

# Differentiation and Quantification of *Theileria sergenti* Piroplasm Types Using Type-Specific Monoclonal Antibodies

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**ABSTRACT.** Antigenic properties of two representative allelic products of the major piroplasm surface protein (MPSP) of *Theileria sergenti* were studied. Sera from cattle infected with either of Ikeda and Chitose types of the parasite reacted strongly with homologous but weakly with heterologous recombinant antigens in immunoblotting. Monoclonal antibodies (MoAbs) produced against the both allelic products of MPSP parasites reacted only to the immunizing antigen. These results suggested that crossreactivity between two allelic products is very low in spite of relatively high homology in their amino acid sequences. Double staining of parasitized erythrocyte smear using type-specific MoAbs by an indirect immunofluorescent assay revealed that the set of MoAbs was useful for quantitative and differential detection of each type of parasite in mixed population. — **KEY WORDS:** monoclonal antibody, piroplasm, *Theileria sergenti*.

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*Theileria sergenti* is a tick-borne hemoprotozoan parasite of cattle. It occurs as intraerythrocytic piroplasms which are central to erythrocyte damage and consequent anemia. Infected cattle die occasionally in severe cases because of elevated parasitemia which is triggered by stress factors or coinfection with other pathogen [12, 13, 22, 23]. For the control of bovine theileriosis in Japan, several methods including vector control with acaricides and treatment with anti-piroplasm drugs are currently being used [12]. However, these methods sometimes face problems such as possible induction of drug-resistant ticks and parasites and low cost-effectiveness for treating infected animals. Therefore, alternative prevention methods are being sought. Effective vaccines for *Babesia bovis*, *T. annulata* and *T. parva* are available [15], however only basic research towards developing immunological control of *T. sergenti* infection have been conducted [2, 19].

In order to understand pathogenesis and to develop effective control methods against piroplasmosis, most of immunological studies have been focused on the analysis of piroplasm surface proteins at this stage and their genes. The piroplasms abundantly express a surface protein of molecular mass between 32 and 34 kDa which is strongly recognized by antibodies produced in infected animals [4, 6, 14, 16]. The gene encoding this major piroplasm surface protein (MPSP) exhibits genetical diversity as analyzed by Kawazu *et al.* [3, 5], Kubota *et al.* [7], and Matsuba *et al.* [10]. The benign theilerial parasites distributed in Japan and other Asian countries, often referred to as *T. sergenti/buffeli/orientalis* are divided into three major genetic groups according to their allelic types of the molecule, Chitose type (C-type; [10]), Ikeda type (I-type; [3]), and *T. buffeli*-

type (B-type; [3]). Amongst these, nucleotide and amino acid sequence homology levels are about 90% and 86%, respectively. Minor variations within each of the allelic types have been reported [10]. Most of the parasite stocks and isolates were revealed to contain mixed parasite populations bearing different allelic types of MPSP gene [7, 8]. Kawazu *et al.* [2, 4] compared antigenicity of piroplasm proteins among *T. buffeli* (Warwick stock), *T. orientalis* (Essex stock) and *T. sergenti* (Ikeda stock) by using sera from infected cattle and demonstrated that MPSP has low cross reactivity among these stocks.

Interestingly, population shift between I- and C-type parasites was generally observed during persistent infection in cattle [8]. Mutation in surface molecules recognized by host immune systems is generally considered to be one of mechanisms of parasite evading host immune responses in malaria [1]. The presence of mixed parasite population bearing antigenically different surface molecules and population shift from one to another allelic types may be one of the mechanisms of *T. sergenti* to disturb host immune surveillance system.

In this study, we produced recombinant products of two allelic types of MPSP and analyzed their immunogenic reactivity and cross-reactivity by polyclonal and monoclonal antibodies (MoAbs). The application of type-specific MoAbs for analysis of parasite population is also described.

## MATERIALS AND METHODS

*Parasite stocks of T. sergenti and polyclonal sera:* *T. sergenti* Shintoku stock had been maintained in our laboratory by blood or tick passages by *Haemaphysalis longicornis* in splenectomized calves [10]. Sera from cattle infected with Fukushima or Ikeda stock were used for immunoblotting. These two stocks have been revealed to consist of a single parasite population with either of C- or I-type MPSP alleles, respectively [7]. These sera were kindly

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provided by Dr. Kawazu, National Institute of Animal Health, Japan.

