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DNA Probes for Detection of *Babesia ovata* or the *Babesia* sp. l, A Newly Isolated Bovine *Babesia* Parasite in Japan

MASATO OHTA, SHIN-ICHIRO KAWAZU, NAOTOSHI TSUJI, YUTAKA TERADA, TSUGIHIKO KAMIO AND KOZO FUJISAKI*

National Institute of Animal Health, Tsukuba, Ibaraki 305, Japan *Present Address: Japan International Research Center for Agricultural Sciences, Tsukuba, Ibaraki 305, Japan

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ABSTRACT

We have recently reported the presence of a *Babesia ovata* like large intraerythrocytic parasite, *Babesia* sp.l in the cattle population of Hokkaido in Japan. A 5.5 kilobase (kb) DNA fragment that showed cross-hybridization with the BOZAP6, a DNA probe derived from *B. ovata* genome, was cloned from the *Babesia* sp.l genome and characterized. Beside a sequence that conferred the cross-hybridization, the fragment contained the parasite-specific sequence that did not hybridize with DNAs from *B. ovata*, *B. bigemina*, *B. bovis*, *Theileria sergenti*, *Anaplasma marginale*, *A. centrale*, *Eperythrozoon wenyoni* and bovine white blood cells. The *Babesia* sp.l specific region on the 5.5 kb fragment was excised and subcloned into a plasmid vector as parasite-specific DNA probe (SpS7). *Babesia ovata*-specific DNA probe (OvS9) was also obtained by deleting a sequence that confers the cross-hybridization with *Babesia* sp.l from original BOZAP6 DNA. SpS7 or OvS9 was sensitive enough to detect 5 ng of DNA either from *Babesia* sp. 1 or *B. ovata*, respectively. These DNA probes, sensitive and either specific for *B. ovata* or *Babesia* sp.l, could be useful tools in epidemiological studies to analyze the variations in the bovine *Babesia* species in Japan.

INTRODUCTION

Recently, a large intraerythrocytic parasite was isolated from the cattle population of Hokkaido in Japan (Ohta et al. 1995). The isolated parasite, tentatively referred to as *Babesia* sp.l differs in the morphological characteristics of

piroplasms from the description of *B. ovata*, a major causative agent of bovine babesiosis in Japan (Minami and Ishihara 1980). Besides the morphological differences, *Babesia* sp.1 showed unique biochemical and immunological characteristics compared with other *Babesia* species in cattle, such as *B. bovis*, *B. bigemina*, *B. major* and *B. ovata* (Ohta et al. 1995). Further characterization of the newly isolated parasite, *Babesia* sp.1 is now in progress in our laboratory, but the taxonomic status of the parasite remains unclear at present. The epidemiology of the parasite in relation to the distribution in cattle population of Japan and intra-specific variation within the species *B. ovata* should be considered for a precise classification of the parasite. However, the characterization of *Babesia* parasites from different geographical areas in Japan is hampered by the difficulty in isolating the parasites showing a low parasitaemia from mixed infection with *Theileria* species.

In recent years, the hybridization technology with DNA probes has been found to be a valuable means of characterizing species and stocks of parasites in animals and in vector ticks (Jasmer et al. 1990). Combination of the parasite-specific DNA probes and the hybridization technology that is applicable for samples with a small number of parasite (Buening et al. 1990, Petchpoo et al. 1992) may enable to overcome the difficulties in stock characterization of *Babesia* parasites in Japan.
In this paper, we describe the preparation of DNA probes either specific for *B. ovata* or *Babesia* sp.1. These probes could be useful tools in epidemiological studies to clarify the taxonomic status of the newly isolated *Babesia* parasite, *Babesia* sp.1, in Japan.

MATERIALS AND METHODS

Parasite stocks and preparation of parasite DNA

The parasite stocks used were as follows: *Babesia* sp.1 isolated from a Japanese Brown in Oshima area of Hokkaido prefecture, Japan (Ohta et al. 1995), *B. ovata* (Miyake Stock; Minami and Ishihara 1980), *B. bigemina* (Kochinda stock; Fujinaga et al. 1980), *B. bovis* (Australian stocks; Minami and Ishihara 1980), *T. 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). Blood was collected from calves experimentally infected with either of the four *Babesia* stocks, and piroplasms were purified by the nitrogen cavitation method (Shimizu et al. 1988). Genomic DNA of these *Babesia* stocks was prepared from purified piroplasms by treatment with sodium dodecyl sulfate (SDS) proteinase K solution, and phenol extraction (Sambrook et al. 1982). The genomic DNA of the other parasites used was prepared in the same manner.

