle. The specificity of the PCR was checked using 30 stocks of *T. annulata*, all of which were detected. Three stocks of *T. lestoquardi*, 4 of *T. equi* and 1 each of *T. buffeli*, *T. parva*, *B. bigemina*, *B. bovis* and *B. divergens* were used to ascertain there were no cross-reactions. A nested PCR using separate primers for the first reaction and the same primers for the second reaction detected *T. annulata* to the same sensitivity and specificity in saponin-extracted DNA samples stored for long periods at −20 °C.

Key words: *Theileria annulata*, cattle, *Hyalomma anatolicum anatolicum*, PCR.

INTRODUCTION

Tropical theileriosis is a severe, often fatal disease of cattle caused by the intracellular protozoan parasite *Theileria annulata* (Neitz, 1957; Robinson, 1982). The parasite, which is transmitted trans-stadially by the bite of *Hyalomma* spp. ticks, is distributed from Morocco eastwards across north Africa, the Near and Middle East to India, central Asia and China (Neitz, 1957; Purnell, 1978). Effective implementation of available control measures (Brown, 1990) depends, to a large extent, on accurate diagnosis of the parasite in cattle and vector ticks.

Clinical *T. annulata* infection is traditionally diagnosed by the demonstration of schizont-infected cells in the superficial lymph node draining the site of the tick bite or in the liver, or of piroplasms in peripheral blood (Anon, 1997). In recovered carrier animals, only the piroplasm stage can be demonstrated, often with great difficulty. In many areas infection with the morphologically similar parasite *T. buffeli* (used synonomously with *T. mutans*; Barnett, 1966; Mimioglu *et al*. 1972) can confuse accurate diganosis of *T. annulata*. Infection rates in ticks are traditionally determined by staining dissected salivary glands with methyl green pyronin (MGP) (Walker *et al*. 1979). This method, although relatively simple, can only be performed on freshly collected ticks and does not allow differentiation between the closely related parasites *T. lestoquardi* (= *T. hirci*) (Hooshmand-Rad & Hawa, 1973) and *T. equi* (= *Babesia equi*) (Friedhoff, 1988) which can be transmitted by the same tick but do not infect cattle (Friedhoff, 1988; Leemans *et al*. 1998*b*).

Antibodies to *T. annulata* can be detected by the use of the Indirect Fluorescent Antibody Test (IFAT) (Anon, 1997). However, this test is subject to cross-reactions with antibodies against related parasites such as *T. buffeli*, *T. parva* and *Babesia* spp. Another disadvantage of this method is that antibody titres may wane with time in the absence of challenge (Pipano, 1974*a*), even though the animal remains immune to reinfection.

In order to complement the existing diagnostic tests, 2 PCR tests which detect *T. annulata* sensitively and specifically have been developed with particular emphasis on distinguishing it from *T.*

* Corresponding author: Moredun Research Institute, International Research Centre, Pentlands Science Park, Bush Loan, Penicuik (Near Edinburgh), Scotland, UK. Tel: +44 131 445 5111/454. Fax: +44 131 445 6111. E-mail: kirve@mri.sari.ac.uk

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buffeli and *T. parva* in cattle and from *T. lestoquardi* and *T. equi* in ticks. The PCR tests described in this paper utilize primers complementary to sequences of the *T. annulata* 30 kDa major merozoite surface antigen gene Tams1 (Shiels *et al*. 1995). These sequences are conserved in all the *T. annulata* stocks analysed to date but show variability for other *Theileria* species (Katzer *et al*. 1998).

MATERIALS AND METHODS

Parasites

T. annulata *piroplasm-infected blood*. Sporozoite stabilates of 3 stocks of *T. annulata* from Turkey (Ankara stock, Schein, Buscher & Friedhoff, 1975), Morocco (Gharb stock, Ouhelli, 1985) and India (Hisar stock, Gill, Bhattacharyulu & Kaur, 1976), were thawed and used to infect 3 cattle. Blood was taken into EDTA when piroplasm parasitaemia was 1–5 % and serially diluted with uninfected bovine blood taken from a different animal to give 10-fold dilutions of piroplasm material from 1 % to 10^{-9} %.

T. annulata *infected cell line*. Peripheral blood mononuclear cells (PBM) were isolated from cattle diagnosed with clinical tropical theileriosis in the county of Aydin, western Turkey and cultured to provide 25 immortal, macroschizont-infected cell lines (Ilhan, unpublished results) according to standard techniques (Brown, 1983). Additionally previously isolated and cryopreserved cell lines from Morocco (Gharb), Tunisia (Battan: Ben-Miled, 1993), Spain (Caceres: de Kok, d'Oliveira & Jongejan, 1993), Sudan (Soba: Melrose *et al*. 1984), Turkey (Ankara), Israel (Tova: Pipano, 1974*b*), Iran (Razi: Hooshmand-Rad & Hashemi-Fesharki, 1968) and India (Hisar) were resuscitated and grown as above.

