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Allelic Analysis of Immunodominant Major Piroplasm Surface Protein Genes of Benign *Theileria* Parasites in Australian Cattle

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ABSTRACT

Benign bovine *Theileria* parasite, which is presumed to be of *T. sergenti/T. buffeli/T. orientalis* group, is distributed in Asia, Europe and Australia. An immunodominant major piroplasm surface protein (MPSP) gene of the parasites was analyzed and most of the parasite stocks and isolates were found to contain more than two different allelic types of MPSP. Parasite populations of a field isolate of benign *Theileria* species collected from Australian calf was analyzed by using allele-specific polymerase chain reaction (PCR), DNA restriction fragment length polymorphism (RFLP) and DNA sequencing. MPSP types of B1- and C-parasites were detected in the Australian samples. Nucleotide sequence of the gene of Australian C-type MPSP was similar to that of Japanese C-type parasite (T. *sergenti*) with 98.5% homology at nucleotide level. The study showed that Australian benign *Theileria* parasites are composed of a combination with at least 2 types of parasite one of which is similar to C-type of *T. sergenti*.

INTRODUCTION

Benign *Theileria* species in cattle are distributed in Asian countries such as China, Taiwan, Korea, Japan and also in Australia and in European countries. The classification and nomenclature of these parasites are still indefinite. The specific name of *T. sergenti* has been adopted for the parasites in Japan and Korea (Minami et al. 1980; Baek et al. 1990a and 1990b) and *T. buffeli* for Australian

isolates (Callow 1984). The parasites isolated in many other countries, including Europe, are sometimes called *T. orientalis* (Uilenberg et al. 1985). To avoid further confusion, these parasites are often referred to as the *T. sergenti/buffeli/orientalis* group (Fujisaki 1992), although *T. orientalis* and *T. sergenti* are considered to be a synonym for *T. buffeli* by Uilenberg (1995).

A PCR method for amplification of the genes of major piroplasm surface protein (MPSP) in a type- or allele-specific manner was developed (Kubota et al. 1995 and 1996b). By using this method and subsequent restriction fragment length polymorphism (RFLP) analysis, the presence of 4 different forms MPSP genes in parasites isolated from cattle in Japan and Korea was demonstrated (Kakuda et al. submitted). The majority of *T. sergenti*-infected calves in these countries harbored a mixed parasite population bearing at least two different allelic forms of MPSP (Kubota et al. 1995). Predicted amino acid sequences of MPSP alleles found in Asian *T. sergenti/buffeli* isolates showed 75 to 91% homologies (Tsuji et al. submitted).

Theileria parasites were previously identified in cattle which had been imported from Australia and grazed in Japan. The nucleotide sequences of their MPSP genes were identical to that of *T. buffeli* Warwick stock. Because parasites with C-type MPSP (*T. sergenti* Chitose stock Clone L9-1 type, Kubota et al. 1996a) was also detected from the same cattle, there is a need to determine whether these parasites with C-type allele of MPSP was transmitted from local cattle in Japan during their short grazing period in Japan, or they were already infected with a mixture of B1 and C type parasites. In this study, *Theileria* parasites in a blood sample collected in a farm in Australia was analyzed by allele-specific PCR and DNA sequence of its MPSP gene was compared to those reported in Japanese parasites.

MATERIALS AND METHODS

Parasites

Parasite stocks and isolates used in this study are listed in Table 1.

Table 1. *Theileria* parasite stocks and isolates used in this study.

Parasites	References
Japan	
T. sergenti Chitose stock	Matsuba et al. 1993 b
Australia	
Six isolates from imported cattle	Kubota et al. 1996b
T. *buffeli* Warwick stock	Stewart et al. 1987
England	
T. *orientalis* Essex stock	Morzaria et al. 1974

Preparation of DNA and PCR

Parasite DNA was prepared from purified piroplasms according to the method of Tanaka et al. (1993). The oligonucleotide primers (20-25 mers) for PCR, Ts-I, Ts-C, Tb and Ts-reverse (Ts-R) primers were previously described by Kubota et al. (1995). For the reaction, 50 μl of a mixture consisted 50 ng of parasite DNA as a template, primers (1 μM each), deoxynucleotide triphosphates (200 μM each) and 1.25 U of *Taq* polymerase (GIBCO BRL Life Technologies, Inc., MD, USA) in 1 X PCR buffer [20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.0 mM $MgCl_2$] were used. The reactions proceeded in a programmed temperature control system (Model PC-700 ASTEC Co., Ltd., Fukuoka, Japan) for 35 cycles. Each cycle consisted of 1.5 min of denaturation at 94°C (4 min for the first cycle), 1.5 min of annealing at 57°C and 1.5 min of polymerization at 73°C, with an additional 3 min at 73°C, after the last cycle. After amplification, each sample was subjected to RFLP analysis.

