ORIGINAL PAPER

R.P. Bishop · B.K. Sohanpal · S.P. Morzaria

Cloning and characterisation of a repetitive DNA sequence from *Theileria mutans*: application as a species-specific probe

Abstract Repetitive DNA sequences were isolated from a *Theileria mutans* genomic library by screening with *T. mutans* total DNA. DNA sequence analysis demonstrated that a section of one of the clones contained a complex series of overlapping perfect repeats ranging between 99 and 20 bp in size. The *T. mutans* repetitive sequence did not contain large open reading frames (ORFs), unlike *T. parva* Tpr repetitive DNA sequences. When used as a hybridisation probe the repetitive sequence revealed restriction-fragment-length polymorphisms (RFLPs) between the *Eco*RI-digested DNAs of two *T. mutans* stocks. The *T. mutans* repetitive probe gave a signal with 1 ng of purified *T. mutans* piroplasm DNA and detected *T. mutans* sequences in whole-blood DNA isolated from an experimentally infected animal when the piroplasm parasitaemia was equal to or above 0.4%. Oligonucleotide primers derived from the repetitive sequence allowed more sensitive detection of *T. mutans* DNA by polymerase chain reaction (PCR) amplification. Using the PCR, *T. mutans* DNA was amplified from an experimentally infected animal with a parasitaemia of <0.1%.

Introduction

Theileria mutans, a protozoan parasite that multiplies in the leukocytes and erythrocytes of cattle and African buffalo (Young et al. 1978a; Uilenberg 1981), is widely distributed in sub-Saharan Africa. The parasite is transmitted by several species of *Amblyomma* ticks. *T. mutans* is responsible for anaemia and productivity losses in cattle, particularly when present in mixed infections with other tick-transmitted protozoa or rickettsias (Paling et al. 1981; Saidu 1982; Flach et al. 1989). Outbreaks of severe anaemia attributed to *T. mutans* have also been observed in East Africa (Irvin et al. 1972; Purnell 1977; Young et al. 1978b), but the prevalence of pathogenic *T. mutans* is unknown.

Current diagnostic methods, based on identification of the piroplasm stage in Giemsa-stained blood smears or detection of antibodies against *T. mutans* using the indirect immunofluorescent antibody (IFA) test (Lohr and Ross 1969; Burridge 1971), are unsatisfactory due to their confusion of *T. mutans* with other species of *Theileria* and to their limited sensitivity (Katende et al. 1990). More recently, an antibody- and antigen-detection enzyme-linked immunosorbent assay (ELISA) has been developed for *T. mutans* (Katende et al. 1990) and is currently undergoing validation. Species-specific repetitive DNA probes, useful both for species identification and for characterisation of stocks and strains within species, have been isolated from many protozoan parasites, including *Trypanosoma* (reviewed by ole-Moi Yoi 1987), *Plasmodium* (Aslund et al. 1985; Enea 1986; Zolg et al. 1987) and *T. parva* (Conrad et al. 1987a; Allsopp and Allsopp 1988). DNA probes have advantages over serological methods for detection of parasites in their arthropod vectors (Chen et al. 1991) and, when coupled to polymerase chain reaction (PCR) amplification of DNA (Mullis and Faloona 1987), for sensitive detection of carrier animals with low piroplasm parasitaemias.

In this paper we describe the cloning and characterisation of a *T. mutans*-specific repetitive DNA sequence. The cloned sequence is useful as a probe for detection of *T. mutans* in infected cattle, and PCR primers derived from the repetitive sequence allow sensitive and specific detection of *T. mutans* DNA.

R. P. Bishop (✉) · B. K. Sohanpal · S. P. Morzaria
International Laboratory for Research on Animal Diseases,
P.O. Box 30709, Nairobi, Kenya

Materials and methods

Organisms and DNA preparation

Details of the parasite stocks used are given in Table 1. Purification of piroplasms and preparation of piroplasm and schizont-infected lymphocyte DNAs were carried out as described by Conrad et al. (1987a). Dissected salivary glands of *Amblyomma variegatum* (Kiboko stock) ticks were supplied by the ILRAD Tick Unit, and DNA was prepared from these by standard methods (Sambrook et al. 1989). Whole-blood DNA was prepared as previously described (Sambrook et al. 1989) from 100-μl aliquots of blood after four washes in lysis buffer containing Triton X-100 and sucrose (Higuchi 1989).

Infection and monitoring of cattle with *Theileria mutans*

Susceptible, splenectomised Friesian (*Bos taurus*) or Boran (*B. indicus*) cattle, aged between 1 and 2 years and kept under strict tick control, were infected intravenously with thawed blood stabilates of *T. mutans*. Following inoculation the cattle were monitored daily by examination of Giemsa-stained blood smears to detect piroplasms. The piroplasm parasitaemia was determined by counting the number of infected erythrocytes per 1000.

Construction of libraries and isolation of genomic clones

A library of sheared *T. mutans* (Kipange) genomic DNA fragments was constructed in λgt11 as described by Young et al. (1985) using purified *T. mutans* piroplasm DNA. The unamplified library was screened with radiolabeled *T. mutans* total DNA, and after DNA isolation the inserts from two strongly positive bacteriophage clones were subcloned into the *Eco*RI site of pUC19. All recombinant DNA techniques were carried out as described by Sambrook et al. (1989).

Blotting and DNA hybridisation

For slot-blotting of DNA a Bio-dot SF apparatus (Bio-Rad Laboratories) was used according to the manufacturer's instructions. The DNA was processed as described by Saiki et al. (1986). Fixation of blots onto nylon membranes was accomplished by UV treatment (2 min). Gel electrophoresis, Southern transfer of gels onto nylon membranes (Hybond N, Amersham) and hybridisation solutions were used as described by Sambrook et al. (1989). Probes were radiolabeled by random priming (Feinberg and Vogelstein 1983) using a Prime-It kit (Stratagene). The specific activity of probes ranged between 5 and 7×10^8 cpm/μg. All filters were washed in 0.1 X SSC/0.1% SDS at 65° C for 30 min.