T. annulata *infected ticks*. Uninfected *H.a. anatolicum* nymphs were fed on the ears of a single calf which was infected with the Ankara stock of *T. annulata*. Engorged nymphs dropped over a period of 4 days when the piroplasm parasitaemia of the calf ranged between 3·4 % and 70·8 %. These ticks were moulted either immediately at 28 °C for 28 days at 85 % relative humidity (RH) or kept at 18 °C at the same humidity for up to 1 year before being moulted as above (Walker *et al*. 1985). Engorged nymphs which had fed on the infected blood were also kept aside for inclusion in the PCR tests.

To access the efficiency of the PCR for whole ticks, engorged nymphs or unfed adult ticks were crushed in liquid nitrogen in 1·5 ml Eppendorf tubes using a commercially available Eppendorf grinder. DNA was extracted from the powder as described below.

At least 20 ticks from each batch of moulted adults were selected and their infection rate determined by extraction of their salivary glands and staining with MGP (Walker *et al*. 1979). The degree of correlation between the infection rates of the left and right salivary gland of 120 ticks was determined statistically using the Spearman Rank Correlation test. Lightly infected adult ticks (mean < 20 infected acini/tick) were chosen to compare the infection rate in the left salivary gland by MGP staining with the intensity of the PCR signal from DNA isolated from the right salivary gland. For this, equal numbers of lightly infected *T. annulata* male and female ticks were either kept unfed or allowed to feed for between 1 and 6 days on the ears of a female New Zealand White rabbit to stimulate various degrees of maturation of the parasite (Walker & McKellar, 1983). On each day, starting from day 1, 20 ticks (10 male, 10 female), were removed from the rabbit, washed in Roccal then twice in 70 % ethanol. The washed ticks were embedded individually in pink dental wax (TAAB) cut into approximately 3 × 3 cm squares which had been melted onto individual small sterile plastic Petri dishes. The salivary glands of each tick were dissected out in sterile PBS (pH 7·2), using different scalpel blades to prevent contamination between samples. The forceps used in the dissections were immersed for 5 sec each in 5 M HCl followed by 5 M NaOH to remove any contaminating DNA and then washed twice with sterile distilled water. Every 5th tick dissected was a negative tick from the colony maintained at the Centre for Tropical Veterinary Medicine (CTVM) to check for contamination in the PCR. The left gland was placed on a microscope slide for MGP staining and the other was placed into sterile PBS in a 1·5 ml Eppendorf tube. The PBS was later removed and the reagents from the Qiagen kit added for lysis of the sample in preparation for the PCR (see below).

To determine the sensitivity of detection in tick salivary glands, the DNA extracted from individual salivary glands was serially diluted 10-fold using uninfected tick DNA. For this, 100 pooled uninfected ticks were crushed in liquid nitrogen using a sterile pestle and mortar pre-chilled to −70 °C and the DNA from the resulting powder extracted as described below.

Other parasites

Three *T. lestoquardi*-infected sheep cell lines from Iran, isolated at Lahr, Shiraz and Kemalabad (Hooshmand-Rad, Magnusson & Uggla, 1993) and 1 *T. parva*-infected bovine cell line from Kenya (Muguga: Brocklesby, Barnett & Scott, 1961) were used in this study. *T. buffeli* (Australia) was provided as a blood stabilate from the Tick Fever Research

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Centre, Wacol, Queensland, Australia. A splenectomized calf was infected with this stabilate. Piroplasm-infected blood was obtained in EDTA at a piroplasm parasitaemia of 1% and stored at −20 °C until used for DNA extraction. *T. equi* parasites used in this study originated from South Africa (Kwa Zulu isolate, Zweygarth, personal communication, and Onderstepoort stock, Phipps, personal communication), USA (USDA or Florida stock: Knowles *et al*. 1991), and North Africa (Gerstenberg, Allen & Phipps, 1998). They were supplied in fresh equine blood in EDTA, blood stabilate in dimethyl sulphoxide (Me_2SO) or as isolated merozoites. *B. bigemina* (Muguga, Kenya), *B. bovis* (Nigeria) and *B. divergens* (Stormont, Ireland), kept at CTVM as bovine blood stabilates in Me_2SO were thawed and the blood used directly to extract DNA.

Uninfected samples

Bovine blood was collected into lithium–heparin containing vacutainers and the PBM isolated according to standard procedures using Ficoll-Paque (Brown, 1983). Washed cells were then used for DNA extraction. Uninfected adult *H.a. anatolicum* ticks were obtained from the colony maintained at CTVM.