RFLP analysis

PCR products which were amplified by using each primers were purified with GENECLEAN II (BIO 101 Inc., CA, USA). Fragments obtained by digestion with *Bgl* I, *Dra* I, *Eco* T14I, *Eco* RV and *Hind* III were subjected to agarose gel electrophoresis, and RFLP analysis of amplified DNA was carried out as described by Kubota et al. (1996a).

DNA cloning and sequencing

PCR products which had been amplified from Australian field isolate using Ts-C and Ts-R primers were cloned into the plasmid pGEM-T vector (Promega, WI, USA) and the recombinant plasmid DNA was used for nucleotide sequencing. The nucleotide sequence of plasmid inserts was determined by using T7 and SP6 promoter primers (Promega, WI, USA), gene-specific primers and the Taq DyeDeoxy™ Terminator Cycle Sequencing System (Applied Biosystems, Foster City, CA, USA) with an automated DNA sequencer (Applied Biosystems model 373A). In order to obtain the complete sequence, sequencing was carried out twice.

RESULTS AND DISCUSSION

The results of population analysis of 6 isolates from Australian imported cattle, *T. orientalis* and *T. buffeli* stocks are summarized in Table 2. Six isolates from Australian imported cattle consisted of a mixture of B1 type and C type, while that of *T. buffeli* Warwick stock and *T. orientalis* Essex stock contained single parasite population with B1 type. This mixed pattern of mixture is same as

those observed in stocks from Taiwan (Kakuda et al. submitted). Since parasites with the C-type MPSP were detected in cattle imported from Australia but not in piroplasm preparation of *T. buffeli* Warwick stock, we could not exclude the possibility that these cattle might have been infected with this C type parasite after their arrival in Japan can not be excluded. Thus, parasite populations in a field isolate collected in a farm in Australia were analyzed and consequently C-type parasite in this sample was detected.

Table 2. Analysis of *Theileria* parasite stocks and isolates by allele-specific PCR.

Parasites	MPSP alleles			
	I	C	B1	B2
Six isolates from imported Australian cattle	-	+	+	-
Australian field isolate	-	+	+	-
T. buffeli Warwick	-	-	+	-
T. orientalis Essex stock	-	-	+	-

Nucleotide sequence of PCR product of an Australian field isolate amplified by Ts-C and Ts-R primers was determined and compared with that of Japanese C-type (L9-1) (Matsuba et al. 1993a). The nucleotide size of coding region for predicted MPSP region of Australian C-type (clone T/AUST-C) was 786 bp which was equal to the size of L9-1 (Fig. 1). Comparison between these two sequences using computer program GENETYX-Homology resulted in 98.5% homology at nucleotide level (Fig. 1) and 96.6% homology at amino acid level (Fig. 2). The potential glycosylation sites and putative erythrocyte binding motifs, Lys-Glu-Lys and Lys-Glu-Leu, or Lys-Glu ion pair (Molano et al. 1992) were conserved between these two clones (Fig. 2).

Serological and genetical comparisons between *T. buffeli* Warwick and *T. orientalis* Essex stocks revealed that these two stocks shared the same antigenic and genetic properties (Kawazu et al. 1992a and 1992b). Benign *Theileria* parasites distributed in Australia has been often referred to as *T. buffeli* or *T. orientalis*. In East Asia, such as China, Korea and Japan, I- and C-types of *T. sergenti* were generally detected, while C-type and B1-type parasites were present in Taiwan and Australia (Table 2). From the results of systemic comparative studies of these parasites it was concluded that Japanese *T. sergenti* belongs to a different taxonomic group from Australian *T. buffeli* (Fujisaki 1992).

