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Rapid and Sensitive Method for Detection of Newly Isolated *Babesia* Parasite (*Babesia* sp.1) in the Anticipated Vector-Tick Using the Polymerase Chain Reaction Technique.

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ABSTRACT

We recently reported that the presence of a *Babesia ovata*-like large intraerythrocytic parasite, *Babesia* sp.1 in cattle population of Hokkaido area in Japan, and the development of DNA probe (SpS7) for the parasite. A nucleic acid probe is a powerful tool for direct detection and identification of parasites in animals and vector insects. However, polymerase chain reaction (PCR) technique has advantages over hybridization technique. In this paper we report the development of PCR and nested-PCR techniques for amplification of the SpS7 sequence from *Babesia* sp. 1 DNA. The PCR with one set of primers was specific for *Babesia* sp.1, since no amplification detected with DNA from bovine white blood cell, *Theileria sergenti, Anaplasma marginale, A. centrale, Eperythrozoon wenyoni, B. bovis, B. bigemina*, and *B. ovata*. The nested-PCR, which reamplified 470-base-pair (bp) in the internal region of the first primer set (670 bp), enabled the detection of less than 10 parasites on calculation. The technique was 10-fold more sensitive than the one-step PCR, and detected *Babesia* sp.1 in larval ticks that had been generated from the parasite-infected adults *Haemaphysalis longicoronis*.

INTRODUCTION

Recently isolated large intraerythrocytic parasite of cattle, *Babesia* sp.1, showed unique biochemical and immunological characteristics compared with other *Babesia* species in cattle such as *B. ovata, B. bigemina* and *B. bovis* (Ohta et al.

1995b). Further studies on the epidemiology and tick vector of *Babesia* sp. 1 should be conducted to clarify the taxonomic status of the parasite. For these studies, sensitive and specific method to detect the parasite is required. Recombinant DNA technology has provided hybridization reagents which can be used for direct detection and identification of some parasitic organisms in their vectors, reservoir hosts, domestic livestock and in humans(Ihler and Rice-Ficht 1989; Wirth et al. 1989). The DNA probes are used in a radioactive form in a majority of hybridization assays. However, the use of radioactivity in labeling DNA probes makes them less attractive for practical application (Majiwa et al. 1994). The polymerase chain reaction (PCR) is a rapid procedure for in vitro enzymatic amplification of a specific segment of DNA (Saiki et al. 1988). Saiki et al. (1985) using the procedure, demonstrated a 220,000-fold amplification of a 110-base-pair (bp) region of the 13-globin gene. The advent of this technique allowed the detection and identification of a small number of parasite without radioactive reagent (Barker 1990). For the southern hybridization, we have developed the DNA probe (SpS7) for detection of *Babesia* sp.1 (Ohta et al. 1995a). In order to increase the availability of the detection method, we attempted to amplify the SpS7 sequence using PCR and nested-PCR techniques. This allowed more sensitive and rapid detection of *Babesia* sp.1 DNA as compared with the hybridization techniques. In particular, transovarial transmission of *Babesia* sp.1 in tick was evaluated using the nested-PCR technique.

MATERIALS AND METHODS

Parasite stocks and ticks

The parasite stocks used were as follows: *Babesia* sp.1 isolated from Japanese Brown in Oshima area of Hokkaido prefecture, Japan(Ohta et al. 1995b), *B. ovata* (Miyake stock; Minami and Ishihara 1980), *B. bigemina* (Kochinda stock; Fujinaga et al. 1980), *B. bovis* (Australian stocks; Minami and Ishihara 1980), *Theileria sergenti* (Ikeda stock; Fujisaki et al. 1992), *Anaplasma marginale* (Kochinda stock; Nakamura et al. 1991), *A. centrale* (Aomori stock; Nakamura et al. 1991) and *Eperythrozoon wenyoni* (Natural infection; Kawazu et al. 1990). A bovine calf was infected with *Babesia* sp. 1 by syringal passage of infected blood. After the level of parasitized erytrocytes had reached >1.5%, adult ticks of a parthenogenetic Okayama strain of *Haemaphysalis longicoronis* (Fujisaki et al. 1976) were fed on the calf in an ear bag. Engorged adults were collected and allowed to lay eggs in an incubator under the controlled conditions (25°C, 100% RH continuous darkness). Larvae that hatched from these eggs were used in this study.