Collection of field samples from Turkey

A pilot study was carried out in Aydin county, western Turkey to assess the suitability of the PCR for field samples. Over a period of 5 weeks 151 blood samples were collected in EDTA from the local exotic cattle with a history of *T. annulata* infection. The blood was used to prepare thin blood smears and to extract DNA for PCR analysis. The blood smears were fixed in methanol, stained with Giemsa and 200 fields of view under high-power light microscopy were examined for the presence of blood protozoa. The blood smears were recorded as negative for *T. annulata* if no piroplasms were observed in 200 high power fields.

Extraction of DNA

For samples of uninfected or infected blood, either fresh or thawed stabilate in Me_2SO, the QIAamp Blood Kit, Qiagen Ltd, UK, for DNA extraction was used according to manufacturer's instructions. The DNA extracted from 200 μl of blood was eluted in a final volume of 200 μl of elution buffer (buffer AE). Standard phenol chloroform extraction was used to extract DNA from macroschizont-infected cell lines, uninfected bovine PBM and pooled uninfected ticks previously crushed to a powder in liquid nitrogen (Sambrook, Fritsch & Maniatis, 1989). The purified DNA from 100 uninfected ticks was dissolved in 5 ml of TE buffer, pH 7·6. The QIAamp Tissue Kit, Qiagen Ltd, UK, for DNA extraction was used for individual whole ticks or individual tick salivary glands. The powder obtained from nymphs and adult ticks was treated according to manufacturer's instructions. To individual salivary glands, lysis buffer and proteinase K supplied in the Qiagen Tissue kit were added directly and incubated for at least 20 h, vortexing frequently to aid the breakdown of the glands. Individual ticks or salivary gland DNA was dissolved in a final volume of 100 μl of buffer AE.

The DNA from blood samples taken during a field survey of cattle in western Turkey was extracted by the saponin lysis method (Barker *et al*. 1992). As a control for contamination, blood from known uninfected cattle was included as a negative sample in every 11 test samples during DNA extraction.

The extracted DNA samples produced were used in the PCR tests. Samples were used either fresh or after storage at −20 °C for up to 1 month. The DNA samples extracted from Turkish field samples were stored at −20 °C for 1 year.

Polymerase chain reactions

For the standard PCR, primers Tams1F (5′ ATG CTG CAA ATG AGG AT) and Tspms1R (5′ GGA CTG ATG AGA AGA CGA TGA G) amplifying a 785 bp fragment of the *T. annulata* 30 kDa major merozoite surface antigen gene, Tams1, were used. The reagents for the PCR were 0·5 μM each primer, 0·2 mM each dNTP, 20 mM Tris–HCl, pH 8·55, 16 mM $(NH_4)_2SO_4$, 3·0 mM $MgCl_2$, 150 μg/ml BSA, *Taq* Polymerase (Ultrotaq DNA 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 simulated tube setting and heated lid. Cycling conditions were 94 °C for 3 min followed by 40 cycles at 94 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min. A final extension step of 72 °C for 10 min was also used. In addition to the test DNA samples, a positive control (DNA from *T. annulata*-infected blood or cell lines), a negative sample control (containing bovine DNA or 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 by electrophoresis on a 1·5% agarose gel containing 0·5 μg/ml ethidium bromide according to standard methods (Sambrook *et al*. 1989).

For the nested PCR, the first amplification was carried out in a final volume of 50 μl using primers Tams1-T3 (5′ GAT AAG TTG TTA CGA ACA TGG GTT) and Tams1-T5 (5′ ATT TAA ATC GCT CAC TAG TCT GC) (Katzer *et al*. 1998) at

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0·5 μM each; 0·2 mM each dNTP, 20 mM Tris–HCl, pH 8·55*, 16 mM $(NH_4)_2SO_4$*, 2·5 mM $MgCl_2$*, 150 μg/ml bovine serum albumin* (*all contained in the 10× reaction buffer supplied with the *Taq* polymerase enzyme), 0·025 U/μl *Taq* Polymerase (Ultrotaq DNA Polymerase, Thermometric Ltd) and 2 μl of extracted DNA. For the second amplification the Tams1F and Tspms1R primers described earlier were used. The reagents for the PCR were at the same final concentrations as the first amplification of the nested PCR in a 20 μl final volume. Two μl of DNA from the first amplification were used as template. Cycling conditions were identical to those described earlier. Electrophoresis was carried out using 10 μl of products as described above.

In order to demonstrate that the PCR product bands were specific to the 30 kDa gene, a 20-mer sequence (5′ ATC TGH CTG TGA CAT TTG HA3′) complementary to the Tams1 gene was end-labelled with digoxigenin-11-ddUTP (Cat. No. 1 362 372, Boehringer Mannheim) and used as a probe following Southern blotting of the gel (Sambrook *et al*. 1989). Hybridization was carried out overnight at 40 °C; washes were performed twice in 2×SSC, 0·1% SDS at room temperature for 5 min and twice in 0·5×SSC, 0·1% SDS at 45 °C. The hybridized probe was detected colorimetrically using the digoxigenin nucleic acid detection kit (Cat. No. 1 175 041, Boehringer Mannheim).