**Expression of recombinant MPSP using *Escherichia coli*:** I- and C-types of MPSP were expressed in *Escherichia coli*. The gene encoding I-type MPSP was amplified by polymerase chain reaction (PCR) by using a set of I-type specific primers from genetic DNA of piroplasm [7], and cloned into pBluescript. Its nucleotide sequence was determined in order to confirm the identity with that of I-type MPSP gene [3]. The insert in the pBluescript was ligated to an expression vector pET 32 (Novagen Inc., Madison, WI). The plasmid was transfected to host cells (BL21{DE3}pLys S, Novagen) by a heat shock procedure, and expression of MPSP was induced by the addition of 1 mM isopropyl- $\beta$ -thiogalactoside (IPTG) to the culture. Expressed fusion protein was purified using pET His-tag systems (Novagen Inc.) according to the manufacturers' protocol. C-type MPSP cDNA (clone L9-1, [11]) was also expressed in the same vector system.

**Production of monoclonal antibodies:** Piroplasms were purified from erythrocytes of an infected calf by the method of Sugimoto *et al.* [18]. Allele-specific PCR [7] was used to determine the purified material which contained only I-type parasites. Balb/c mice were subcutaneously inoculated with the purified piroplasms emulsified with Freund's complete adjuvant (Difco, Detroit, MI). Three booster inoculations were at the interval of two weeks with the antigen emulsified with Freund's incomplete adjuvant (Difco). Three days after the last immunization, mice were sacrificed and their spleen cells were fused with myeloma cells, P3U1, using polyethylene glycol 1500 (Boehringer Mannheim GmbH, Mannheim, Germany). Hybrid cells were selected by a medium containing hypoxanthine, aminopterin, and thymidine. Production of MoAbs was examined by ELISA using the recombinant fusion proteins as antigen according to the methods described by Zhuang *et al.* [24, 26]. Hybridomas producing antibodies against MPSP were cloned twice in a soft agar culture.

**Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting:** Protein analysis by SDS-PAGE was carried out by the method of Laemmli [9]. The fusion proteins were solubilized with a SDS sample buffer (10 mM Tris-HCl [pH 6.8] containing 2% SDS and 5% 2-mercaptoethanol) at 100°C for 2 min and separated electrophoretically in a 12% polyacrylamide gel and the gel was stained with Quick CBB (Wako Purechemical Industries Ltd., Osaka, Japan). For immunoblot analyses, the proteins were electrophoretically transferred to a polyvinylidene difluoride membrane sheet (Millipore Corporation, Bedford, MA) as described by Towbin *et al.* [21]. The sheet was blocked with 5% gelatin (Sigma, St. Louis, MO), and probed with calf serum or MoAbs for 1 hr at room temperature followed by peroxidase-conjugated anti-bovine Ig G (H+L) (Organon Teknika N. V.-Cappel Products, West Chester, PA) or anti-mouse Ig G (H+L) (Jackson ImmunoResearch Laboratory Inc., West Grove, PA) diluted in 1:1,000. 3–3–

diaminobenzidine tetrahydrochloride was used for color development.

**Surface immunofluorescent assay:** Unfixed piroplasms purified as described above were incubated with a monoclonal antibody for 30 min at room temperature. They were washed three times with phosphate-buffered saline (PBS), and stained with fluorescein isothiocyanate (FITC)-conjugated anti-mouse Ig G (H+L) (Organon Teknika N. V.-Cappel Products) for 30 min at room temperature. Stained parasites were mounted on a slide glass in a carbonate-buffered glycerol, and observed under a fluorescent microscope.

**Double staining in immunofluorescent assay:** Erythrocytes collected from a calf infected with *T. sergenti* Shintoku stock were washed three times with PBS, smeared on a slide glass, and air-dried. This stock contained mixed parasite population with I- and C-type alleles [7]. The smear was fixed with acetone for 5 min at -20°C, washed with PBS, and incubated with a MoAb C9 [24] for 30 min at room temperature in a moisture chamber. After incubation, the smear was washed three times with PBS and stained with tetramethylrhodamine B isothiocyanate (TRITC)-conjugated anti-mouse Ig G for 30 min at room temperature. The smear was washed again before and after double staining with FITC-conjugated MoAb 4G10 produced in this study, and red and green fluorescences were observed under UV-microscope (Olympus, BXFLA, Tokyo) with suitable combinations of filters.