Table 1 Parasite stocks

Species	Stock	Stage	Country	Origin/reference
Theileria mutans	Kipange	Piroplasm	Tanzania	Unguja island/Morzaria et al. 1990
Theileria mutans	Intona	Piroplasm	Kenya	Mara/Morzaria et al. 1990
Theileria parva	Muguga	Piroplasm	Kenya	Central Highlands/Brocklesby et al. 1961
Theileria parva	Mariakani	Piroplasm	Kenya	Coast/Irvin et al. 1983
Theileria parva	Marikebuni	Piroplasm	Kenya	Coast/Irvin et al. 1983
Theileria taurotragi	East African orphanage	Schizont	Kenya	Central Highlands/Stagg et al. 1983
Theileria taurotragi	Kitengela	Piroplasm	Kenya	From eland 7695, which was experimentally infected by ticks fed on naturally infected eland 7696/T. Dolan and S. Morzaria, unpublished data
Theileria annulata	Tova	Schizont	Israel	Tsur 1945
Theileria sergenti	Ikeda	Piroplasm	Japan	Fujisaki et al. 1985
Theileria sp. Marula	Marula	Piroplasm	Kenya	Rift valley/Young et al. 1992
Theileria sp. buffalo	Buffalo 6834 B (clone 11)	Schizont	Kenya	Laikipia/Conrad et al. 1987b
Babesia bigemina	Muguga	Piroplasm	Kenya	Central Highlands/S. Williamson and P. Ngumi, unpublished data
Babesia bigemina	Kikuyu	Piroplasm	Kenya	Central Highlands/J. Katende, unpublished data
Babesia bovis	Brisbane	Piroplasm	Australia	W. Jorgensen, unpublished data
Anaplasma marginale	Kikuyu	Blood stages	Kenya	Central Highlands/J. Katende, unpublished data

DNA sequencing

Plasmid clones in pUC19 were subjected to BAL 31 or Exo III and mung-bean nuclease digestion and subcloned into SmaI/EcoRI cut M13 for sequencing by the dideoxy chain termination method (Sanger et al. 1977). A Sequenase kit (United States Biochemical Corporation) incorporating a modified T7 polymerase was used for all sequencing reactions. The sequence was determined on both strands.

PCR amplification of DNA

Oligonucleotides to act as PCR primers were synthesised on an Applied Biosystems 381 A synthesiser and were purified using OPC columns supplied by Applied Biosystems. DNA was amplified in 100-μl reactions containing 10 mM TRIS-HCl (pH 9.0), 50 mM KCl, 2.5 mM, MgCl$_2$, 0.01% gelatin, 1% Triton X-100, 200 μM deoxynucleoside triphosphates (dNTPs), 100 ng of each primer, 2.5 units Taq polymerase (Promega) and either 5 ng of purified piroplasm DNA or 50 ng of schizont-infected lymphocyte or uninfected bovine DNA. Reactions were performed on a Perkin Elmer Cetus DNA thermal cycler. Cycling conditions included denaturation at 94°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 10 s, for a total of 30 cycles.

Results

Characterisation of cloned *Theileria mutans* repetitive DNA sequences

T. mutans total DNA was used to screen a λgt11 library of *T. mutans* (Kipange) piroplasm DNA. The inserts from two strongly positive clones were subcloned into pUC19. In both plasmids the cloned DNA fragments were approximately 3.8 kb in size and they exhibited identical restriction maps. The plasmids were designated pTmr 1 and pTmr 1 A. The *T. mutans* DNA fragment cloned in pTmr 1 hybridised to multiple fragments in *Eco*RI-digested *T. mutans* DNA (Fig. 1, lanes 1 and 2), indicating that the sequence was repeated in the genome. Several restriction-fragment-length polymorphisms (RFLPs) were apparent between the DNAs of the two *T. mutans* stocks used, Intona (Fig. 1, lane 1) and Kipange (Fig. 1, lane 2). The pTmr 1 probe did not hybridise to *Eco*RI-digested DNA isolated from three *T. parva* stocks (Fig. 1, lanes 3–5).

The DNA sequence of a 2253-bp section at one end of the 3.8-kb *T. mutans* DNA fragment in pTmr 1 was determined. The arrangement of the sequenced region of the pTmr 1 clone, indicating the position of a major repeat, is shown in diagrammatic form in Fig. 2 together with a partial restriction enzyme map. The sequence contained a complex series of repeats involving 48 perfect direct repeats of > 20 bp situated between bases 9 and 1525. The size of the repeats varied from 99 to 20 bp and the spacing between the start of the repeats varied between 939 and 22 bp. In some instances the end of one copy of the repeat overlapped the starting point of the duplicate sequence (e.g. Fig. 3, repeat numbers 43–48). The first 1525 bases of the sequence are shown in Fig. 3 together with a table showing all perfect direct repeats
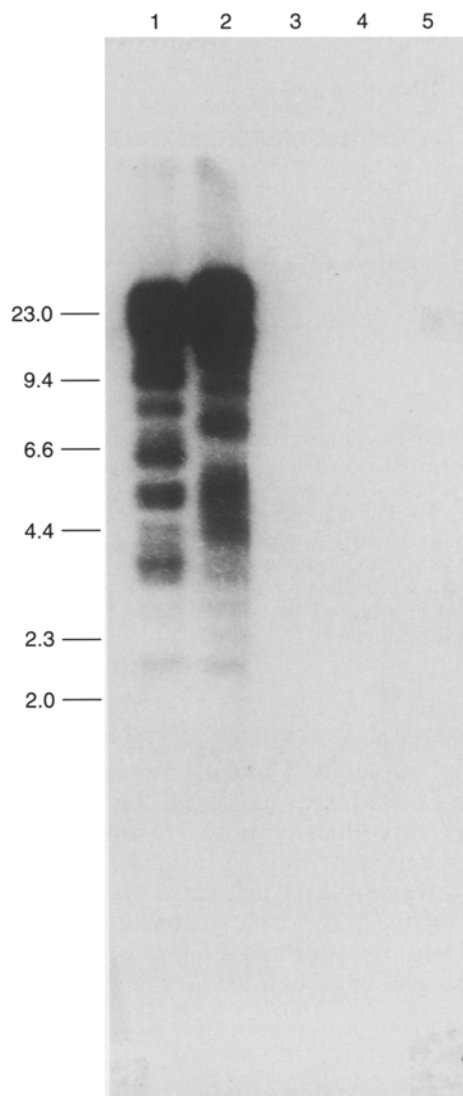