RESULTS

Detection of T. annulata *in bovine blood*

T. annulata Gharb piroplasm infected blood with parasitaemias ranging from 1% at 10-fold dilution steps to 10^{-9}% was used to test the sensitivity of the PCR. The lowest concentration of piroplasms detected was 10^{-5}%, both by PCR (Fig. 1A) and by subsequent Southern blot hybridization (Fig. 1B). Assuming that cattle have 10^7 red blood cells per μl of blood, this figure is equivalent to the detection of 1 piroplasm in 1 μl of blood and is therefore approximately 100 times more sensitive than searching 200 fields in high-power light microscopy. A similar sensitivity was obtained using the Ankara stock of *T. annulata* (results not shown).

Detection of different stocks of T. annulata

Using *T. annulata* stocks isolated from widely different areas of the geographical range of the parasite and from the same geographical region, this PCR was demonstrated to detect all stocks tested (Fig. 2A). This was confirmed by Southern blot hybridization (Fig. 2B). The brightness of the PCR products were of the same intensity for all the stocks tested. The signal generated following hybridization was strong for the majority of the stocks tested; however, 3 stocks produced a weaker signal.

Detection of T. annulata *in ticks*

The number of infected acini in the right and left salivary glands of *H.a. anatolicum* ticks as assessed by MGP staining revealed a very strong correlation (Rs value of 0·91) which was significant at the $P < 0·01$ level. Therefore for the purposes of the comparison between MGP staining in one gland and PCR analysis on the other, it can be assumed that each salivary gland has the same number of infected acini.

Heavily infected (mean > 100 infected acini/tick) adult ticks were initially used to test the PCR. The strong bands obtained reflected the high infection rates of the ticks as determined by MGP staining of salivary glands. Uninfected ticks failed to produce a band of the correct size under the conditions described for the PCR; however, some non-specific amplification was seen. This, however, was not detected in the Southern blot of this gel (results not shown).

A time-course study was carried out on lightly infected ticks, to show that the PCR was sensitive enough to detect low levels of parasites. Lightly infected ticks were kept either unfed, or fed on a rabbit for between 1 and 6 days. The number of infected acini in the left hand salivary gland was determined by MGP staining. Of the 165 ticks dissected, 80 were chosen and the DNA from their other salivary gland was extracted and amplified by PCR. There was a very good agreement between the number of acini infected in the left salivary gland and the formation of a detectable PCR product in the right salivary gland. A histogram showing PCR signal intensity (numbers were assigned arbitrarily; 0 = no band, 1 = weak band, 2 = moderate band, 3 = strong band) against number of infected acini for each tick can be seen in Fig. 3. In 3 samples, a detectable PCR product was observed in the right salivary gland whereas infection was not demonstrated by MGP staining in the left gland. Conversely, there are 6/37 occasions when the PCR was negative in the right salivary gland when there were 1 or 2 infected acini in the left salivary gland. This may be explained by the fact that ticks with low infection rates often have 1 or 2 infected acini in one gland but no infection in the other (unpublished observations). At higher infection rates in the left salivary gland, the PCR was always positive for the right salivary gland.

To determine the sensitivity of detection more accurately, the DNA extracted from selected salivary glands was serially diluted 10-fold using DNA extracted from uninfected ticks. The dilutions were assigned the letters A (undiluted), B (10× diluted),

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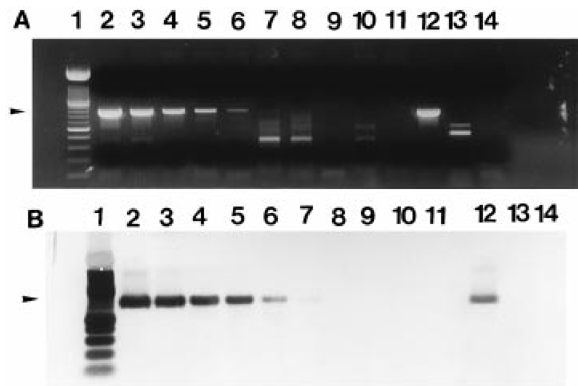

Fig. 1. Ethidium bromide-stained gel (A) and Southern blot (B) of the PCR to determine the level of sensitivity of the test using DNA extracted from *Theileria annulata*–Gharb infected blood, serially diluted with uninfected bovine blood. Lane 1, XIV Marker (Boehringer Mannheim) hereafter referred to as Marker; Lanes 2–10, 1% piroplasm parasitaemia, decreasing in 10-fold dilution steps to 10^{-8}%; Lane 11, uninfected bovine blood; Lane 12, *T. annulata*–Ankara cell line; Lane 13, *T. buffeli*–Australia and Lane 14, no DNA control. Arrows indicate 785 base pairs.