Nucleic acid techniques

For Southern blotting, 0.3 µg of genomic DNA or a certain amount of plasmid DNA was digested with restriction enzymes and separated on 0.8% agarose gel. The gel was treated with ultraviolet light at 254 nm, and DNA was transferred to GeneScreen Plus (NEN Research Products, USA) in 0.5 N NaOH and 1.5 M NaCl. To evaluate the specificity of DNA probe candidates, 0.1 µg of parasite DNA was blotted onto the GeneScreen Plus after short-run agarose gel electrophoresis. The membrane was hybridized at 65 °C overnight with a DNA probe radio labeled by the random hexanucleotide primer method (Ready-To-Go system; Pharmacia, Sweden) in 5% sodium dextran sulfate, 0.5% SDS and 0.5 M NaCl. The membrane was washed twice in 2X SSC (1x SSC= 150 mM NaCl, 15 mM sodium citrate) with 0.1% SDS, and then twice in 0.2X SSC with 0.1% SDS at 65 °C and examined by autoradiography using Fujix Bio-image Analyzer BAS 2000 (Fuji Photo Film, Japan). Construction of library and screening: *Babesia* sp.l genomic DNA was completely digested with EcoRI. One micro gram of digested DNA was inserted into the phage vector, lZAPII EcoRI arms (Stratagene, USA). A total of 1,000 recombinant phages were plated out on *Escherichia coli* strain XLl-Blue (Stratagene, USA) with 250 plaques per petri dish, and grown at 37 °C ovemight. DNA from phage plaques was transferred onto nylon membranes (Colony/Plaque Screen; NEN Research Products, USA), and screened by using a 3.6 kilobase (kb) insert of BOZAP6 (Kawazu et al. 1993) as hybridization probe. After further four screenings, recombinated pBluescript plasmid was excised out from the phage DNA by in vivo excision technique (Stratagene, USA). The plasmid DNA was prepared on a large-scale according to the method previously described (Sambrook et al. 1982).

RESULTS

Southern blot analysis of *Babesia* DNAs with BOZAP6: A 3.6 kb insert of BOZAP6 hybridized with genomic DNA from *B. ovata* and *Babesia* sp.l, but did not hybridize with genomic DNAs from *B. bigemina, B. bovis* and bovine white blood cells. Various lengths of EcoRI fragments from *B. ovata* and *Babesia* sp.l genomic DNA hybridized with the DNA probe. There were differences in the number and sizes of the hybridized fragments between *B. ovata* and *Babesia* sp.l genomic DNA. BOZAP6 hybridized with *B. ovata* genomic DNA more intensely than *Babesia* sp.l genomic DNA (Fig. 1). The pattern of hybridization observed indicated the presence of a cross-hybridizing sequence in the form of multiple copies in *B. ovata* and *Babesia* sp.l genomes. Since differences were observed in the sizes of the hybridized fragments and their hybridization intensities between *B. ovata* and *Babesia* sp.l genomic DNA, we assumed that the cross-hybridizing

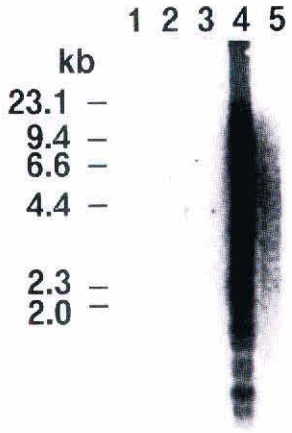

Fig. 1. Hybridization patterns of cloned genomic DNA of *Babesia ovata*, BOZAP6 with EcoRI-digested genomic DNAs (0.3 μg/lane) from *Babesia bigemina* (lane 1), *B. bovis* (lane 2), bovine white blood cells (lane 3), *B. ovata* (lane 4), and *Babesia* sp.l (lane 5). Sizes are given in kilobases (kb) on the left.

