J. Protozool. Res., 2, 34-39 (1992)

Biotin-labeled Genomic DNA Probe for the Detection of *Theileria sergenti* and its Nucleotide Sequence

MASAYUKI TANAKA[1,2] ,TAKASHI MATSUBA[3], SADAO ONOE[4], HIROMI YONEMICHI[4], TATSUJI OKABE[2], NORIMASA SASAKI[2], CHIHIRO SUGIMOTO[1], and MISAO ONUMA[1]

[1]Department of Epizootiology, Faculty of Veterinary Medicine, Hokkaido University, Sapporo, Hokkaido 060, [2]Division of Veterinary Microbiology, Kyoto Biken Laboratrories, Uji, Kyoto 611, [3]Department of Veterinary Microbiology, School of Veterinary Medicine, Rakunou Gakuen University, Ebetsu, Hokkaido 069, [4]Hokkaido Prefectuaral Shintoku Animal Husbandry Experimental Station, Shintoku, Hokkaido 081, JAPAN

Recieved 17 May 1991 / Accepted 28 October 1991

Key words: DNA probe diagnosis, nucleotide sequence, *Theileria sergenti*

Bovine theileriosis is economically one of the most important tick-borne protozoan parasite disease of grazing cattle in Japan. It is caused by *Theileria sergenti*, and brings about mild hyperthermia and anemia in cattle. The diagnosis of *T. sergenti* has routinely relied upon laborious microscopical examination of Giemsa-stained blood smears.

Recent advances in molecular biology have made it possible to develop diagnostic assays that use nucleic acid probes to detect pathogens directly, specifically and sensitively in clinical specimens. The utility of nucleic acid probes for routine diagnosis of pathogens has been limited by the use of radiolabeled probes for their detection. However, this limitation has been overcome by the development of non-radioactive detection systems (Langer et al. 1981, Leary et al. 1983). We previously reported the molecular cloning of genomic DNA of *T. sergenti* (Kajiwara et al. 1990). One of the cloned genomic DNA fragments (pTs-11-D1) was used to detect *T. sergenti* in a dot blot hybridization assay (Hirano et al. 1991). This pTs-11-D1 probe did not react with DNA of *Babesia ovata* or *Anaplasma centrale* by dot blot and Southern hybridization as reported by Hirano et al.(1991). However, the same probe hybridized to DNA of *Theileria buffeli* which may be taxonomically related to *T. sergenti* (Matsuba et al. 1991). In the present study, we evaluated a dot blot hybridization assay using a genomic DNA pTs-11-D1 probe for the detection of *T. sergenti* as a practical diagnostic method. The nucleotide sequence of this probe is also described.

T. SERGENTI GENOMIC DNA PROBE

We compared the dot blot hybridization using a pTs-11-D1 probe for *T. sergenti* with routine microscopic diagnosis. A splenectomized calf was infected with *T. sergenti* (Chitose stock) by nymphal ticks of *Haemophysalis longicornis* which had been infected with *T. sergenti*. The calf was kept under daily clinical and hematological observations and treated with blood transfusion or anti-parasite drug if it showed severe anemia. The piroplasm DNA was prepared from parasitized erythrocytes as described previously (Kajiwara et al. 1990) with slight modifications; infected erythrocytes were lysed by *Aeromonas hydrophila* hemolysin (Sugimoto et al. 1990) instead of 0.2% NaCl, and purified parasites were digested with proteinase K (0.3 mg/ml) instead of pronase E. The DNA was spotted on a sheet of cellulosenitron (E) filter (Schleicher & Schuell, F. R. G.), denatured in 0.5N NaOH, and fixed onto the membrane by cross-linking with short wavelength ultraviolet light. Genomic DNA pTs-11-D1 probe was labeled with biotin 16-dUTP (Boehringer, USA) by random priming (Feiberg and Vogelstein 1983). Hybridization was performed as described previously (Kajiwara et al. 1990). The hybridization solution containing 4x SSPE (0.6M NaCl, 0.04M NaH_2PO_4 and 4mM EDTA) , 10x Denhardt's solution (0.2% Ficoll, 0.2% polyvinylpyrrolidone and 0.2% bovine serum albumin), 150 μg/ml salmon sperm DNA, and 50% formamide was used to prehybridize filters at 42 ^{O}C for 2 h. After prehybridization, the buffer was removed and fresh hybridization solution was added with the biotinylated probes. After incubation at 42 ^{O}C for 18 h, the filters were washed three times at room temperature for 5 min and twice at 55 ^{O}C for 15 min each in 0.2x SSC (0.03M NaCl and 3mM sodium citrate) containing 0.1% SDS. The biotinylated probes were visualized using the BluGENE nonradioactive nucleic acid detection system (BRL, USA).