Preparation of PCR templates

Genomic DNA of the parasite stocks was prepared with previous procedure

(Ohta et al. 1995). In brief, partially purified parasite from infected blood by the nitrogen cavitation method (Shimizu et al. 1988) was treated with sodium dodecyl sulfate (SDS)-proteinase K solution, and phenol extraction (Sambrook et al. 1982). The pooled larval ticks were frozen with liquid nitrogen and grin to fine powder in mortar and pestle keeping the tissue frozen throughout this procedure by occasionally adding liquid nitrogen (Richards et al. 1994). The total DNA was extracted from frozen powder for PCR amplification. Nucleotide sequence determination: The SpS7 fragment (700 bp) was subcloned into the pBlueccriptII plasmid. Sequence analysis was performed by the dideoxy chain termination method using the recombinant plasimid DNA as a template (Sanger et al. 1977) and the entire sequences were obtained on both strands. DNA sequence was analyzed by genetic information processing program (GENETYX; Software Development Co. Ltd., Japan).

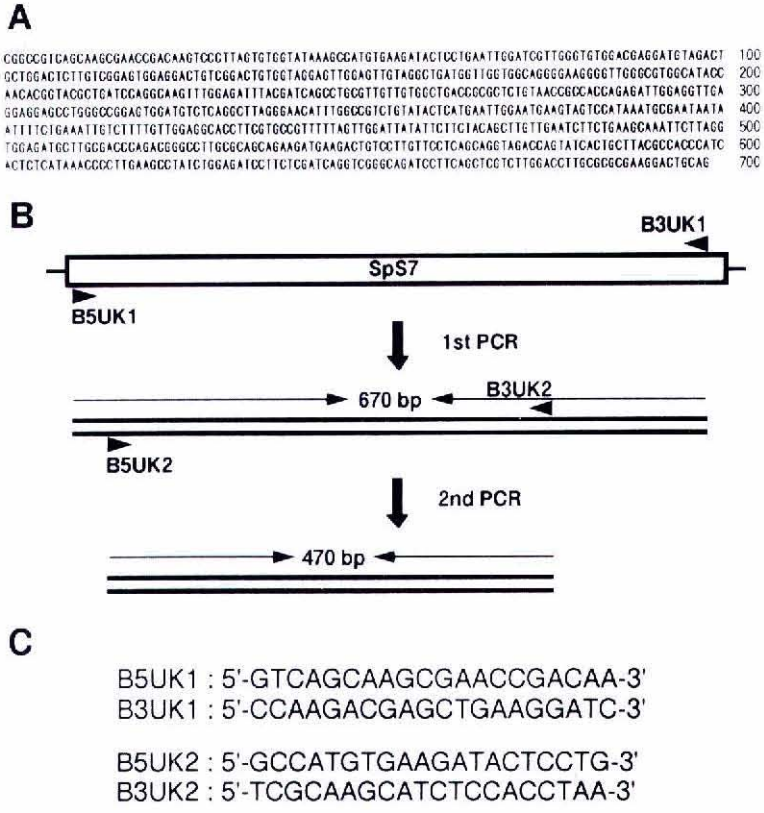

Figure 1. The nucleotide sequence of SpS7 (panel A). Schematic diagram of amplification of the segment in the SpS7 by nested-PCR technique (panel B). The open box in top corresponds to the SpS7 region. The thickly double lines correspond to the first-PCR product (middle) and the second PCR product(bottom). The sequence of oligonucleotide primers used in this study (panel C).

Primers design

The first primer set (B5UK1 and B3UK1) was designed to amplify 670 bp region of the SpS7 sequence. The second primer set (B5UK2 and B3UK2) was designed to amplify 470 bp region in between the first primer set (Fig. 1).

PCR amplification

PCR amplification was performed in a 100 μl reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM each of the four dNTPs, 0.5 μM each of the oligonucleotide primers, and 2.5 units of Taq DNA polymerase (Perkin Elmer Cetus, USA). Positions and sequences of the primer used are indicated in Figs. 1B and C, respectively. The template DNA added to the reactions was indicated in the figure legend. The reaction mixture was overlaid with paraffin oil and subjected to 35 cycles of amplification in a programmable heating block (Perkin Elmer Cetus). The program used was followed; 2 min of denaturation at 94°C(5 min for the first cycle), 2 min of annealing at 60°C and 3 min of polymerization at 72°C, with additional 5 min after the last cycle. For reamplification of the samples amplified with primers B5UK1 and B3UK1, a 1 μl portion of the primary amplification reaction was amplified as above with primers B5UK2 and B3UK2 (the nested-PCR technique; Fig. 1).