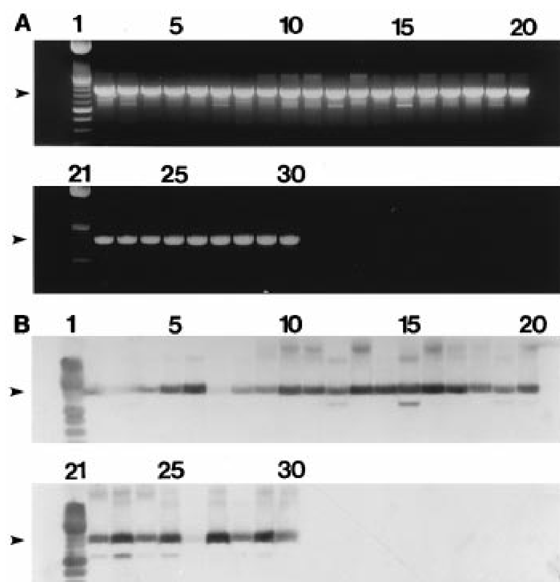

Fig. 2. Ethidium bromide-stained gel (A) and Southern blot (B) of the PCR demonstrating the specificity of the test with cell lines of 28 different stocks of *Theileria annulata* across its geographical range. Lanes 1 and 21, Marker; Lanes 2–9, Caceres, Gharb, Battan, Soba, Tova, Ankara, Razi, Hisar; Lanes 10–20 and 22-30 are isolates from different villages around Aydin, Turkey; Lane 31, uninfected ticks; Lane 32, uninfected bovine PBM; Lane 33, no DNA control. Arrows 785 bp.

C (100× diluted), D (1000× diluted) and E (10000× diluted). Five μl samples of each dilution was tested by PCR (Fig. 4). Since the number of infected acini in the opposite salivary gland was known, it was possible to make an accurate determination of the sensitivity of the PCR in infected

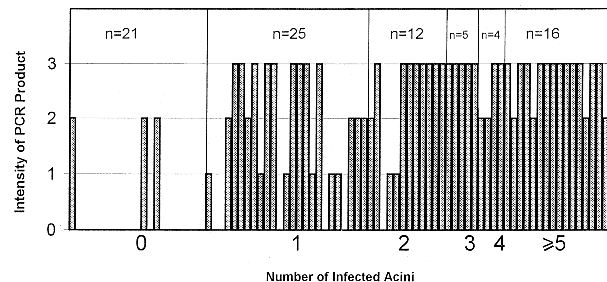

Fig. 3. Histogram showing the relationship between the number of infected acini in the left salivary gland by MGP staining and the intensity of the PCR signal obtained from DNA extracted from the right salivary gland of *Hyalomma a. anatolicum* ticks infected with *Theileria annulata*–Ankara. *n* = Number of ticks tested.

ticks. The results obtained are shown in Table 1. Sensitivities of detection varied between 0·1 and 0·001 infected acini.

The PCR was also tested with DNA extracted from engorged nymphs. These nymphs had been infected a year earlier and had been kept at 18 °C, 85% RH to prevent moulting. All 10 nymphs were shown to be infected though the intensity of the bands was not very strong (results not shown).

The results pertaining to the sensitivity of detection in ticks were obtained using the standard PCR only.

Cross-reactions

The DNA from closely related parasites, listed in the Materials and Methods section, which can be mistaken for *T. annulata* using traditional methods of diagnosis was not amplified using the primers described here. In some circumstances there was non-specific amplification possibly due to the prolonged number of cycles (Fig. 5). These extra bands were not detected by Southern blotting (not shown).

Field survey around Aydin, Turkey using the nested PCR

Blood smear examinations of 151 cattle from around the county of Aydin revealed that 100 were positive for piroplasms which, based on morphology and clinical symptoms of the cattle were assumed to be *T. annulata*. There was a concurrent infection of *Anaplasma marginale* in only 1 animal. There were 2 animals in which *A. marginale* alone and 2 animals in which *Babesia* spp. alone was observed by light microscopy. These animals were negative for the nested PCR. Of all the animals tested 121/151 were positive in the nested PCR whereas *T. annulata* piroplasms were seen in 100/151 cattle. In all animals in which *T. annulata* piroplasms were detected by light microscopy, a positive PCR product was obtained, whereas no piroplasms were seen by light