T. sergenti piroplasms were detected at day 11 after inoculation by the microscopical examination of Giemsa-stained blood smear. *T. sergenti* DNA was first detected at day 7 after inoculation by using pTs-11-D1 probe (Table 1.).

Table 1. Detection of *Theileria sergenti* from calf erythrocytes

Assay	Days after inoculation															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	21	26
Giemsa staining (parasitemia %)	0	0	0	0	0	0	0	0	0	0	0.01	0.05	0.05	0.10	8.15	23.2
Dot blot hybridization	ND	ND	ND	ND	-	-	+	+	+	+	+	+	+	+	+	+

ND: Not done

T. SERGENTI GENOMIC DNA PROBE

Since clone pTs-11-D1 was derived from DMA of *T. sergenti* (Chitose stock) which was isolated in Hokkaido, we examined the reactivity of the DNA probe with DNA of *T. sergenti* isolates from 11 geographically different districts in Japan. Purified piroplasm DNA was applied onto the membrane with HYBRI-SLOT^{TM} MANIFOLD (BRL, USA) and probed with pTs-11-D1. The probe hybridized with DNA from all the isolates at a similar intensity. This result suggested that the sequence detected by the DNA probe is conserved among the *T. sergenti* isolates in Japan (Fig. 1.). The probe did not hybridized with DNA prepared from bovine erythrocyte infected with *Babesia ovata* or *Anaplasma centrale* under the same hybridization condition (data not shown).

Fig. 1. Reactivity of pTs-11-D1 for DNA of *T. sergenti* isolates from geographically different districts. Each piroplasm DNA was extracted from purified parasites and 0.1-0.2 μg applied onto the membrane. The filters were hybridized with biotin 16-dUTP-labeled pTs-11-D1. Slots 1 to 12 correspond to *T. sergenti* isolates collected from 1; Hokkaido (Chitose), 2; Hokkaido (Hiyama), 3; Hokkaido (Oshima), 4; Hokkaido (Shintoku), 5; Hokkaido (Abashiri), 6; Aomori, 7; Fukushima, 8; Tochigi, 9; Shizuoka, 10; Okayama, 11; Fukuoka, 12; Kagoshima.