Analysis of amplified DNA

One tenth of reaction (10 μl) was fractionated by 2.0% agarose gel electrophoresis and visualized by UV fluorescence after staining with ethidium bromide.

RESULTS

Sequence analysis

The SpS7 sequence consisted of 697 nucleotides, and did not have significant homology with other known sequences stored in the GenBankTM database. This sequence contained 200 bp of open reading frame, and the analysis of messenger RNA from the SpS7 sequence is now in progress. Specificity and sensitivity of the PCR and nested-PCR technique: The first primers B5UK1 and B3UK1 generated a PCR product of 670 bp from *Babesia* sp.1 DNA (Fig. 2, lane 9). The primers did not generate any detectable band by ethidium bromide staining, when 100 ng of DNA isolated from *T. sergenti*, *A. marginale*, *A. centrale*, *E. wenyoni*, bovine white blood cell, *B. bovis*, *B. bigemina*, or *B. ovata* was used as template in the PCR (Fig. 2, lanes 1-8). The PCR with second primers B5UK2 and B3UK2 produced a 470 bp DNA band from *Babesia* sp.1 DNA, and did not produce a visible DNA band from other parasite DNAs except for *A. centrale* DNA, in which a weakly visible band of different size was seen (data not shown). To determine the lower limit of detection of *Babesia* sp. 1 DNA in our experiments, 10-fold serial

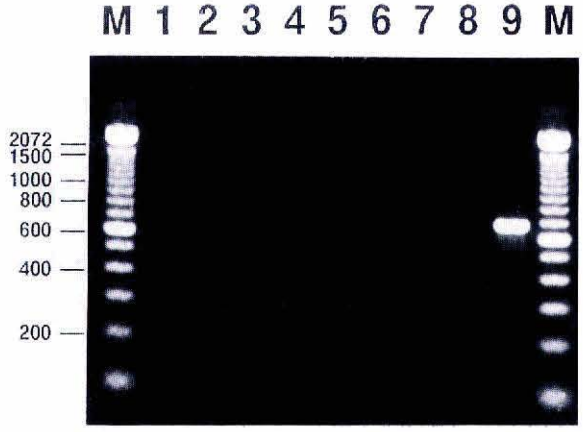

Figure 2. The specificity of the polymerase chain reaction with primers B5UK1 and B3UK1. The reactions were conducted on 100 pg of total DNA from *Theileria sergenti*, *Anaplasma marginale*, *A. centrale*, *Eperythrozoon wenyoni*, bovine white blood cell, *Babesia bovis*, *B. bigemina*, *B. ovata*, and *Babesia* sp.1 (lanes 1-9, respectively). The left and right lanes (M) contain size markers (The 100 bp DNA Ladder; GIBCO BRL), the sizes are indicated in base pair(bp).

dilution of purified genomic DNA of the parasite was analyzed by PCR with primers B5UK1 and B3UK1. A visible DNA band of expected size of 670 bp was observed under ethidium bromide fluorescence in all samples containing an initial quantity of ≥1 pg of DNA (Fig. 3A). The PCR with primers B5UK2 and B3UK2 were also sensitive enough to detect ≥1 pg of the parasite DNA (data not shown). After the PCR amplification with primers B5UK1 and B3UK1, each reaction mixture was reamplified with primers B5UK2 and B3UK2. A 470 bp DNA band was detected in the sample containing an initial quantity ≥100 fg of DNA (Fig. 3B). The nested-PCR was 10-fold more sensitive than the one-step PCR for the detection of *Babesia* sp.1 DNA.