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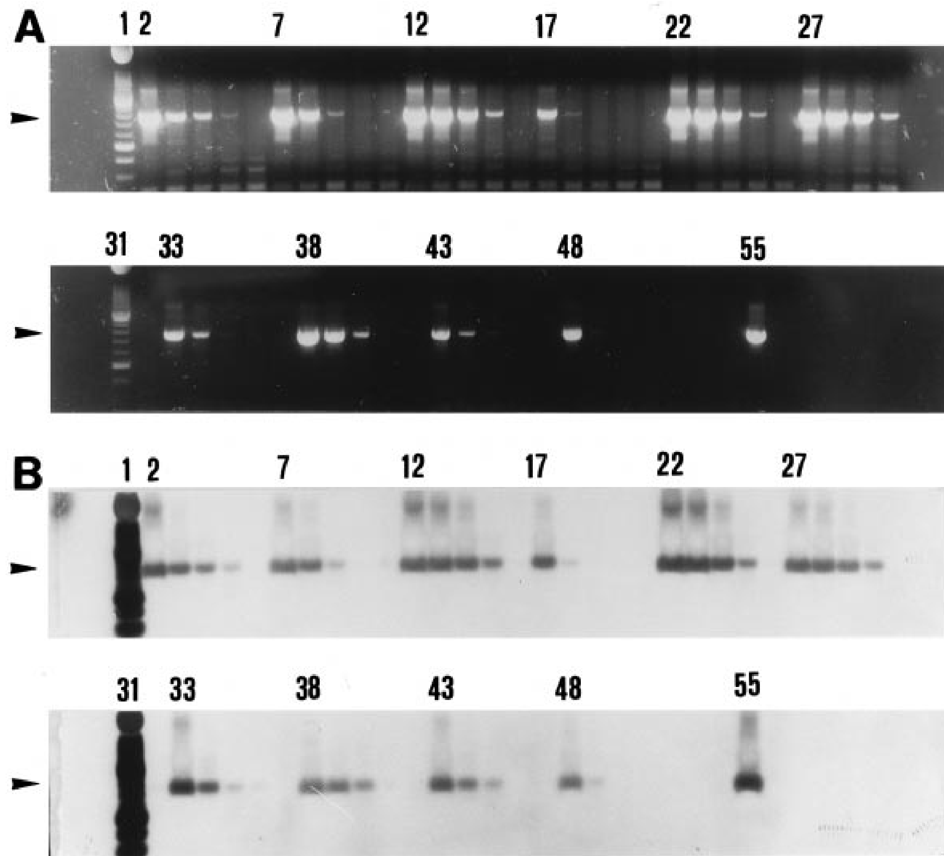

Fig. 4. Ethidium bromide-stained gel (A) and Southern blot (B) of the PCR to determine the level of sensitivity in *Hyalomma a. anatolicum* ticks. Ten-fold serial dilutions (A, B, C, D and E – see text) of the DNA from selected infected ticks were prepared using uninfected tick DNA. The number of infected acini in the opposite salivary gland as determined by MGP staining is also given. Lanes 1 and 31, marker; Lanes 2–6, unfed tick, 3 acini, A–E; Lanes 7–11, one-day fed tick, 6 acini, A–E; Lanes 12–16, one-day fed tick, 26 acini, A–E; Lanes 17–21, one-day fed tick, 1 acinus, A–E; Lanes 22–26, two-day fed tick, 15 acini, A–E; Lanes 27–30 and lane 32, two-day fed tick, 1 acini, A–E; Lanes 33–37, three-day fed tick, 1 or 2 acini, A–E; Lanes 38–42, four-day fed tick, 1 acinus, A–E; Lanes 43–47, five-day fed tick, 1 acinus, A–E; Lanes 48–52, six-day fed tick, 1 acinus, A–E; Lanes 53 and 54, uninfected ticks; Lane 55, *Theileria annulata*–Hisar cell line; Lane 56, no DNA control. Arrows 785 bp.

Table 1. The detection limit of *Theileria annulata* in infected *Hyalomma a. anatolicum* ticks expressed as the number or proportion of infected acini

(Individual adult ticks were unfed or partially fed for 1–6 days and the salivary glands dissected. The left salivary gland of each tick was stained with MGP and DNA was extracted from the right salivary gland. The extracted DNA was serially diluted 10-fold with uninfected *H.a. anatolicum* DNA and the greatest dilution to give a signal in the PCR was considered to be the limit of detection.)

Day of feeding	Tick sex	Number of infected acini in left hand salivary gland (MGP staining)	Greatest dilution of sample positive by PCR	Number or proportion of infected acini detected
0	Female	3	1000 ×	0·003
1	Female	6	100 ×	0·06
1	Female	26	1000 ×	0·026
1	Female	1	10 ×	0·1
2	Female	15	1000 ×	0·015
2	Male	1	1000 ×	0·001
3	Female	1 or 2	1000 ×	0·001–0·002
4	Female	1	1000 ×	0·001
5	Female	1	100 ×	0·01
6	Male	1	10 ×	0·1

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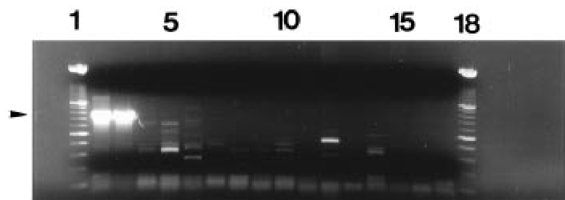