## RESULTS

**Reactivities of recombinant MPSP with polyclonal and monoclonal antibodies:** Since biological techniques to produce clonal parasite of *T. sergenti* have not been established, only the practical way to produce monospecific antigen relies on expression systems of recombinant products in *E. coli* or other prokaryotic or eukaryotic cells. Although C-type and I-type recombinant products have been produced by Matsuba *et al.* [10, 11] and Kawazu *et al.* [5], respectively, we expressed them as histidine-tagged recombinant forms to facilitate purification step. The C- and I-type recombinant products expressed in *E. coli* and purified by Ni affinity chromatography were analyzed by SDS-PAGE as shown in Fig. 1A (lanes 1 and 2) and subjected to immunoblot analysis with the sera of cattle infected with either C or I type parasites (lanes 3–8). Sera from cattle infected with I- or C-type *T. sergenti* were reacted much stronger with the homologous antigen.

To analyze antigenic differences of these allelic products more precisely, we established hybridomas producing MoAbs (4G10, 5H9) against I-type MPSP. The reactivity of these MoAbs against the recombinant antigens was compared with that of MoAb C9. The later MoAb had been produced against piroplasms of Fukushima stock of which MPSP allele was C-type [24]. Two clones, 4G10 and 5H9 were revealed to be specific for the I-type MPSP and MoAb C9 specifically reacted with C-type MPSP as shown in Fig.

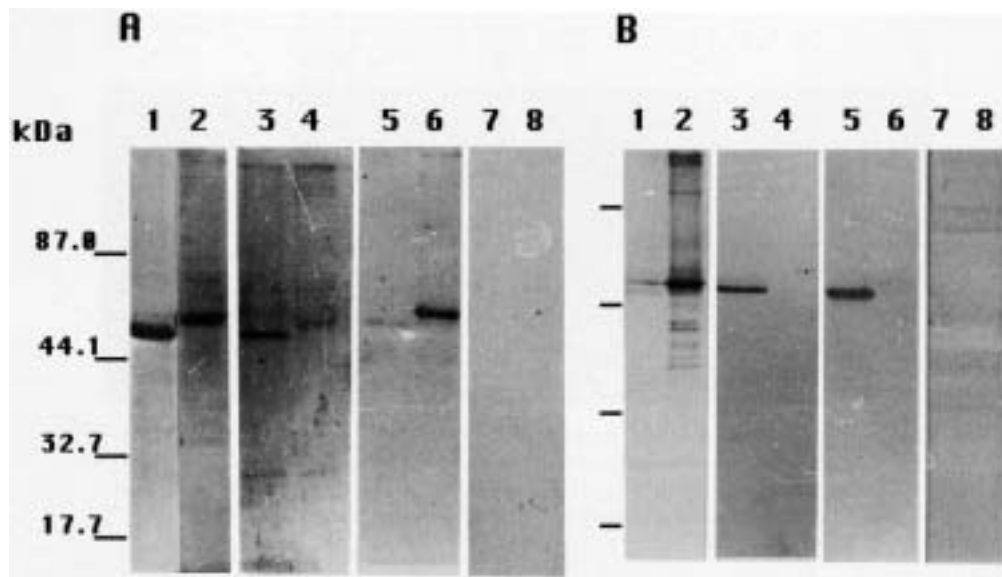


Fig. 1. Immunoblot analysis of C- and I-type recombinant MPSP products with polyclonal and monoclonal antibodies. A: Immunoblot analysis with infected bovine sera. Affinity purified C-type (lanes 1, 3, 5, 7) and I-type (lanes 2, 4, 6, 8) products were stained with Coomassie brilliant blue G-250 (lanes 1 and 2) or probed with serum from a calf infected with Fukushima stock which contains only C-type parasites (lanes 3 and 4, Kubota *et al.* [7]), or with Ikeda stock which contains only I-type parasites (lanes 5 and 6, Kubota *et al.* [7]). Serum from an uninfected calf was used for lanes 7 and 8. B: Immunoblot analysis with monoclonal antibodies. Affinity purified C-type (lanes 1, 3, 5, 7) and I-type (lanes 2, 4, 6, 8) products were probed with MoAb C9 (lanes 1 and 2), MoAb 4G10 (lanes 3 and 4) and MoAb 5H9 (lanes 5 and 6). MoAb against chicken lymphocyte surface antigen was used for lanes 7 and 8.

1B. MoAb C9 also produced weak reactions against several bands other than the fusion protein (Fig. 1B, lane 2), which was possibly due to partial breakdown of the recombinant product in bacterial cells, and due to interaction of the products with other bacterial proteins.

**Epitope localization of MoAbs:** To determine accessibility of these MoAbs to native MPSP molecule expressed on the surface of the parasites, purified piroplasms were incubated with the MoAbs under unfixed condition. MoAb 4G10 stained the surface of the I-type piroplasms, but 5H9 did not (data not shown). C-type specific MoAb C9 reacted with the surface of C-type piroplasms (data not shown).