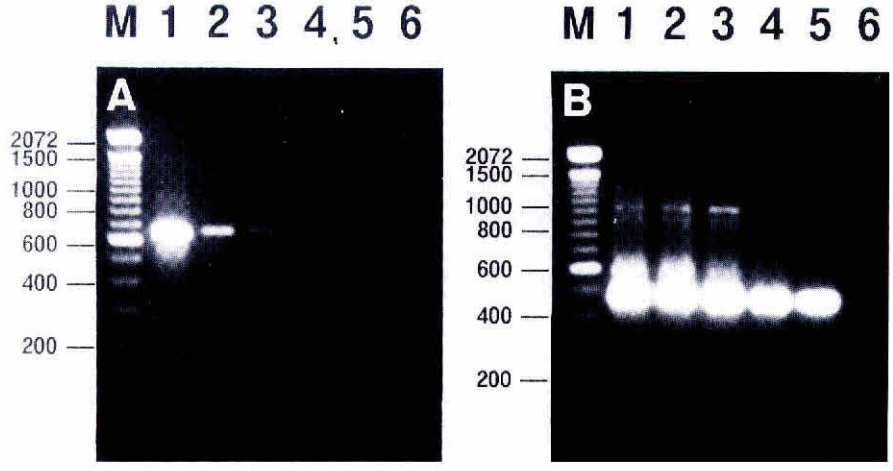

Figure 3. The sensitivity of the polymerase chain reaction(PCR) and nested-PCR. Purified *Babesia* sp. 1 DNA were amplified with the first primer set B5UK1 and B3UK1 (PCR: panel A). The reactions were conducted in the presence of 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, and 10 fg of *Babesia* sp.1 DNA(lanes 1-6, respectively). One percent (1 μl) of the first-PCR products in panel A were reamplified with the second primer set B5UK2 and B3UK2 (nested-PCR: panel B). The left lane (M) contains size markers (The 100 bp DNA Ladder; GIBCO BRL).

Detection of Babesia sp.1 in the parasite infected-ticks

Ten-fold serial dilution of DNA from larval tick sample was analyzed by PCR. The first primer set B5UK1 and B3UK1 generated many sizes PCR products (Fig. 4A), and the second primer set B5UK2 and B3UK2 did not generated detectable PCR products (Fig. 4B) in samples containing an initial quantity of >325 pg of the DNA. When each of the PCR products generated with the first primer set B5UK1 and B3UK1 was reamplified with the second primer set B5UK2 and B3UK2, sample containing initial quantity of 3.25 μg of the DNA produced a visible DNA band of expected size of 470 bp by ethidium bromide fluorescence (Fig. 5).

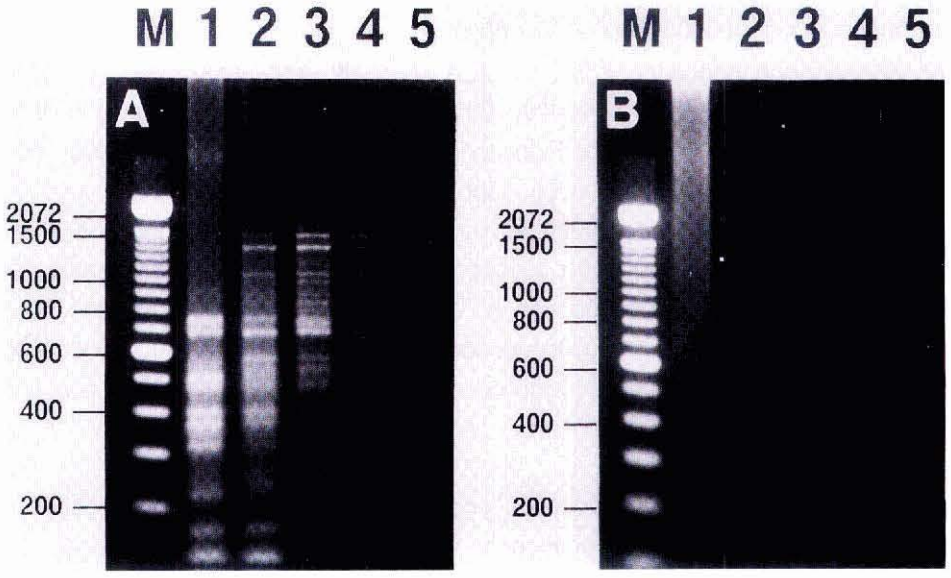

Figure 4. The polymerase chain reaction conducted with primers B5UK1 and B3UK1 (panel A) or B5UK2 and B3UK2 (panel B) on the tick DNA, prepared from progeny of Haemaphysalis longicoronis which had fed on the *Babesia* sp. 1-infected calf. The reactions were conducted in the presence of 3.25 µg, 325 ng, 32.5 ng, 3.25 ng, and 325 pg of the DNA(lanes 1-5, respectively). The left lane (M) contains size markers (The 100 bp DNA Ladder; GIBCO BRL).