Fig. 1 Hybridisation of pTmr 1 to *Eco*RI-digested *Theileria mutans* and *T. parva* genomic DNA. In all, 1 μg of each purified piroplasm DNA sample was digested with *Eco*RI, electrophoresed through a 0.8% agarose gel and transferred onto a nylon filter by Southern blotting. The filter was hybridised with radiolabeled pTmr 1 and washed twice in 0.1 X SSC/0.1% SDS at 65°C for 30 min (*1 T. mutans* [Intona], *2 T. mutans* [Kipange], *3 T. parva* [Muguga], *4 T. parva* [Mariakani], *5 T. parva* [Marikebuni])

of > 20 bp. The sequence from bases 1525–2523 did not contain repeats. The sequence also exhibited an imperfect major repeat in which almost all of the 683 bases from 9 to 692 were repeated between bases 824 and 1525, the main exceptions being the 15 bases between 284 and 300 and the 42 bases between 539 and 582. The relative position and copy number of consituent sequences often varied between the two copies of the major repeat. An 873-bp *Bgl*II restriction fragment (bases 74–927), containing most of the first copy of the major repeat and part of the second, and a 206-bp PCR product (bases 2019–2224) derived from the "unique" region of the pTmr 1 clone (see Fig. 2 for the location of
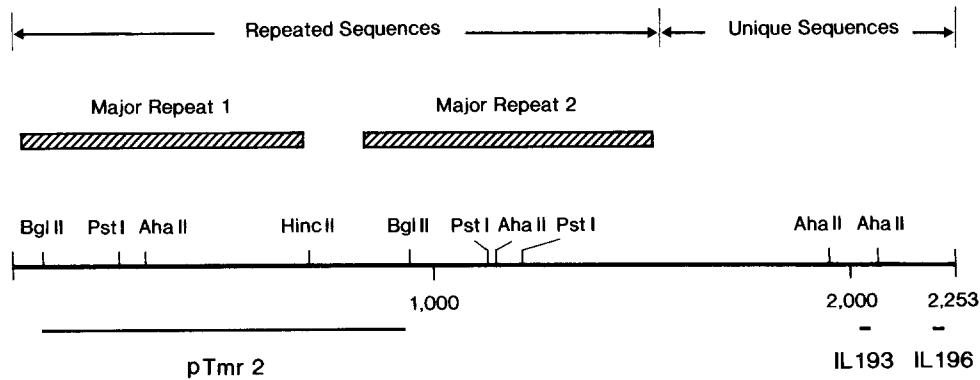
Fig. 2 Physical organisation of cloned *T. mutans* repetitive DNA sequences. A partial restriction map is shown of a 2253-bp sequence at the 3' end of the 3.8-kb *T. mutans* DNA fragment cloned in plasmid pTmr 1. The complete DNA sequence of this section of the clone was determined. The extent of the repeated and unique DNA sequences within the clone is indicated, as is the position of an imperfect major repeat within the region of repeated sequences. The location of subclone pTmr 2 and of the PCR primers IL 193 and IL 196 is also indicated

these sequences) were hybridised to *Eco*RI-digested *T. mutans* piroplasm DNA. The *Bgl*II fragment hybridised to the same *Eco*RI fragments as did the whole pTmr 1 clone. The PCR product hybridised to multiple genomic *Eco*RI fragments but only to a subset of those homologous to the whole pTmr 1 clone (data not shown). The region of the pTmr 1 clone that did not contain DNA sequence repeats was therefore also repeated in the *T. mutans* genome. The *T. mutans* repeated sequence

did not exhibit significant homology to any sequences in the GenBank or EMBL databases and did not contain large open reading frames (ORFs).

Application of cloned *T. mutans* repetitive DNA as a species-specific probe

The pTmr 1 probe exhibited weak cross-hybridisation with uninfected bovine DNA. An 873-bp *Bgl*II fragment between bases 74 and 927 of the sequenced region of the pTmr 1 clone was therefore subcloned into pUC19. This clone was designated pTmr 2 (Fig. 2). The pTmr 2 probe hybridised only to *T. mutans* genomic DNA (Fig. 4, row B) and not to DNA isolated from six other species of *Theileria* (Fig. 4, row C, lanes 4–6; row D, lanes 1–6), from *Babesia bigemina* (Fig. 4, row C, lane 2) or from *Anaplasma marginale* (data not shown). The pTmr 2 probe also did not hybridise to excess quantities (5 µg)

Fig. 3a, b Detailed arrangement of repeated sequences in one section of the 3.8-kb cloned *T. mutans* DNA fragment. The DNA sequence of bases 1–1525 in the sequenced section of the *T. mutans* repetitive DNA fragment cloned in plasmid pTmr 1 (a) is shown together with a table detailing the positions of all perfect DNA sequence repeat units of > 20 bp (b). The length of each individual repeat and the number of bases separating the start of the repeats is given in the table. The repeats 1–48 are numbered according to the decreasing distance separating their starting points. An imperfect major repeat is present in the sequence. The major repeat involves bases 9–692 and 824–1525. The nucleotide sequence data presented in this figure have been submitted to the GenBank database with accession number L16682

```
   1   GGCTGAAGCCAGGATCGCGGTGTAGGTCTTCGATGGCCAATATACACCGGCTAAGGTGGG
  61   ACGTAGCGCGTTTAGATCTTCGATGGCCAATCTACACCGGCTAAGGTGGGACGTAGCGCG
 121   GTGTAGGTCTTCGTCGCTTCTAAGAAGTGACCGTCATCGGCTCCAGGTGGACGACGCGAA
 181   GGGCTAAAGCCCAGGATCGCGAAGGGCAAAAGCCCAGGATCGCGAAGGGCAAAAGCCCAG
 241   GATCGCGAAGGGTTCGAAGCCCACGACCGCAGAGTCGCGATCGGCTCGATAGCGCACAGT
 301   TTCCGTCTTCAAGGGCGCCGATGCTACAAAGGGAACACGCTTGCGCGATAACCGCAGAGT
 361   CGCGATCGGTTCGATAGCCCGCAGTTCCCGTCTTTAAGGCCGCCGATGCTACAAAGGGAA
 421   CACGCTTGCGCGATAACCGCAGAGTCGCGATCGGTTCGATAGCCCGCAGTTCCCGTCTTT
 481   AAGGCCGCCGATGCTACAATGGGAACACGCTTGCGCGATAACCGCAGAGTCGCGATCGGC
 541   TCGATAGCGCACAGTTTCCGTCTTCAAGGCCGCCGATGCTACAATGGTCAGGATAATCCC
 601   AGGGCCGAATGTAGAGTTACACTGGCCAGGGTAATCCCAGGGCCGAATGTAGAGTTACAC
 661   TGGCCAGGGTAATCCGACGACCGAATGTAGAGATCGAATCGTTCCGTAGTCGCTCCTAAG
 721   TTGACCGTCATCGGCTCGAATACCAGGATCGCGAAGGGCTCGAAGCCCATAACCGGACGG
 781   GCTGAAGCCCAGGGCCGTGACGGCTGAAGCCCAGGATCGCGAAGGCAAAAGCCCAGGATC
 841   GCGGTGTAGGTCTTCGATGGCCAATATACACCGGCTAAGGTGGACACAGCGCGGTGTAGG
 901   TCTTCGATGGCCAATATACACCGGCTAAGGTGGACGTAGCGCGTTTAGATCTTCGATGGC
 961   CAATCTACACCGGCTAAGGTGGGACGTAGCGCGGTGTAGGTCTTCGTCGCTTCTAAGAAG
1021   TGACGGTCATCGGCTCCAGGTGGACGACGCGAAGGGCAAAAGCCCAGGATCGCGAAGGGC
1081   AAAAGCCCAGGATCGCGAAGGGTTCGAAGCCCACGACCGCAGAGTCGCGATCGGATTTGT
1141   TCGCCTGCAGATTTCCGTCTTCAAGGGCGCCGATGCTACAAAGGGAACACGCTTGCGCGA
1201   TAACCGCAGAGTCGCGATCGGTTTGTTCGCCTGCAGTTTCCGTCTTCTAAGGGCGCCGAT
1261   GCTACAAAGGGAACACGCTTGCGCGATAACCGCAGAGTCGCGCGATCGGTTCGATAGCCC
1321   GCAGTTCCCGTCTTTAAGGCCGCCGATGCATCAATGGTCAGGATAATCCCAGGGCCGAAT
1381   GTAGAGTTACACTGGCCAGGGTAATCCGACGACCGAATGTAGAGTTACACTGGCCAGGGT
1441   AATCCCAGGGCCGAATGTAGAGTTACACTGGCCAGGGTAATCCCAGGGCCGAATGTAGAG
1501   CTACACTGGCCAGGGTAATCCGACG
```
a