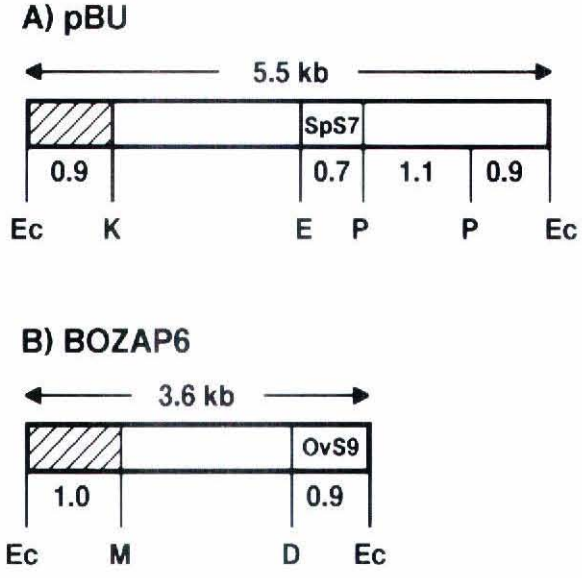

Fig. 2. Restriction maps of cloned *Babesia* sp.l genomic DNA, pBU (A) and cloned *B. ovata* genomic DNA, BOZAP6 (B). Restriction enzymes sites are designated as follows: EcoRI, Ec; MluI, M; DraII, D; KpnI, K; EagI, E; PstI, P. The hatched areas indicate the cross-hybridizing regions. The region selected for the parasite-specific probe is indicated as SpS7 (*Babesia* sp.l) in A or OvS9 (*B. ovata*) in B, respectively. Sizes of the rgions are indicated in kilobases (kb).

fragments on either parasite genomes contained both the cross-hybridizing sequence and the sequence which did not confer the cross-hybndization. Cloning of DNA fragments either specific for *B. ovata* or *Babesia* sp.l: For cloning the sequences that do not confer cross hybridization with *B. ovata* genomic DNA, a subgenomic library was constructed in lZAPII from EcoRI-digested *Babesia* sp.l genomic DNA. Four phage clones that showed strong hybridization signals in screening with BOZAP6 were selected from the library. Recombinated plasmids were excised out

from the phage DNA and analyzed for their restriction enzymes sites. One of the four clones, pBU that contained a 5.5 kb insert with suitable restriction enzymes sites, was selected for further characterization. To delineate the region which did not confer cross-hybridization in the pBU, the plasmid DNA was digested with various restriction enzymes and hybridized again with BOZAP6. A 0.7 kb EagI/PstI fragment where cross-hybridization with BOZAP6 was not observed (SpS7 in Fig. 2A) was subcloned from pBU DNA into the plasmid vector. A 0.9 kb EcoRI/KpnI fragment of pBU DNA showed cross-hybridization with BOZAP6 (hatched area in Fig. 2A). The region which did not confer cross-hybridization was also delineated in the same manner in BOZAP6. A 0.9 kb DraII/EcoRI fragment which did not show cross-hybridization with pBU (OvS9 in Fig. 2B) was subcloned from BOZAP6 DNA into the plasmid vector. A 1.0 kb EcoRI/MluI fragment of BOZAP6 DNA showed cross-hybridization with pBU (hatched area in Fig. 2B).

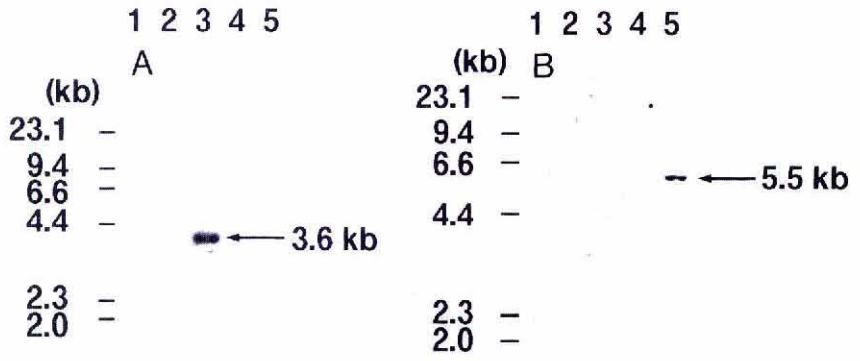

Fig. 3. Hybridization patterns of cloned genomic DNA of *Babesia* sp.1, SpS7 (panel A) and cloned genomic DNA of *B. ovata*, OvS9 (panel B) with EcoRI-digested genomic DNAs (0.3 µg/lane) from *Babesia bigemina* (lane 1), *B. bovis* (lane 2), bovine white blood cells (lane 3), *B. ovata* (lane 4), and *Babesia* sp.1 (lane 5). Sizes are given in kilobases (kb) on the left.

