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Development of DNA Probes for the Detection and Identification of *Babesia ovata*

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Babesia ovata is one of the important organism that promotes anaemia in the presence of mixed infection with Theileria species in cattle on pasture in Japan (Minami and Ishihara 1980). The pathogenicity of B. ovata infection has generally been found to be lower than that of other pathogenic species of Babesia such as B. bovis and B. bigemina. However, with increasing international trade of cattle there is a high possibility that the latter two pathogenic species may spread in the cattle population of Japan. Animals diagnosed as positive for B. bovis or B. bigemina are culled to facilitate the national control and eradication programme. Consequently, it is imperative that the accurate identification of the causative organisms is done rapidly. Although diagnosis of babesiosis using light microscopy of stained blood smear is one of the most reliable techniques, it requires a skillfulness and experience enough to detect and identify the Babesia parasite at low parasitaemic levels (Lanar et al. 1989). Serological diagnosis of babesiosis may fail to detect infections and to identify the species in some cases, especially the early stage of infection (Minami et al. 1979, Fujinaga et al. 1980). Thus, it is important to develop improved techniques which can diagnose B. ovata infection for surveillance and epidemiological studies so that reliable control measures can be implemented to control and eradicate the more pathogenic disease, B. bovis and B. bigemina infections, as well as help in early treatment of B. ovata infections in cattle. With the advent of biotechnology, it is now possible to use DNA probes as a rapid and sensitive tool for the detection and identification of other Babesia species (Buening et al. 1990, Petchpoo et al. 1992). In this paper we attempted to use the DNA probe to detect and identify B. ovata.

B. OVATA DNA PROBE

Babesia ovata (Miyake stock; Minami and Ishihara 1980) was used in this study. Blood was collected from a calf experimentally infected with the parasite stock, and merozoites were purified by the nitrogen cavitation method (Shimizu et al. 1992). Genomic DNA of the parasite was prepared from purified merozoites by treatment with sodium dodecyl sulfate (SDS) -proteinase K solution, and phenol extraction (Sambrook et al. 1989). The genomic DNA of other parasites used were prepared in the same manner. The parasite stocks used were as follows: B. bovis (Australian stock; Minami & Ishihara 1980) ; B. bigemina (Kochinda stock; Fujinaga et al. 1980); Theileria sergenti (Ikeda stock; Fujisaki et al. 1992); T. buffeli (Warwick stock; Fujisaki et al. 1992); T. orientalis (Essex stock; Fujisaki et al. 1992); Anaplasma marginale (Kochinda stock; Nakamura et al. 1991); A. centrale (Aomori stock; Nakamura et al. 1991); Eperythrozoon wenyoni (Natural infection; Kawazu et al. 1990). The genomic DNA of B. ovata (2.7μg) was digested with 80 units of EcoRI at 37°C for 2 h. The digested DNA was then fractionated by the size fractionation column (Amersham Japan, Japan), and fractions containing the fragments of more than 1 kilobase (kb) were pooled. The DNA fragments were concentrated by precipitation under ethanol at -80°C with 0.3M sodium acetate, and were inserted into λZAP II EcoRI arms (Stratagene, U.S.A.). The library size packaged in vitro was 1.1×10^7. Approximately 99.7% of the resulting plaques were recombinant phages as shown on 5-bromo-4-chloro-3-indolyl-b-D-galactoside indicator plate. A total of 894 recombinant phages were plated out on Escherichia coli XL1-Blue (Amersham Japan, Japan) with 100-200 plaques per petri dish, and grown at 37°C overnight. DNA from phage plaques were absorbed onto nylon filters (Hybond-N+; Amersham Japan, Japan) in replicate, and screened by using B. ovata and B. bovis genomic DNA as hybridization probes. The DNA was labelled by random hexanucleotide priming method with digoxigenin (DIG)-dUTP (DNA labeling and detection kit, nonradioactive; Boehringer Mannheim-Yamanouchi, Japan). Hybridization and immunological detection of hybridized DIG-labelled probe were also performed with this kit. Two phage clones, namely, BOZAP6 and BOZAP7 that showed intensive signal when hybridized to B. ovata genomic DNA but not detectable to B. bovis genomic DNA were selected as possible candidates for probe. Recombinated pBluescript plasmid was excised out from the phage DNA by the in vivo excision technique (Stratagene). BOZAP6 and BOZAP7 had 3.6kb and 5.0kb insert, respectively (data not shown). The plasmid DNAs were prepared in large-scale (Sambrook et al. 1989), and approximately 1mg of DNA was labelled with DIG-dUTP.