of uninfected bovine DNA (Fig. 4, row C, lane 1) or *Amblyomma variegatum* DNA (Fig. 4, row C, lane 3). The cloned repetitive DNA sequence in pTmr 2 therefore hybridised specifically to *T. mutans* DNA. The pTmr 2 probe detected approximately 1 ng of purified *T. mutans* piroplasm DNA after a 16-h exposure (Fig. 4, row B, lane 3). The intensity of hybridisation to 1 ng of *T. mutans* genomic DNA was approximately equivalent to that of hybridisation to 10 pg of the pTmr 2 clone (Fig. 4, row A, lane 4), suggesting that the cloned sequence represented approximately 1% of the total *T. mutans* genome.

To test the usefulness of the probe for detection of *T. mutans* in cattle blood, pTmr 2 was hybridised to whole-blood DNA samples (prepared from 100-μl aliquots of blood) isolated from an animal experimentally infected with a *T. mutans* blood stabilate. The probe was hybridised to samples taken at ten different dates during the course of the infection. The piroplasm parasitaemia of these samples, determined by light microscopic analysis of blood smears, varied from <0.1% to 46%. The probe detected *T. mutans* in samples with parasitaemias of 0.4% (Fig. 5, row A, lane 3) and above. The probe exhibited no hybridisation to two uninfected bovine DNA samples (Fig. 5, row D, lanes 2 and 3). There was a general correlation between the percentage of parasitaemia determined microscopically and the intensity of hybridisation of the pTmr 2 probe to the whole-blood DNA samples. For example, the sample that had the highest parasitaemia (46%) also showed the strongest hybridisation to the probe (Fig. 5, row C, lane 1), whereas the sample with the lowest detectable parasitaemia (0.4%) showed the weakest hybridisation to the probe (Fig. 5, row A, lane 3).

PCR amplification primers derived from cloned *T. mutans* repetitive DNA

Synthetic oligonucleotide primers derived from the major repeat region of the cloned *T. mutans* repetitive sequence did not amplify unique products when used in PCR reactions with *T. mutans* genomic DNA as a target (data not shown). Two oligonucleotide primers, IL 193 (25-mer) and IL 196 (21-mer), designed to amplify 206 bp between nucleotides 2019 and 2024 in the "unique" sequence region of the pTmr 1 clone, were therefore synthesised (Fig. 6).

Primers IL 193 and IL 196 generated a PCR product of approximately 200 bp from DNA isolated from the two available stocks of *T. mutans* (Fig. 7A, lanes 1 and 2). The primers did not generate specific PCR products, detectable by ethidium bromide staining, from DNA isolated from *T. parva* (Fig. 7A, lanes 3 and 4), *T. taurotragi* (Fig. 7A, lanes 5 and 6), *Theileria sp.* Marula (Fig. 7A, lane 7), *T. annulata* (Fig. 7A, lane 8), *T. sergenti* (not shown) or *Theileria sp.* buffalo (not shown). Additionally, the primers did not generate specific PCR products from *B. bovis* (Fig. 7A, lane 9), *B. bigemina* (Fig. 7A, lane 10), uninfected bovine DNA (Fig. 7A, lane 11) or *A. marginale* DNA (not shown). Probing of a blot of the gel shown in Fig. 7A with radiolabeled pTmr 1 DNA revealed hybridisation only to the PCR products derived from *T. mutans* DNA (Fig. 7B, lanes 1 and 2). This result demonstrated that the observed PCR products resulted from the amplification of the *T. mutans* repetitive DNA sequences cloned in pTmr 1 and confirmed the absence of PCR products amplified from the DNA of the other parasite species tested. The