For the evaluation of specificity and sensitivity, SpS7 and OvS9 were used as DNA probes in Southern blotting with genomic DNA from *Babesia* sp.1, *B. ovata, B. bigemina, B. bovis* and bovine white blood cells. SpS7 or OvS9 detected either a 5.5 kb EcoRI fragment of *Babesia* sp.1 genomic DNA or a 3.6 kb EcoRI fragment of *B. ovata* genomic DNA, respectively. No hybridization was detected for both the

probes with genomic DNA from *B. bigemina*, *B. bovis* and bovine white blood cells (Fig. 3). Specificity of SpS7 and OvS9 for 0.1 μg of genomic DNA from other haemoparasites, such as *T. sergenti*, *A. marginale*, *A. centrale* and *E. wenyoni* was also confirmed by hybridization (Fig. 4). SpS7 or OvS9 was sensitive enough to detect 5 ng of genomic DNA from either *B. ovata* or *Babesia* sp.1 (Fig. 5).

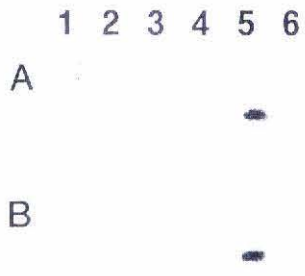

Fig. 4. Reactivity of cloned genomic DNA of *Babesia* sp.1, SpS7 (panel A) and cloned genomic DNA of *B. ovata*, OvS9 (panel B) with various haemoparasites DNA at 0.1μg. The DNA samples are aligned as follows: lane 1, *Theileria sergenti*; lane 2, *Anaplasma marginale*; lane 3, *A. centrale*; lane 4, *Eperythrozoon wenyoni*; lane 5, *Babesia* sp.1 (panel A) and *B. ovata* (panel B); lane 6, bovine white blood cells.

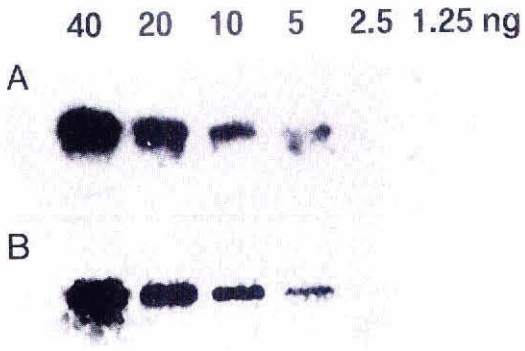

Fig. 5. Sensitivity of cloned genomic DNA of *Babesia* sp.1, SpS7 to purified B. sp.1 genomic DNA (column A) and cloned genomic DNA of *B. ovata*, OvS9 to purified *B. ovata* genomic DNA (column B). The amounts of DNA blotted are indicated above.

DISCUSSION

The cross-hybridization observed in Southern blotting of *Babesia* sp.1 genomic DNA with BOZAP6, a DNA probe derived from *B. ovata* genome suggested the presence of a genetical relationship between these two parasites. Since the cross-hybridization was less pronounced than homologous hybridization, both in the number of hybridized fragments and in their hybridization intensity, the results also indicated the presence of a difference in the genome arrangement between *Babesia* sp.1 and *B. ovata*. Based on the hybridization profiles observed in Fig. 1, we assumed that the cross-hybridizing fragments on either parasite genomes contained both the cross-hybridizing sequence and the sequence which did not confer cross hybridization. Therefore, DNA probes either specific for *Babesia* sp.1 or *B. ovata* were prepared by separating and subcloning the regions which did not confer the

cross-hybridization from the original fragments. The obtained probe, SpS7 or OvS9, was highly specific for *Babesia* sp.1 or *B. ovata*, respectively, because no hybridization was detected with genomic DNA from *B. bigemina*, *B. bovis*, bovine white blood cells and other haemoparasites that may have contaminated the blood samples. These probes were sensitive enough to detect 5 ng of purified parasite DNA in 50 µl, which is equivalent to a blood sample with about 0.5% parasitaemia estimated from the calculation of McLaughlin et al. (1986). Bovine babesiosis is distributes throughout the country in Japan, and except for the southern area where *B. bovis* and *B. bigemina* are endemic, *B. ovata* is considered to be a major *Babesia* species of cattle (Minami and Ishihara 1980). However the variation in bovine *Babesia* parasites in Japan has not been fully investigated, because of the difficulties in the isolation of the parasites showing a low parasitaemia from a mixed infection with *Theileria* species. The DNA probes sensitive and either specific for *B. ovata* or *Babesia* sp.1 could be useful tools in epidemiological studies to analyze the variation in bovine *Babesia* species in Japan.

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