As the pTs-11-D1 fragment hybridized with more than 14 *Bam*H I restriction fragments of *T. sergenti* genomic DNA, it detects a repetitive sequence of the genome (Hirano et al. 1991). Furthermore, it hybridized with a 6 kb fragment of piroplasm mRNA by Northern blotting (unpublished observation). To elucidate whether there are internal repeats within the probe sequence as known in diagnostic DNA probes of other parasitic organisms (Barker 1990, Klion et al. 1991), and whether this DNA fragment of 2 kbp in length contains protein coding region, we sequenced the pTs-11-D1 fragment. Clone pTs-11-D1 was subcloned into the Bluescript KS^{+} vector and deletion clones were generated for dideoxy sequencing (Henikoff 1984). Sequencing by the dideoxy method was performed using [α-^{32}P]dCTP and 7-deaza-sequencing kits (TOYOBO Co., Ltd., Japan). Sequence analysis verified the cloned fragment to be 1,998 base pairs in length (Fig. 2.). The A/T content was 63%, and the G/C content was 37%. The pTs-11-D1 did not contain portions of a long open reading frame in both strands (Fig. 3.). No internal repeats or inverted repeats were recognized. Significant homology was not detected with sequences stored in the GenBank data bases using the DNASIS program (Hitachi Software Engineering Co., Ltd., Japan). Recently, a genomic DNA encoding a 41 kDa protein of *Plasmodium falciparum* was cloned and sequenced (Knapp et al. 1991).

```
GAAAATATAT TTATGGCTTG AAGGATTATC GAATAAATAA ATCTAGGGAA ATAATTTCAC ATTAAACTTA   70
ATTGACAGAT GTGCATGGAC GACATTGCAC ATAACATTAT AACGAGCACA AGGAATAACG AGCTCATGTT  140
ACTTTTTTTA AGATTTTTAC TATCGTTCCT CAATAGGTTG TTTGTAAACA TTCTTACTTC GTTTTCAGTT  210
TATCATGTTC TAGATGATTT GGTAAAAGAG ACAATTAAAA CACAGGATCT TAAAAAATTT GAAACAGCTC  280
CTATGGTTGC AACTATGACG GTAATGCTCA AGATTTCAGA TTCGATGTTG TTCGTTCCAG ATGCAATGAA  350
ATTTTTCACT TATTCATTGA TGCTATATCT CAGAGCTGAG TACTACATGA ATGACTGTTC TCCTAACTTT  420
TATATCAGTG GTAACAAATT CACTGGTTCC ACTCAAACAT CAAATGTGGT AACTGAGGTA ACCAATGAAA  490
TTCCCAATGA TGTTGTTGTA TATTATAAGG ACGCACTTTC ACATGGGTCA CACACGCGAA GACCTAATGA  560
ATAAGAACTA TGAACCTTAT TTGAAAAACA TCATTTCGAA CTAAAACGTG GCGAGATGGC CATTGTCACT  630
GGTTCCAAGG GTTCTGGGAA ATCAAATTTT ATCAAATCAT ATACTTGGTG AGATGACCCT GGTTGGGGGT  700
TCCATGGCTG TAGTACCATT ATACACCTCA ATGCCATATT CTATGCATCT CAAGACATAT GGCTTCACGT  770
GGCACTATCA GATCCATATT ACATTTGGTT ATCGATTCGA TGAACATCTG TACATGCTGT GTTGAAGGCA  840
GTTGAACTTG AGTATGACAT ATCGACCTGG GAGAAGGGTG ACCTCAGAGT AGTCTCTGAT AATGCTCATT  910
CATTGAGTGG TGGTCAGAGA GTGAGATTGG AGTTAGCTCG TGCCGTCTAT GCTTATTTGG TGTTCCACAA  980
AGTCAACGAG GAGTATAATA ACAGTCAATG TTCCTACCTG ATGTGCTTGG ATTGTTCGTT CCATGGTCTA 1050
GATCCTTATG TATCCAAAAC CATATTCAAT AACCTGTTTA ATCTTAAAGA TGGAATATTA GTAAATGATG 1120
ATCTATGTGT TGTCTTATGT TCTACAAAAA GCGAACTTAT AGGACTTTCA TCAGCTGAAG TATCGTTCCT 1190
AAACATAACT TTGTACAACA TTGCGAATCA AAAACCTGAA TAAAATCAAG GATATTCGAC ATACGGAGAC 1260
GGCTAGAAAG CCTTTGCCCT CGGAATACAG GCTATACCAA CTGATGAAAT AATAAAGTTG TGTGAATCGG 1330
GTGAAACACC GCGAAGGGAG AATAGCATTA GTAAAAAAAA AGTACGAGGA TTCGTTGATA ATAAGTCCCT 1400
GAATCCAAAG TAACAAAGCA GAAGATCGGA TTGGCTTTTA AGCCGTATCT CATGTACGCC AAATCATTGG 1470
ATTCCTATTC GTGCTGTTTA TAATATTCAC CATTGTTTAC GAATCCATTG AAACGTCCAA ATACGTGCTT 1540
GCTGGTGATT TAGGAGCGGT AATATTGACT ACGTCAGGAA AAATATCAGA TCGGAAGCAA TACATTGAAG 1610
CATTCCAAAA GATTAGAAAT TCATGTGATG ATACTCTATT GAAAATTAAG AAACTTACAG TGGCTCTTCT 1680
AGTTTTCTGC TTTTAGCCAA TTTCGTTTTG ACGTTCTGCT GCCGTGCGTG GATGCAAAAG GATACACGAG 1750
TACTGTATCA ATTCACTGAT AAATAACCAA GTCGTCGATC GTGAAGATTA AGAAATACAG CAGTGAAATC 1820
GTAACATTCT TGGCTTCCGA CGTCTTCATC ATAGACGAGA ACTTTGGCCC TTGTCTATCT TCAATGTTGA 1890
TACTGTTTAT AGAGGTGCTC CTTCAGACTT TAGTTCTGGC ATACTATTTT CCCATTTGCA TACCAGTCAT 1960
AGTAATATGC CTTTTCGTAA TCATTAACTT TATTTTTC                                     1998
```