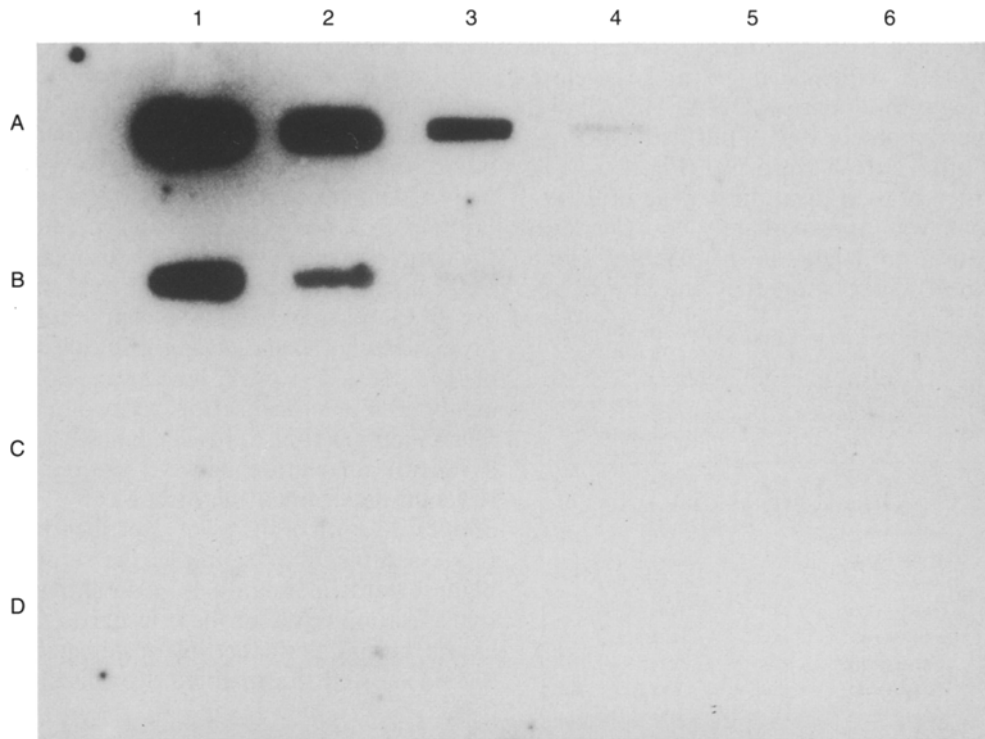| Repeat number	First copy: Bases	Second copy: Bases	Length of repeat	No. of bases separating start of repeats
1	361-409	1301-1349	49	939
2	311-364	1249-1302	54	937
3	43-70	966-993	28	922
4	186-206	1082-1102	21	895
5	594-616	1478-1500	23	883
6	618-637	1502-1521	20	883
7	16-59	890-933	44	873
8	59-151	932-1034	93	872
9	153-184	1026-1057	32	872
10	186-229	1059-1102	44	872
11	446-494	1301-1349	49	854
12	401-449	1254-1302	49	852
13	300-371	1152-1223	72	851
14	198-284	1048-1134	87	849
15	594-654	1440-1500	61	845
16	656-679	1502-1525	24	845
17	221-252	1048-1079	32	826
18	9-59	833-883	51	823
19	605-675	1413-1483	71	807
20	582-637	1352-1407	56	769
21	643-675	1413-1445	33	769
22	50.-534	1269-1302	34	767
23	401-456	1168-1223	56	766
24	632-692	1364-1424	61	731
25	501-539	1183-1221	39	681
26	205-224	824-843	20	618
27	228-247	824-843	20	595
28	266-314	521-569	49	254
29	824-843	1078-1097	20	253
30	824-843	1055-1074	20	230
31	331-369	501-539	39	169
32	1364-1368	1478-1500	23	113
33	1388-1411	1502-1525	24	113
34	1163-1216	1249-1302	54	85
35	316-414	401-499	99	84
36	416-454	501-539	39	84
37	481-502	566-587	22	84
38	1364-1407	1440-1483	44	75
39	1426-1445	1502-1521	20	75
40	43-70	93-120	28	49
41	840-885	890-935	46	49
42	186-206	232-252	21	45
43	594-637	632-675	44	37
44	1375-1407	1413-1445	33	37
45	1413-1462	1451-1500	£0	37
46	1464-1483	1502-1521	20	37
47	186-229	209-252	44	22
48	1048-1079	1071-1102	32	22

b

38

Fig. 4 Specific hybridisation of a cloned *T. mutans* repetitive DNA fragment to *T. mutans* genomic DNA. A slot-blot of genomic DNAs isolated from *T. mutans*, 7 other species of apicomplexan parasites, an uninfected bovine and *Amblyomma variegatum* ticks is shown. The blot was hybridised with radiolabeled pTmr 2 and washed in 0.1 X SSC/0.1% SDS at 65° C for 30 min (*row A* pTmr 2 plasmid: *1* 10 ng, *2* 1 ng, *3* 100 pg, *4* 10 pg, *5* 1 pg; *row B T. mutans* piroplasm: *1* 100 ng, *2* 10 ng, *3* 1 ng, *4* 100 pg; *rowC 1* uninfected bovine, 5 μg; *2 Babesia bigemina* [Muguga] piroplasm, 1 μg; *3* uninfected *A. variegatum*, 5 μg; *4 T. parva* [Marikebuni] piroplasm, 1 μg; *5 T. parva* [Muguga] piroplasm, 1 μg; *6 T. parva* [Mariakani] piroplasm, 1 μg; *row D 1 Theileria* sp. [Marula] piroplasm, 1 μg; *2 Theileria* sp. buffalo [Buffalo 7684 B, clone 11] schizont-infected lymphocyte, 20 μg; *3 T. annulata* [Tova] schizont-infected lymphocyte, 20 μg; *4 T. taurotragi* [East African orphanage] schizont-infected lymphocyte, 20 μg; *5 T. taurotragi* [Kitengela] piroplasm, 1 μg; *6 T. sergenti* [Ikeda] piroplasm, 1 μg)

primers IL 193 and IL 196 therefore specifically amplified *T. mutans* DNA sequences.

The oligonucleotide primers were tested on whole-blood DNA samples from the *T. mutans*-infected animal, with piroplasm parasitaemias ranging from <0.1% to 46%. The primers generated *T. mutans*-specific PCR products from all ten DNA samples, including those with piroplasm parasitaemias of 0.2% and <0.1% as determined by light microscopy, which had not given signals with the radiolabeled pTmr 2 probe.

Discussion

This paper describes the cloning and characterisation of a repetitive DNA sequence from *Theileria mutans*. The

sequence contained a complex series of short overlapping repeats. The repetitive sequence was specific for *T. mutans* and did not hybridise to the DNA of six other *Theileria* species, *Babesia bigemina* or *Anaplasma marginale*. The probe will therefore be useful for identification of *T. mutans* in animals with mixed infections of *T. mutans* and other tick-borne parasites, which are often observed in the field (Katende et al. 1990). Oligonucleotide primers derived from a section of the repetitive sequence allowed sensitive and specific detection of *T. mutans* DNA by PCR amplification.