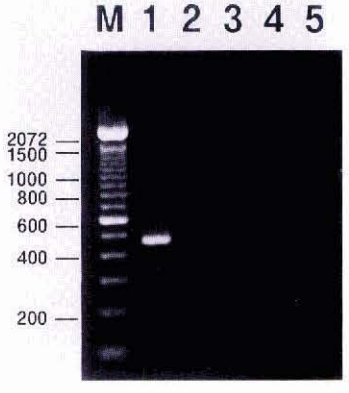

Figure 5. The detection of *Babesia* sp.1 DNA from the ticks DNA sample by nested-PCR. The second-PCR reactions, in lanes 1-5, were conducted on the presence of one percent (1 μl) of the first-PCR products in Fig. 4 (panel A). The left lane(M) contains size markers (The 100 bp DNA Ladder; GIBCO BRL).

DISCUSSION

Majiwa et al.(1994) described the application of non-radioactive hybridization system in combination with the PCR to break through the limitations of non-radioactive probe. However, hybridization technique is expensive, time-consuming and involves several critical steps. We previously demonstrated that the detection limits for *Babesia* sp.1 DNA was 5 ng by radioactive hybridization assay using SpS7 (Ohta et al. 1995a). The nested-PCR which was $5x10^4$-fold more sensitive than hybridization assay. It may enable the detection of parasites in 10 μl of blood with 0.00002% parasitaemia (Tanaka et al. 1993), if the genome size of *Babesia* sp.1 is assumed to be about 10^7 bp, similar to that of *T. parva* (Morzaria and Young 1992). This level of sensitivity is useful for detection of the parasite not only in actually infected animals, but also in carrier animals with low piroplasm parasitaemias. The nested-PCR can be completed in 1 day even if both PCR cycles are 35. Moreover, it was also produced excellent results when the second PCR cycle was reduced to 15 (data not shown).

The first primer set B5UK2 and B3UK2 produced a weakly visible band in A. centrale. However, the size of band seen was different from the size expected (670 bp). The specificity is increased by nested-PCR, since requiring four separate priming events to take place (Mullis et al. 1986). The nested-PCR may be more effective in some cases: the one-step PCR generates no visible products because of absolute or relative quantity of target is small, or it generates visible unsatisfiable bands.

In this study one-step PCR with primer set B5UK2 and B3UK2 did not generate PCR products, detectable by ethidium bromide staining, from 100 fg of *Babesia* sp.1 DNA. In reamplification for PCR products generated with the first primer set B5UK1 and B3UK1, the second primer set B5UK2 and B3UK2 generated visible PCR products. Successive amplification in the second PCR could be a proof of the presence of second primer sequences, which are specific for the parasite, within the first PCR product.

A transovarial transmission of *Babesia* sp.1 in *H. longicoronis* was evaluated using SpS7 sequence as the parasite-specific DNA marker. DNA sample was prepared from larval progeny of female ticks which had fed on *Babesia* sp.1-infected calf. No detectable signal was observed in tick samples by southern hybridization analysis with radiolabelled SpS7 probe (data not shown). The primer set B5UK2 and B3UK2 did not generated visible products in one-step PCR. The expected band was observed from the tick sample by nested-PCR technique. Therefore, it was indicated that *Babesia* sp. 1 was transovarially transmitted from adults of *H. longicoronis* to eggs and resultant larvae. After same colony of the larvae were fed on a calf which had been splenectmized, the appearance of

piroplasms were recognized in blood smears stained with Giemsa (data not shown). A parasite infection rate in ticks are usually low, and large number of ticks and transmission experiments are required in vector studies. Preliminary assessment of tick-batches using the PCR would allow us to select the ticks with higher infection rate for the parasite transmission experiments.

The characterization of the Japanese *Babesia* parasite regarding the parasite distribution in cattle population of Japan is hampered by the difficulty in isolating the parasites showing a low parasitaemia. The PCR and nested-PCR should be powerful tools in such epidemiological studies, because of their sensitivity. They should be also useful tools in the elucidation for the life cycle of *Babesia* sp.1. In particular, to combine the extreme sensitivity PCR/nested-PCR with the cell localizing ability of in situ hybridization (Nuovo et al. 1991) would be greatly enhance our understanding of these. The PCR and nested-PCR technique reported in this study, should facilitate studies aimed at the classification of this *Babesia* parasite.

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