**Double immunofluorescent staining method for the analysis of parasite population:** The ability of type specific MoAbs to differentiate parasite types under fluorescent microscope was tested by double immunofluorescent assay. The smear was prepared from Shintoku-stock infected erythrocytes with contained almost equal number of I- and C-type parasites as estimated allele-specific PCR. MoAb 4G10 (I-specific) and C9 (C-specific) conjugated with FITC and TRITC, respectively, were used. As shown in Fig. 2 A and B, in mixed population of I- and C-type parasites, piroplasms exhibited either green or red fluorescent. No piroplasm showing double fluorescence was seen. Ratio between the number of C- and I-type parasites was determined to be about 2:3.

## DISCUSSION

In the present study, we clearly demonstrated antigenic difference between two major allelic products of MPSP. Both of polyclonal antibodies from infected cattle and MoAbs were demonstrated to react in a type-specific manner. Although homology in amino acid sequences between these two types was more than 85%, they do not share common B cell epitope(s). The epitope(s) recognized by these antibodies are now being characterized by a peptide scanning method.

Since these MoAbs reacted in a type-specific manner, and MPSP is the most abundant protein on the parasite surfaces [4, 12], we investigated whether these MoAbs can be used for differential detection of each type of piroplasm using a double staining immunofluorescent assay. When we used two type-specific MoAbs, 4G10 and C9, each parasite cell was stained with either of the MoAbs, and none of them were double-stained with them. A PCR amplification system with allele-specific primer sequences has been used to analyze parasite populations both in bovine bloods and in salivary glands in ticks [7, 20]. Although this method is very sensitive, quantitative analysis of parasite populations with this technique is limited. The double staining method using type-specific MoAbs was demonstrated to be quantitative method and can, thus, used to analyze parasite population dynamics.

*Theileria sergenti* seemingly undergoes antigenic

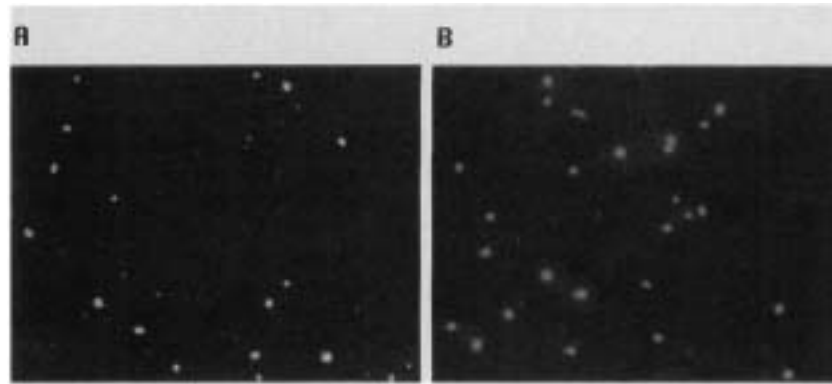


Fig. 2. Double immunofluorescent staining with type-specific MoAbs. Blood smears used for this test were prepared with erythrocytes obtained from a calf infected with Shintoku stock which contain both of C- and I-type parasites [7]. Panel A and B represent observations of the same microscopic field. Parasites were stained separately with type-specific MoAbs.

variation. Zhuang *et al.* [25] observed that parasites obtained at different points of infection sometimes lost reactivity with MoAb, C9, but it had not been clarified whether this antigenic change was due to antigenic variation or to other mechanisms. Kubota *et al.* [8] demonstrated that parasite population changes between I- and C-type parasites occurred during persistent infection in cattle. Results in this study provide further evidence that population shift from parasite expressing one allelic type of MPSP to those expressing another type, rather than mutation in the gene, result in apparent antigenic changes of the parasites. Keeping these observations in view, parasite population shift appears to be one of the mechanisms of parasite evasion from host immune responses.

MoAbs, 4G10 and C9, reacted with unfixed piroplasms, which indicates that epitope(s) recognized by these MoAbs are accessible on intact parasite surface. Passive immunization with MoAb C9 are reported to induce partial protective effects in cattle against challenge with piroplasm-infected erythrocytes [19], implying that the MoAb directed to surface-exposed epitope(s) on MPSP may be able to interfere parasite invasion into and growth in host cells. Therefore, the MoAbs developed in this study will be useful to analyze molecular interaction between parasite surface molecule and host cells.

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