DNA sequence analysis of the *T. mutans* repetitive sequence revealed numerous short direct repeats ranging between 20 and 99 bp in size (Fig. 3); it was therefore very different in structure from the repetitive sequences, designated *Tpr*, isolated from *T. parva* in which the repeat units are up to 1.4 kb in size (Baylis et al. 1991). The *Tpr* repetitive sequences also contain large open reading frames (ORFs), the largest observed being 2.4 kb in size, which are predicted to encode proteins (Baylis et al. 1991). By contrast, the largest ORF present in the *T. mutans* repetitive sequence was 408 bp, and this ORF was not predicted to encode a protein by the criteria of Fickett (1982). The G/C content of the *T. mutans* sequence was 54%, suggesting that the *T. mutans* genome is more G/C-rich than *T. parva*, whose average G/C content is estimated to be approximately 30% based on melting-curve analysis of genomic DNA (Allsopp and Allsopp 1988). A relatively higher G/C content than that in *T. parva* is also suggested by the observation that the restriction enzymes *Sfi*I and *Not*I, whose recognition sites contain only G/C bases, are frequent cutters in the *T. mutans* genome (Morzaria et al. 1990).
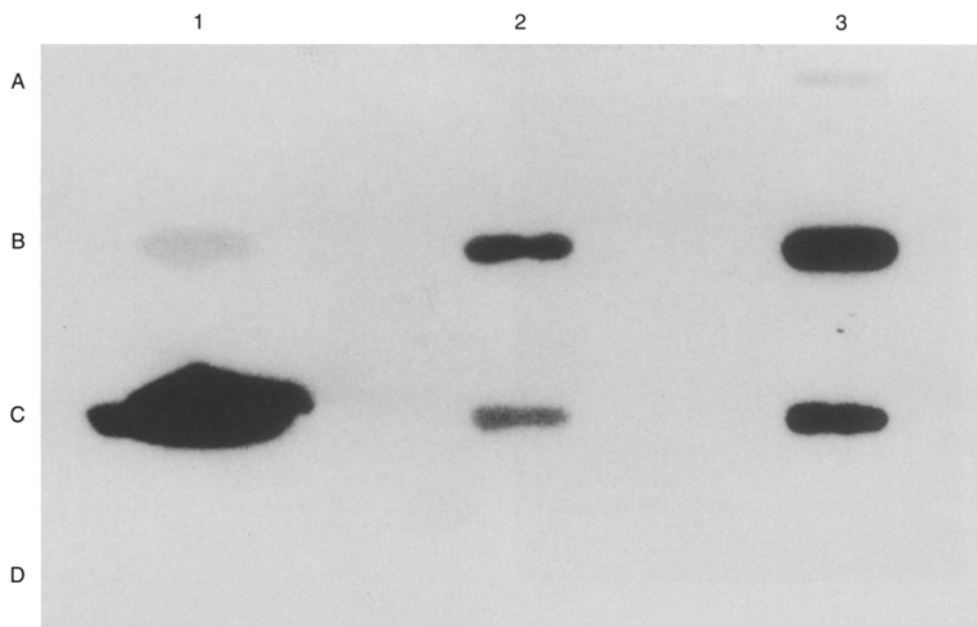
Fig. 5 Detection of *T. mutans* DNA sequences in cattle blood by hybridisation with a cloned *T. mutans* repetitive DNA probe. The figure shows a slot-blot of DNAs made from blood samples (100 μl), isolated on 10 different dates, from an animal that was experimentally infected with *T. mutans*. Two uninfected bovine DNA samples (5 μg/slot) are also shown. The blot was hybridised with radiolabeled pTmr 2 and washed in 0.1 X SSC/0.1% SDS at 65° C for 30 min. Whole-blood DNA from animal BH 159 is shown (*row A 1* isolation date 10/7/90, piroplasm parasitaemia in red blood cells <0.1%; *2* 14/7, 0.2%; *3* 17/7, 0.4%; *row B 1* 20/7, 1.6%; *2* 23/7, 4.6%; *3* 25/7, 10%; *row C 1* 31/7, 46%; *2* 17/8, 4.8%; *3* 19/8, 4.8%; *row D 1* 23/8, 0.4%; *2, 3* uninfected bovine DNA samples)

```
                  IL 193
      1   CGGGTATATAATGTAAATGAAGAAGAATATGAAAATGATAAATTGGGAGGGTATGACGTC

     61   GGTTATTTATGCAGATTTGCATGTTGCCGATTATTCTTTTTGATAATTTTTAACCTCCCC

    121   ATCCCTGGTGGATTGTGTGGCGGTTAGGATCATCGGTACTCGCTGCTTATCGAAAACCGT
                  IL 196
    181   GTTTACACATTCCTACACACCTGAGA


      IL 193 5'  CGGGTATATAATGTAAATGAAGAAG 3'

      IL 196 5'  TCTCAGGTGTGTAGGAATGTG 3'
```

Fig. 6 PCR amplification primers derived from *T. mutans* repetitive DNA sequences. Primers IL 193 and IL 196 were designed to amplify a 206-bp fragment between bases 2019–2224 of the sequenced region of the cloned *T. mutans* repetitive DNA sequence present in plasmid pTmr 1 (Fig. 2). The whole of the amplified sequence is shown in the figure; the sequences from which the primers IL 193 (bases 2019–2043) and IL 196 (bases 2224–2204) were derived are *underlined*

The *T. mutans* repetitive probe pTmr 2 detected approximately 1 ng of *T. mutans* DNA, which is equivalent to approximately 10⁴ parasite genomes, if the genome size of *T. mutans* is assumed to be about 10⁷ bp, similar to that of *T. parva* (Allsopp and Allsopp 1988; Morzaria et al. 1990). This value lies in the lower range of sensitivities reported for *Plasmodium* repetitive DNA probes (Anonymous 1986) but is similar to the sensitivity observed for a repetitive probe isolated from a related apicomplexan parasite, *B. equi* (Posnett and Ambrosio 1989). The pTmr 2 probe detected *T. mutans* sequences in whole-blood DNA from an experimentally infected animal with piroplasm parasitaemias of 0.4% and