To investigate the genomic organization of cloned sequences, we performed Southern hybridization using two probes. The genomic DNAs of B. ovata, B. bigemina, and B. bovis were digested with EcoRI and electrophoretically fractionated in a 0.8% agarose gel in duplicate. The DNAs were transferred onto nylon filters (Nytran-N; Schleicher & Schuell, F.R.G.) and hybridized with BOZAP6 and BOZAP7 probes. The patterns of homologous hybridization between the probes and B. ovata genome suggested that B. ovata genome contained multiple copies of the sequence (Fig. 1). There was no detectable hybridization between the probes and the DNA of other two Babesia species (Fig. 1).

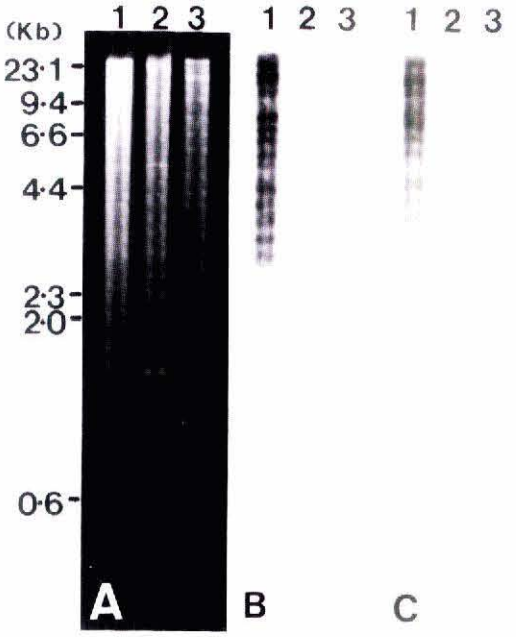

Fig.1 Agarose gel electrophoresis of EcoRI-digested genomic DNA (0.3μg / lane) from Babesia ovata (lane 1), B. bigemina (lane 2) and B. bovis (lane 3); (panel A). Hybridization patterns of Southern blots of panel A with BOZAP6 probe (panel B) and BOZAP7 probe (panel C). Filters were washed with 0.1x saline sodium citrate (SSC; 1xSSC=150mM NaCl, 15mM sodium citrate), 0.1% SDS at 68°C. Sizes are given in kilobases (kb) on the left.

The specificity of these probes with 100ng of DNA from various organisms that may contaminate into a field sample and from bovine white blood cells were assessed by dot blot hybridization. The DNA was blotted in duplicate onto the Hybond-N+ filters by the Bio-Dot apparatus (Bio-Rad Laboratories Japan, Japan). As shown in Fig. 2, strong hybridization signal is noted only with B. ovata DNA.

The B. ovata DNA was dot blotted in duplicate onto the Hybond-N+ filters in serial dilutions and hybridized with BOZAP6 and BOZAP7 probes. The limit detection in this assay was at 62pg and at 125pg for BOZAP6 and BOZAP7 probes, respectively (Fig. 3).

Fig. 2. Reactivity of cloned genomic DNA of Babesia ovata, BOZAP6 (column A) and BOZAP7 (column B) with various organisms and bovine white blood cell DNA at 100ng. The DNA samples are aligned as follows: lane1, Babesia ovata; lane 2, B. bovis; lane 3, B. bigemina; lane 4, Theileria sergenti; lane 5, T. buffeli; lane 6, T. orientalis; lane 7, Anaplasma marginale; lane 8, A. centrale; lane 9, Eperythrozoon wenyoni; lane 10, bovine white blood cell. Filters were washed in the same condition as Figure 1.

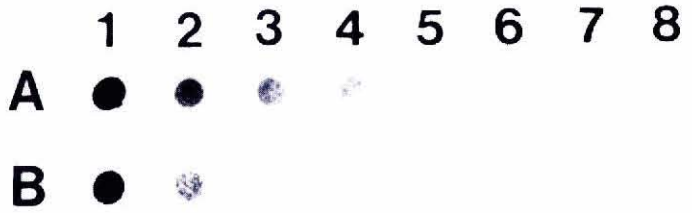

Fig. 3. Sensitivity of cloned genomic DNA of Babesia ovata, BOZAP6 (column A) and BOZAP7 (column B) to decreasing amounts of purified B. ovata genomic DNA. The amount of DNA blotted are as follows; lane 1, 10ng; lane 2, 1ng; lane 3, 500pg; lane 4, 250pg; lane 5, 125pg; lane 6, 62pg; lane 7, 31pg; lane 8, 15pg. Filters were washed in the same condition as Figure 1.

These results indicate that the selected clones, BOZAP6 and BOZAP7 were suitable for probes in the diagnosis of B. ovata infection. These probes are also capable of distinguishing the closely related species with certainty. However, the usefulness of these probes should be evaluated to test samples from the field as well as to follow experimentally induced parasitaemias in cattle especially where the parasitaemic levels are low. These experiments are now in progress in our laboratory to establish a DNA-based diagnosis of B. ovata infections. This will aid in the effective control of the diseases which will in turn reduce losses incurred through babesiosis caused by B. ovata, B. bovis and B. bigemina.

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