Fig. 5. Ethidium bromide-stained gel of the PCR demonstrating lack of cross-reactivity with closely related parasites. Lanes 1 and 18, marker; Lane 2, *Theileria annulata*–Gharb cell line; Lane 3, *T. annulata*–Razi cell line; Lane 4, *T. lestoquardi*-Lahr cell line; Lane 5, *T. lestoquardi*–Shiraz cell line; Lane 6, *Babesia bigemina* (Kenya); Lane 7, *B. bovis* (Mexico); Lane 8, *B. divergens* (Stormont); Lane 9, *T. equi*–USDA; Lane 10, *T. equi*, Onderstepoort; Lane 11, *T. equi*–Kwa Zulu; Lane 12, *T. buffeli*–Australia; Lane 13, bovine PBM; Lane 14, sheep PBM; Lane 15, horse whole blood; Lane 16, adult tick; Lane 17, no DNA. Arrow 785 bp.

microscopy in 21 out of the 121 PCR positive animals (Table 2). These results indicate that a greater degree of accuracy relating to the animals' infection with *T. annulata* can be gauged by the use of PCR.

DISCUSSION

In this study we have designed 2 PCR tests using primers complementary to the *T. annulata* 30 kDa major merozoite surface antigen gene Tams1 and which are sensitive and specific for the detection of *T. annulata* in bovine blood and *H.a. anatolicum* adult ticks. The sensitivity of these PCRs in bovine blood was 1 piroplasm in 1 μl of bovine blood, approximately 100 × more sensitive than searching 200 fields in high-power light microscopy. The sensitivity of the detection of *T. annulata* in adult ticks was demonstrated by comparing MGP staining of 1 salivary gland with the PCR signal intensity obtained from the other. As the numbers of infected acini in each salivary gland are approximately the same, this method gave a useful indication of the sensitivity of the test. To our knowledge this is the first objective assessment of the sensitivity of a *T. annulata*-specific PCR in ticks in which the number of infected acini in the test sample is known. The PCR detected *T. annulata* in unstimulated and 1–6 day fed ticks in most of the glands that had 1 or more infected acini in the corresponding salivary gland and is therefore at least as sensitive as MGP staining. Further assays using *T. annulata*-infected tick DNA diluted with uninfected tick DNA revealed that it was possible to detect one thousandth of an infected acinus.

T. annulata was also detected in engorged nymphs; however, the bands obtained were relatively weak. The difference in the strength of the band obtained from bovine blood at the time the ticks engorged and the weakness of the bands in the engorged nymphs suggests that some piroplasms are being destroyed by the tick. However, when engorged nymphs of this batch were allowed to moult, they gave rise to adults with high infection rates in their salivary glands. One explanation for this observation is that there may be inhibitory substances arising from engorged nymphs which are not completely removed during DNA extraction. Although not tested, it is probable that this PCR could amplify *T. annulata* in unfed nymphs, which are cumbersome to dissect. It would also be possible, judging by the sensitivity of the PCR in ticks, to perform wide-scale surveys to test for the presence of *T. annulata* in pooled groups of 10–100 ticks.

One major advantage of the described PCR tests over traditional diagnostic methods is that they distinguish *T. annulata* from *T. buffeli*, *T. parva* and *Babesia* spp. in bovine blood and *T. annulata* from *T. lestoquardi* and *T. equi* in the tick. Thus this work has gone some way to address the concerns expressed by other workers on accurate parasite diagnosis in *Hyalomma* spp. (Walker *et al*. 1983; Flach *et al*. 1993).

Over 30 stocks of the parasite as macroschizont-infected cell lines, collected from 10 countries across a wide range of *T. annulata* distribution and from within a small region of Turkey were detected by this method. All these parasites have been shown to be phenotypically diverse by glucose phosphate isomerase polymorphism (unpublished observations). In addition the test detected *T. annulata* DNA from at least 20 other isolates made from naturally infected *H. detritum* ticks collected in Turkey and Tunisia. It therefore appears that this PCR detects *T. annulata* across its geographical range. There were slight differences between the intensity of the bands formed following Southern blot hybridization to confirm the identity of the PCR products. This can be explained by small differences in the nucleotide sequence which may exist between the labelled probe and the amplified product of certain *T. annulata* stocks.