above. This level of sensitivity is useful for detection of the parasite in acutely infected animals but not in carrier animals with low piroplasm parasitaemias. The sensitivity could be improved by the removal of lymphocytes from blood prior to DNA extraction and by more efficient DNA extraction procedures (Eriks et al. 1989). Higher specific activity probes in the form of radiolabeled RNA transcripts derived from the cloned repeat sequences (Anonymous 1987) could also be used for improved sensitivity. It seems likely, however, that detection of *T. mutans* sequences in carrier cattle will require the application of the PCR primers described in this study. PCR amplification of parasite DNA could be
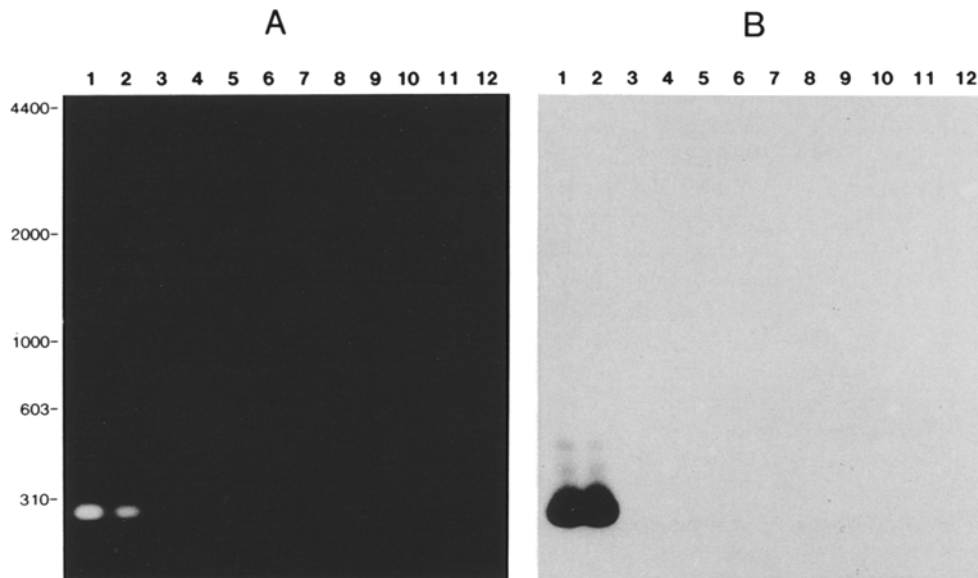
Fig. 7 A, B Specific amplification of *T. mutans* DNA using PCR primers derived from *T. mutans* repetitive sequences. **A** Ethidium bromide-stained 1.2% agarose gel showing PCR amplification products generated from the DNA of *T. mutans*, 4 other *Theileria* species, *B. bovis*, *B. bigemina* and an uninfected bovine using primers IL 193 and IL 196. **B** Southern blot of the gel hybridised with radiolabeled pTmr 1 and washed in 0.1 X SSC/0.1% SDS at 65° C for 30 min (*1 T. mutans* [Kipange] piroplasm, *2 T. mutans* [Intona] piroplasm, *3 T. parva* [Uganda] piroplasm; *4 T. parva* [Muguga] piroplasm, *5 T. taurotragi* [Kitengela] piroplasm, *6 T. taurotragi* [East African orphanage] schizont-infected lymphocyte, *7 Theileria* sp. [Marula] piroplasm, *8 T. annulata* [Tova] schizont-infected lymphocyte, *9 B. bovis* [Brisbane], *10 B. bigemina* [Kikuyu], *11* uninfected bovine, *12* control reaction without DNA)

carried out on rapidly processed whole blood, without DNA extraction, as described by Bishop et al. (1992). Detection of parasites in carrier animals is important since carrier animals are likely to play a major role in the transmission of *T. mutans*. An ELISA test based on monoclonal antibodies directed against species-specific epitopes situated on a 32-kDa antigen has recently been described for detection of *T. mutans* antigen and antibodies in infected cattle (Katende et al. 1990). The antibody-detection ELISA was sensitive but, unlike the *T. mutans* repetitive probe and the PCR primers derived from the repetitive sequence, it may not always detect currently infected animals. Antigenaemia was not always detectable by the antigen-capture ELISA in carrier animals from the field or in long-term experimentally infected animals (Katende et al. 1990). PCR amplification of DNA could be used concurrently with the serological tests so as to evaluate the relative sensitivity and specificity of the two techniques.

The *T. mutans* DNA probe is potentially capable of providing quantitative data concerning levels of parasitaemia in animals infected with *T. mutans*. The quantitative hybridisation of the pTmr 2 probe was much greater to the blood DNA sample taken on August 19,

1990, from an animal experimentally infected with *T. mutans* (Fig. 5, row C, lane 3) than to that taken 2 days earlier (Fig. 5, row C, lane 2), suggesting that numbers of parasites fluctuate in cattle during the acute phase of *T. mutans* infection. Both of these blood samples had piroplasm parasitaemias of 4.8% as determined by light microscopy. The pTmr 2 probe could also be applied to the detection and quantitation of *T. mutans* in the *Amblyomma* tick vectors as has been described for *T. parva* in *Rhipicephalus appendiculatus* ticks by Chen et al. (1991). Transmission of *T. mutans* involves at least three species of *Amblyomma* in East Africa (Paling et al. 1981).

The repetitive probe detected RFLPs between *Eco*RI-digested DNAs isolated from the two available *T. mutans* stocks. The probe can therefore be used for characterisation of *T. mutans* stocks and clones. *Tpr* repetitive probes have been extensively employed for characterisation of *T. parva* stocks (Allsopp and Allsopp 1988; Conrad et al. 1987a, 1989; Bishop et al. 1993). A question that could be addressed using the probe is whether the pathogenic stocks of *T. mutans*, which have been observed in the field (Irvin et al. 1972; Purnell 1977; Young et al. 1978b), differ from non-pathogenic *T. mutans* stocks.

References

Allsopp BA, Allsopp MTEP (1988) *Theileria parva*: genomic DNA studies reveal intraspecies sequence diversity. Mol Biochem Parasitol 28:77–84

Anonymous (1986) The use of DNA probes for malaria diagnosis: memorandum from a WHO meeting. Bull WHO 64:641–652

Anonymous (1987) High specific activity RNA probes. In: Bluescript DNA Sequencing System Instruction Manual. Stratagene, San Diego, pp 7–9

Aslund L, Franzen L, Westin G, Perrson T, Wigzell H, Pettersson U (1985) Highly reiterated non-coding sequence in the genome of *Plasmodium falciparum* is composed of 21 base-pair tandem repeats. J Mol Biol 185:509–516

Baylis HA, Sohal SK, Carrington M, Bishop RP, Allsopp BA (1991) An unusual repetitive gene family in *Theileria parva* which is stage-specifically transcribed. Mol Biochem Parasitol 49:133–142

Bishop R, Sohanpal b, Kariuki DP, Young AS, Nene V, Baylis H, Allsopp BA, Spooner PR, Dolan TT, Morzaria SP (1992) Detection of a carrier state in *Theileria parva*-infected cattle by the polymerase chain reaction. Parasitology 104:215–232

Bishop RP, Sohanpal BK, Allsopp BA, Dolan TT, Morzaria SP (1993) Detection of polymorphisms among *Theileria parva* stocks using repetitive, ribosomal and telomeric DNA probes and anti-schizont monoclonal antibodies. Parasitology (in press)

Brocklesby DW, Barnett SF, Scott GR (1961) Morbidity and mortality rates in East Coast fever (*Theileria parva* infection) and their application to drug screening procedures. Br Vet J 117:529–531

Burridge MJ (1971) Application of the indirect fluorescent antibody test in experimental East Coast fever (*Theileria parva* infection in cattle). Res Vet Sci 12:338–341

Chen PP, Conrad PA, ole-Moi Yoi OK, Brown WC, Dolan TT (1991) DNA probes detect *Theileria parva* in the salivary glands of *Rhipicephalus appendiculatus* ticks. Parasitol Res 77:590–594