Fig.2. Nucleotide sequence of clone pTs-11-D1. clone pTs-11-D1 was subcloned into Bluescript KS^+ vector and deletion clones were generated for dideoxy sequencing.

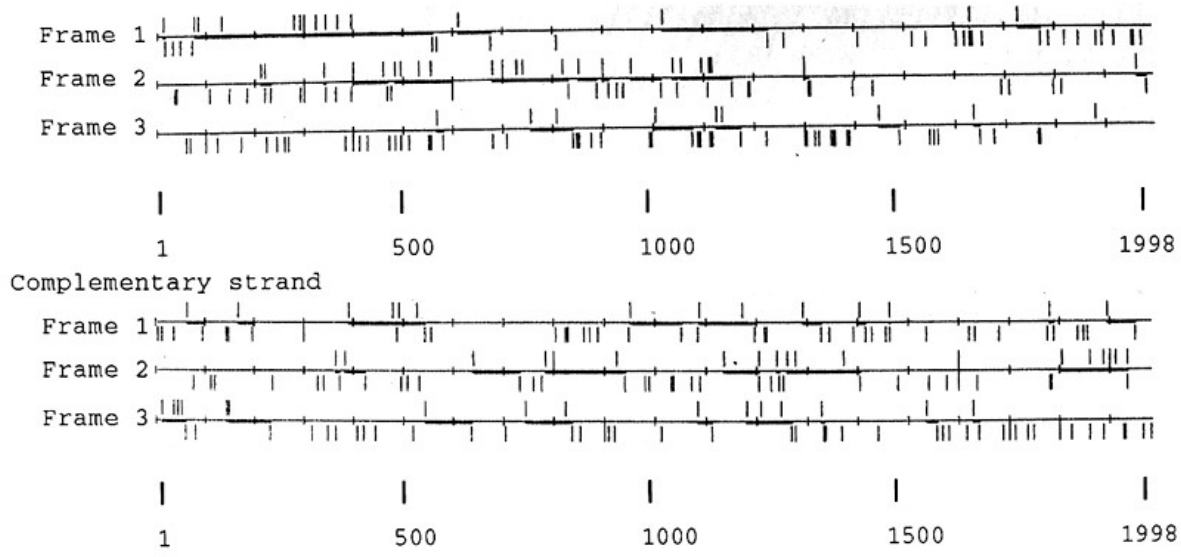

Fig. 3. The occurrence of initiation (ATG) and termination (TAG, TGA, TAA) codons of both strands. Nucleotide numbers are shown at the bottom. Vertical lines initiation (above the horizontal lines) and termination (under the horizontal lines) codons in three frames in both strands.

The gene consists of 9 exons with short open reading frames coding for 23 to 109 amino acids, respectively. If the probe DNA of *T. sergenti* is a part of a gene carrying several intervening sequences, disagreement between our sequence data indicating that the sequence does not contain long open reading frames, and the result of Northern blot analysis could be explained. To elucidate this, cDNA library of *T. sergenti* should be screened with this DNA probe and DNA sequence of the transcript should be compared with that of genomic DNA presented in this paper.