Other PCR assays which detect *T. annulata* have been described. These tests utilize either the gene of the small subunit of ribosomal RNA (ssu-rRNA) (Allsopp *et al*. 1993) or the gene encoding the 30 kDa major merozoite surface antigen (d'Oliveira *et al*. 1995) since both these genes have been sequenced for several stocks of *T. annulata* and closely related haemoprotozoa. However, using the ssu-rRNA PCR, we could not differentiate between *T. annulata* and *T. lestoquardi* DNA and did not detect the same level of sensitivity for piroplasm infected blood as the currently described PCR tests (unpublished results). In the PCR described by d'Oliveira *et al*. (1997), the sensitivity of the detection in ticks utilized tick samples spiked with *T. annulata* DNA. This

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Table 2. A comparison of light microscopy and PCR for the detection of *Theileria annulata* in 151 blood samples obtained from cattle in an endemic area of western Turkey

	Number of blood samples negative by PCR	Number of blood samples positive by PCR	Total
Number of blood smears negative by microscopy	30	21	51
Number of blood smears positive by microscopy	0	100	100
Total	30	121	151

paper demonstrates the sensitivity of the PCR in ticks by dilution of DNA extracted from a known number of infected acini with uninfected tick DNA and demonstrates it with unfed adult and partially fed ticks. In addition, the primers N516 and N517 (d'Oliveira *et al*. 1995) were unable to detect some of the *T. annulata* stocks isolated from around Aydin in Turkey. Despite this, the PCR tests described in this paper were unable to significantly improve upon the previously published sensitivities of *Theileria* PCR assays, these being, for *T. annulata*, 2–3 parasites per μl of bovine blood (d'Oliveira *et al*. 1995), 12 parasites (extrapolation from spiked samples using purified piroplasm DNA in tick DNA) (d'Oliveira *et al*. 1997) and 1 piroplasm per 4 μl of blood (Ilhan *et al*. 1998). For *T. lestoquardi*, sensitivities of 1 piroplasm per μl of bovine blood (Kirvar *et al*. 1998*a*) and 0·022 infected acini/tick (Kirvar *et al*. 1998*b*) were determined. Bishop *et al*. (1992) has estimated that it is possible to detect 1 parasite per μl of blood for *T. parva*. More recently a Reverse Line Blot (RLB) assay has been developed for the simultaneous detection of different species of *Theileria* and *Babesia* in cattle (Gubbels *et al*. 1999). This assay is able to detect *T. annulata* in bovine blood to a level of 10^{-6}% parasitaemia.

In this work, advantage was taken of the relatively large numbers of stocks of parasite at our disposal to select primers from an area of Tams1 that was conserved for all *T. annulata* stocks tested but variable in the closely related parasites listed in the Materials and Methods section. By adjusting the PCR conditions it has been possible to take advantage of these small differences in nucleotide sequences. It has been demonstrated that the ssu-rRNA gene sequences of *T. annulata* and *T. lestoquardi* are very similar (Katzer *et al*. 1998) and to distinguish between these 2 species in ticks, we suggest that the use of the 30 kDa major merozoite surface antigen gene PCR is more appropriate. As further DNA sequences of the parasites are identified and sequenced it may be possible to improve on the sensitivity of detection by the use of multi-copy genes in contrast to the single-copy 30 kDa major merozoite surface antigen gene (Shiels *et al*. 1995). Also it may be possible to utilize genes which represent the biological differences between *T. annulata* and *T. lestoquardi* such as the ability of the latter parasite to infect sheep PBM but not bovine PBM (Leemans *et al*. 1999*a*). These parasites undoubtedly possess greater differences in the sequences of their genes which code for ligands responsible for cell invasion. Similar arguments can be made for *T. equi* and *T. buffeli*.

The majority of this work was carried out on experimental samples and under laboratory conditions with fresh DNA samples. DNA samples repeatedly used for PCR tests become less amenable to amplification and DNA stored at −20 °C is likely to degrade after several weeks (unpublished observations). In a field survey of *T. annulata* infections in cattle in Turkey, DNA was extracted by the saponin lysis method and stored for over 1 year so that samples could be processed together for PCR. These DNA samples were amplified by the nested PCR method. It is possible that much of the extracted DNA had degraded by the time it was analysed but the total of 80 amplification cycles of the nested PCR was sufficient to detect the parasite DNA.

The level of sensitivity, specificity and cross-reactivity in PCR tests is undoubtedly due to a variety of factors such as sequences of primers, amplification conditions, DNA extraction methods, storage of samples and DNA. Standardization of such parameters has been recommended for *Plasmodium* spp. PCR tests (Bjorkman *et al*. 1998). Further work should be performed to address a similar situation now arising in *Theileria* diagnosis.

The PCR methods described in this paper have been shown to work sensitively and specifically under laboratory conditions using commercial kits to extract DNA which, according to manufacturer's guidelines, can remove chemicals present in samples that may inhibit the PCR. It would, however, be useful for wide-scale epidemiological surveys if simpler and cheaper DNA extraction methods could be tested and developed to detect *T. annulata* in bovine blood and in ticks. The use of blood spots dried onto filter paper to detect *Plasmodium* in human blood (e.g. Long *et al*. 1995; Tan *et al*. 1997) has already been described. Other simple yet sensitive methods to extract DNA from ticks, such as tick squashes onto filter paper should be investigated further.

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