Conrad PA, Iams K, Brown WC, Sohanpal B, ole-Moi Yoi OK (1987a) DNA probes detect genomic diversity in *Theileria parva* stocks. Mol Biochem Parasitol 25:213–226

Conrad PA, Stagg DA, Grootenhuis JG, Irvin AD, Newson RM, Njammungeh REG, Rossiter PB, Young AS (1987b) Isolation of *Theileria* parasites from African buffalo (*Syncerus caffer*) and characterisation with anti-schizont monoclonal antibodies. Parasitology 94:413–423

Conrad PA, ole-Moi Yoi OK, Baldwin CL, Dolan TT, O'Callaghan CJ, Njammungeh REG, Grootenhuis JG, Stagg DA, Leitch BL, Young AS (1989) Characterisation of buffalo-derived theilerial parasites with monoclonal antibodies and DNA probes. Parasitology 98:179–188

Enea V (1986) Sensitive and specific probe for detection of *Plasmodium falciparum*. Mol Cell Biol 6:321–324

Eriks IE, Palmer GH, McGuire TH, Allred DR, Barbet AF (1989) Detection and quantitation of *Anaplasma marginale* in carrier cattle using a nucleic acid probe. J Clin Microbiol 27:279–284

Feinberg AP, Vogelstein B (1983) A technique for radiolabelling DNA fragments to high specific activity. Anal Biochem 132:6–13

Fickett JW (1982) Recognition of protein coding regions in DNA sequences. Nucleic Acids Res 17:5303–5318

Flach EJ, Shambwana IA, Dolan TT, Kassim A, Pedersen H, Glass M, Morzaria SP (1989) East Coast fever immunisation trials on Unguja island Zanzibar. In: Dolan TT (ed) Theileriosis in Eastern, Central and Southern Africa. ILRAD, Nairobi, pp 77–79

Fujisaki K, Kamio T, Kitaoka S (1985) The presence of *Theileria sergenti* in *Haemaphysalis longicornis* overwintering in pasture in Japan. Ann Trop Med Parasitol 79:519–524

Higuchi R (1989) Simple and rapid preparation of samples for PCR. In: Erlich HA (ed) PCR technology: principles and applications for DNA amplification. Stockton, New York, pp 31–38

Irvin AD, Brown CGD, Burridge MJ, Cunningham MP, Musoke AJ, Pierce MA, Purnell RW, Radley DE (1972) A pathogenic *Theileria* syndrome of cattle in Narok district of Kenya. I. Transmission studies. Trop Anim Health Prod 4:220–229

Irvin AD, Dobbelaere DAE, Mwamachi DM, Minami M, Spooner PR, Ocama JGR (1983) Immunisation against East Coast fever: correlation between monoclonal antibody profiles of *Theileria parva* stocks and cross immunity in vivo. Res Vet Sci 35:341–346

Katende JM, Goddeeris BM, Morzaria SP, Nkonge CG, Musoke AJ (1990) Identification of a *Theileria mutans*-specific antigen for use in antibody and antigen detection ELISA. Parasite Immunol 12:419–433

Lohr KF, Ross JPJ (1969) Improvement of the indirect fluorescent antibody test for the diagnosis of diseases caused by intra erythrocytic parasites. Z Tropenmed Parasitol 19:427–430

Morzaria SP, Spooner PR, Bishop RP, Musoke AJ, Young JR (1990) *Sfi*I and *Not*I polymorphisms in *Theileria* stocks detected by pulsed field gel electrophoresis. Mol Biochem Parasitol 40:203–212

Mullis KB, Faloona F (1987) Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. Methods Enzymol 155:335–350

ole-Moi Yoi OK (1987) Trypanosome species-specific DNA probes to detect infection in tsetse flies. Parasitol Today 3:371–374

Paling RW, Grootenhuis JG, Young AS (1981) Isolation of *Theileria mutans* from Kenyan buffalo, and transmission by *Amblyomma gemma*. Vet Parasitol 8:31–37

Posnett ES, Ambrosio RE (1989) Repetitive DNA probes for the detection of *Babesia equi*. Mol Biochem Parasitol 34:75–78

Purnell RE (1977) East Coast fever: some recent research in East Africa. Adv Parasitol 15:83–132

Saidu SNA (1982) Bovine theileriosis due to *Theileria mutans*: a review. Vet Bull 52:451–457

Saiki RK, Bugawan TL, Horn GT, Mullis KB, Erlich HA (1986) Analysis of enzymatically amplified β-globin and HLA-DQα DNA with allele-specific oligonucleotide probes. Nature, London 324:163–166

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

Sanger F, Nicklen S, Coulsen AR (1977) DNA sequencing with chain terminating inhibitors. Proc Natl Acad Sci USA 74:5463–5467

Stagg DA, Young AS, Leitch BL, Grootenhuis JG, Dolan TT (1983) Infection of mammalian cells with *Theileria* species. Parasitology 86:243–254

Tsur I (1945) Multiplication in vitro of Koch bodies of *Theileria annulata*. Nature 156:391

Uilenberg G (1981) Theilerial species of domestic livestock. In: Irvin AD, Cunningham MP, Young AS (eds) Advances in the control of theileriosis. Martinus Nijhoff, The Hague, pp 4–37

Young AS, Brown DG, Burridge MJ, Grootenhuis JG, Kanhai GK, Purnell RE, Stagg DA (1978a) The incidence of theilerial parasites in East African buffalo (*Syncerus caffer*). Z Tropenmed Parasitol 29:281–288

Young AS, Purnell RE, Payne RC, Brown CGD, Kanhai GK (1978b) Studies on the transmission and course of infection of a Kenyan strain of *Theileria mutans*. Parasitology 76:99–115

Young AS, Mutugi JJ, Kariuki DP, Lampard D, Maritim AC, Ngumi PN, Linyonyi A, Leitch BL, Ndungu SG, Lesan AC, Mining SK, Grootenhuis JG, Orinda GO, Wesonga D (1992) Immunisation of cattle against theileriosis in Nakuru district of Kenya by infection and treatment and the introduction of unconventional tick control. Vet Parasitol 42:225–240

Young RA, Mehra V, Sweetser D, Buchanan T, Clark-Curtis J, Davis RW, Bloom BR (1985) Genes for the major protein antigens of the leprosy parasite *Mycobacterium leprae*. Nature 316:450–452

Zolg WJ, Andrade LE, Scott ED (1987) Detection of *Plasmodium falciparum* DNA using repetitive DNA clones as species-specific probes. Mol Biochem Parasitol 22:145–151