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L9-1      atgttgtccaagagatcattcaacgtactttgcctaggatacttcctcatcgtctctgca   60
T/Aust-C                                    gcggatcctcatcgtctctgca
          actgcaGAAGAGAAAAAAGAAGCTGCAAAGGCTGATGAGAAGAAGGATTTAGCTCTTGAA  120
          act.....................................................
          GTAAACGCCACCCAGGCTGAAAATTTTACAGTCAATGCAACCAATGCCAACGACGTCGTT  180
          .....................................................
          TTTACTGCCAATGAGGGATACCGTATCAAGACACTTAAGGTTGGAGATAAAACTTTGTAT  240
          ..................T..................................A......
          ACTGTAGATACATCCAAATTCACTCCAACAGTCGCCCACAGACTGAAGCATGCTGAAGAC  300
          .....................................................
          CTGTTCTTAAAGCTCGACCTTTCCCATGCAAAACCACTTTTGTTCAAGAAGAAGAGCGAC  360
          ........C.....A.....................................................
          AAGGAATGGGTACAGTTCAGCTTCGCCCAGTACCTCGATGAAGTTCTCTGGAAAGAAAAG  420
          ....................................C.....................
          AAGGAATCCAAAGACCTCGATGCCTCCAAGTTTGCAGAAGCTGGTCTTTTTGCCCCTGAT  480
          .....................................................
          GCATTCGGTACCGGAAAGGTTTACGACTTCGTCGGAAACTTCAAGGTCACCAAGGTCAAG  540
          .....................................................
          TTCGAGGATAAGGAAGTCGGAGATTCAAAGAAGGCCAAATACACCGGAGTCAAAGTTTAC  600
          .............................................C.......
          GTCGGTACCGATGACAAGAAAATCGTAAGACTCGACTACTTCTATACCGGTGATGAGAGA  660
          ............................G.....................
          TTCAAGGAAGTATACTTCAAATTGGTCGATGGAAAGTGGAAGAAGCTTGAGCAGAGCGAC  720
          ......................A.............A.................G
          GCAAACAAGGATTTGCACGCTATGAACAATGCTTGGCCTTTGGACTACAAGCCTCTTGTC  780
          .....................................................
          GACAAGTTCTCACCACTTGCCGTCCTCAGCGCTGTTCTCATCGCCTTACTCGCAGTATCC  840
          ........................G.....................T........
          TACTATCTCTAG                              852
          .........taggcgcattgagtctcaca
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Fig. 1. Comparison of nucleotide sequences of major piroplasm surface protein genes between Japanese C-type cDNA clone and Australian C-type gene clone. Nucleotide sequences of L9-1 (Japanese C-type) is shown from initiation (ATG) to termination (TAG) codons. Small letters indicate predicted signal peptide region and small italic letters indicate PCR primer sequences which were used for gene amplification and cloning.

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L9-1      EEKKEAAKADEKKDLALEVNATQAENFTVNATNANDVVFTANEGYRIKTLKVGDKTLYTV   60
T/AUST-C  ....................................................F.......
          DTSKFTPTVAHRLKHAEDLFLKLDLSHAKPLLFKKKSDKEWVQFSFAQYLDEVLWKEKKE  120
          ....................F..N.....................................
          SKDLDASKFAEAGLFAPDAFGTGKVYDFVGNFKVTKVKFEDKEVGDSKKAKYTGVKVYVG  180
          ....................................................A.....
          TDDKKIVRLDYFYTGDERFKEVYFKLVDGKWKKLEQSDANKDLHAMNNAWPLDYKPLVDK  240
          ........V.................I.........E.....................
          FSPLAVLSAVLIALLAVSYYL     261
          .......G.......S.....
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Fig. 2. Comparison of predicted amino acid sequences of MPSP except signal peptide region between Japanese (L9-1) (Matsuba et al., 1993a) and Australian (T/AUST-C) C-type. Dots indicate identical amino acids. Underlines indicate charged amino acid-rich regions.

Changes of parasite populations during persistent infection of two types (I- and C-types) of *T. sergenti* in cattle were observed, which suggests the host immune responses can select a certain allelic variant (Kubota et al. 1996a). Coexistence of multiple allelic variant of immunogenic surface molecules in an infected host may disturb host defense mechanisms and facilitate the parasites to evade them. From this point of view, the antigenic differences between 4 allelic products of MPSP are now being analyzed. As MPSP is one of the vaccine candidates, analysis of parasite populations based on PCR-RFLP analysis is essential for developing control methods for *T. sergenti* infection in cattle.

Note: Nucleotide sequence data reported in this paper have been submitted to DDBJ, EMBL and GeneBank™ Nucleotide Sequence Databases with the accession numbers T/AUST-C: D78015.

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REFERENCES

Baek, B. K., Kim, B. S. & Lee, H. I. 1990a. Fine structure of *Theileria sergenti* merozoite in Korean native cattle. *Korean J. Vet. Res. 30:* 465-471 (in Korean with English abstract).