In conclusion, the use of the pTs-11-D1 DNA probe is practical for early diagnosis of *T. sergenti* infection with high sensitivity and specificity. As the probe hybridized with DNA of *T. sergenti* isolates collected from various regions in Japan, it is also useful for epidemiological studies of *T. sergenti* infection in Japan. Furthermore, the use of DNA probes will elucidate taxonomical relationships among benign Theileria species, *T. sergenti*/*buffeli*/ *orientalis*, which are distributed in Asian and European countries and Australia (Stewart et al. 1988, Uilenberg et al. 1985), at the molecular level.

ACKNOWLEDGMENTS :

We are grateful to Dr. S. Mori, Bayer Japan Ltd., for arranging the collection of *T. sergenti*-infected blood from different districts in Japan.

REFERENCES :

Barker, R. H. 1990. DNA probe diagnosis of parasitic infections. *Exp. Parasitol. 70*: 494-499.

Feinberg, A. P. & Vogelstein, B. 1983. A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem. 132*: 6-13

Henikoff, S. 1984. Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. *Gene 28*: 351-359.

Hirano, A., Kirisawa, R., Matsuba, T., Komatsu, R., Tanaka, M., Takahashi, K., Kawakami, Y. & Onuma, M. 1991. Evaluation of high sensitive DNA probe for the detection of *Theileria sergenti* infection in cattle. *J. Vet. Med. Sci. 53*: 933-935.

Kajiwara, N., Kirisawa, R., Onuma, M. & Kawakami, Y. 1990. Specific DNA probe for the detection of *Theileria sergenti* infection in cattle. *Jpn. J. Vet. Sci. 52*: 1199-1204.

Klion, A., D., Raghaven, N., Brindley, P., J. & Nutman, B. 1991. Cloning and characterization of a species-specific repetitive DNA sequence from *Loa loa*. *Mol. Biochem. Parasitol. 45*: 297-306.

Knapp, B., Nau, U., Hundt, E. & Küpper H. A. 1991. Demonstration of alternative splicing of a pre-mRNA expressed in the blood stage form of *Plasmodium falciparum, J. Biol. Chem. 266*: 7148-7154.

Langer, P. R., Waldrop, A. A. & Ward, D. C. 1981. Enzymatic synthesis of biotin-labeled polynucleotides: Novel nucleic acid affinity probes. *Proc. Natl. Acad. Sci. USA. 78*: 6633-6637.

Leary, J. J., Brigait, D. J. & Ward, D. C. 1983. Rapid and sensitive colorimetric method for visualizing biotin-labeled DNA probes hybridized to DNA or RNA immobilized on nitrocellulose: Bio-blots. *Proc. Natl. Acad. Sci. USA. 80*: 4045-4049.

Matsuba, T., Kawakami, Y., Iwai, H. & Onuma, M. 1991. Genomic analysis of *Theileria sergenti* stocks in Japan with DNA probes. *Vet. Parasitol*. (in press)

Stewart, N. P., Devos, A. J., McGregor, W. & Shiels, A. 1988. Observations on the development of tick-transmitted *Theileria buffeli* (syn *T. orientalis* ?) in cattle. *Res. Vet. Sci. 44*: 338-342.

Sugimoto, C., Sato, M., Kawazu, S., Kamio, T. & Fujisaki, K. 1991. Purification of merozoites of *Theileria sergenti* from infected bovine erythrocytes. *Parasitol. Res. 77*: 129-131.

Uilenberg, G., Perie, N. M., Spanjer, A. A. M. & Franssen, F. F. J. 1985. *Theileria orientalis*, a cosmopolitan blood parasite of cattle: demonstration of the schizont stage. *Res. Vet. Sci.* 38: 352-357.

Address corespondence and reprint requests to Dr. Misao Onuma,
Department of Epizootiology, Faculty of Veterinary Medicine, Hokkaido University,
Sapporo/ Hokkaido 060, Japan.