Baek, B. K., Song, H. J., Kim, B. S. & Rhee J. K. 1990b. Study on the antigenicity of *Theileria sergenti* merozoite in Korean native cattle. *Korean J. Vet. Res. 30:* 223-229 (in Korean with English abstract).

Callow, L. L. 1984. Theileriosis. *In: Animal health in Australia*, vol. 5, Australian Government Publishing Service, Canberra. pp. 168-173.

Fujisaki, K. 1992. A review of the taxonomy of *Theileria sergenti/buffeli/orientalis* group parasites in cattle. *J. Protozool. Res. 2:* 87-96.

Kawazu, S., Sugimoto, C., Kamio, T. & Fujisaki, K. 1992a. Analysis of the genes encoding immunodominant piroplasm surface proteins of *Theileria sergenti* and *Theileria buffeli* by nucleotide sequencing and polymerase chain reaction. *Mol. Biochem. Parasitol., 56:* 169-176.

Kawazu, S., Sugimoto, C., Kamio, T. & Fujisaki, K. 1992b. Antigenic differences between Japanese *Theileria sergenti* and other benign *Theileria* species of cattle from Australia *(T. buffeli)* and Britain *(T. orientalis). Parasitol. Res. 78:* 130-135.

Kubota, S., Sugimoto, C. & Onuma, M. 1995. A genetic analysis of mixed

populations in *Theileria sergenti* stocks and isolates using allele-specific polymerase chain reaction. *J. Vet. Med. Sci. 57:* 279-282.

Kubota, S., Sugimoto, C. & Onuma, M., 1996a. Population dynamics of *Theileria sergenti* in persistently infected cattle and vector ticks analyzed by a polymerase chain reaction. *Parasitology 112:* 437-442.

Kubota, S., Sugimoto, C., Kakuda, T. & Onuma, M. 1996b. Analysis of immunodominant piroplasm surface antigen alleles in mixed parasite population of *Theileria sergenti* and *T. buffeli. Int. J. Parasitol., 26:* 741-747.

Matsuba, T., Kubota, H., Tanaka, M., Hattori, M., Murata, M., Sugimoto, C. & Onuma, M. 1993a. Analysis of mixed parasite populations of *Theileria sergenti* using cDNA probes encoding a major piroplasm surface protein. *Parasitology 107:* 369-377.

Matsuba, T., Sugimoto, C., Onoe, S., Kawakami, Y., Iwai, H. & Onuma, M. 1993b. Changes in the hybridization patterns of populations of *Theileria sergenti* during infection. *Vet. Parasitol., 47:* 215-223.

Minami, T., Fujinaga, T., Furuya, K. & Ishihara, T. 1980. Clinico-hematologic and serologic comparisons of Japanese and Russian strains of *Theileria sergenti. Natl. Inst. Anim. Health Q. (Jpn.) 20:* 44-52.

Morzaria, S. P., Barnett, S. F. & Brocklesby, D. W. 1974. Isolation of *Theileria mutans* from cattle in Essex. *Vet. Rec. 94:* 256.

Molano, A., Segura, C., Guzman, F., Lozada, D. & Patarroyo, M. E. 1992. In human malaria protective antibodies are directed mainly against the Lys-Glu-Lys motif of the synthetic vaccine SP66. *Parasite Immunol., 14:* 111-124.

Stewart, N. P., Devos, A. J., Shiels, I. & McGregor, W. 1987a. The experimental transmission of *Theileria buffeli* of cattle in Australia by *Haemaphysalis humerosa. Aust. Vet. J., 64:* 81-83.

Sugimoto, C., Ali, S., Kubota, S., Sako, Y., Yin, H., Zhuang, W. Z., Lu, W., Baek, B. & Onuma, M. Antigenic and genetic comparisons of benign *Theileria* species isolated from cattle in China, Korea and Japan. *Vet. Parasitol.,* In press.

Tanaka, M., Onoe, S., Matsuba, T., Katayama, S., Yamanaka, M., Yonemichi, H., Hiramatsu, K., Beak, B. K., Sugimoto, C. & Onuma, M. 1993. Detection of *Theileria sergenti* infection in cattle by polymerase chain reaction amplification of parasite-specific DNA. *J. Clin. Microbiol. 31:* 2565-2569.

Uilenberg, G. 1995. International collaborative research: significance of tick-borne hemoparasitic diseases to world animal health. *Vet. Parasitol. 57:* 19-41.

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.

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