

# A Genetic Analysis of Mixed Population in *Theileria sergenti* Stocks and Isolates Using Allele-Specific Polymerase Chain Reaction

Shuichi KUBOTA, Chihiro SUGIMOTO, and Misao ONUMA\*

Department of Epizootiology, Faculty of Veterinary Medicine, Hokkaido University, Sapporo 060, Japan

(Received 4 October 1994/Accepted 15 December 1994)

**ABSTRACT.** In order to differentiate parasite populations bearing two allelic forms of p33/32, an immunodominant piroplasm surface protein of *Theileria sergenti*, two sets of oligonucleotide primers were designed to amplify either of the two allele by the polymerase chain reaction (PCR). Each set of the primers differentially amplified either of Ikeda- or Chitose-type p33/32 genes. By using this allele-specific PCR and restriction enzyme digestion of amplified products, parasite populations within field isolates collected from different geographical regions in Japan were analyzed. Both of the allelic forms were detected in 13 samples out of 20 isolates and stocks by PCR. Either of Ikeda or Chitose type of p33/32 allele was detected in the other 7 samples. These results indicated that the majority of *T. sergenti*-infected calves (11/15) in Japan harbored mixed parasite populations bearing at least two different alleles of p33/32.—**KEY WORDS:** allele, piroplasm, polymerase chain reaction, *Theileria sergenti*.

*J. Vet. Med. Sci.* 57(2): 279–282, 1995

*Theileria sergenti* is a tick-borne protozoan parasite in cattle that causes anemia as intraerythrocytic piroplasma [10]. This parasite shows genetic diversities as analysed by probes of genomic DNA clones [9] and cDNA encoding a major piroplasm surface protein, p33/32 [5, 8, 17]. Antigenic diversities of piroplasm surface proteins, p33/32 and p23 were also analyzed by using a panel of monoclonal antibodies [17]. Nucleotide sequences of cDNAs encoding piroplasm surface major proteins of Chitose and Ikeda stocks of *T. sergenti* were determined [4, 8], which showed 86% homology at amino acid level. Within the cDNA clones from Chitose stock, we found two allelic variants with transition of a few nucleotides, one of which resulted in one amino acid substitution [8]. These results indicated the presence of multiple allelic forms of p33/32.

In this study, we designed allele-specific primers for polymerase chain reaction (PCR) amplification of Ikeda- and Chitose-type p33/32 and analyzed parasite populations of field isolates from cattle in different geographical areas in Japan.

## MATERIALS AND METHODS

**Parasite stocks and field isolates of *T. sergenti*:** The Shintoku stock had been maintained in our laboratory by blood or tick passages by *Haemaphysalis longicornis* ticks in splenectomized calves [8, 9]. Chitose [9], Ikeda [3], Shintoku [7], Fukushima [10] and Takahara [7] stocks and field isolates of *T. sergenti* [17] were also used for analysis.

**Preparation of DNA and PCR:** Parasite DNA was prepared from parasitized bovine erythrocytes or purified piroplasms as described previously [15]. The oligonucleotide primers (20–25 mers) for PCR, Ts-universal

(Ts-U) and Ts-reverse (Ts-R) primers were described elsewhere [15]. Ikeda-specific (Ts-I) primer, Chitose-specific (Ts-C) primer were synthesized on a DNA synthesizer (model 391; Applied Biosystems, U.S.A.) as shown in Fig. 2. In the initial reaction, 100  $\mu$ l of PCR mixture consisted of 10–100 ng of *T. sergenti* DNA as a template, primers (1  $\mu$ M each), deoxynucleotide triphosphates (200  $\mu$ M each), and 2.5 U of Taq polymerase (GIBCO BRL, Life Technologies, Inc. U.S.A.) in 1 X PCR buffer (20 mM Tris-HCl [pH 8.4], 50 mM KCl, 2.0 mM MgCl<sub>2</sub>). When samples from infected carrier cattle were tested, the amount of DNA included in the reaction was determined according to the estimated extraction efficiency of the DNA. The reactions proceeded in a program temperature control system (ASTECCo., Ltd., Japan) for 35 cycles. Each cycle consisted of 1 min of denaturation at 94°C (4 min for the first cycle), 1 min of annealing at 55°C, and 1 min of polymerization at 73°C, with an additional 3 min at 73°C after the last cycle. After amplification, 5–10  $\mu$ l of each sample were subjected to agarose gel electrophoresis with or without restriction enzyme digestion [4].

## RESULTS

**Allele-specific PCR:** In order to differentiate two allelic forms of p33/32, we designed allele-specific PCR primers for I- and C-types as shown in Fig. 1. By combination of universal or either of allele-specific primers with Ts-R primer, 836 bp or 831 bp bands were specifically amplified from DNA of Shintoku stock and cDNA clone obtained from Chitose stock (L9–1) (Figs. 2 and 3b). DNA was amplified from L9–1 by combination of Ts-R with Ts-C or with Ts-U. From DNA of Shintoku stock, amplification with Ts-I in addition to Ts-C and Ts-U primers was observed, which indicate the presence of mixed parasite populations with two allelic form of p33/32 in this

\* CORRESPONDENCE TO: ONUMA, M., Department of Epizootiology, Faculty of Veterinary Medicine, Hokkaido University, Sapporo 060, Japan.

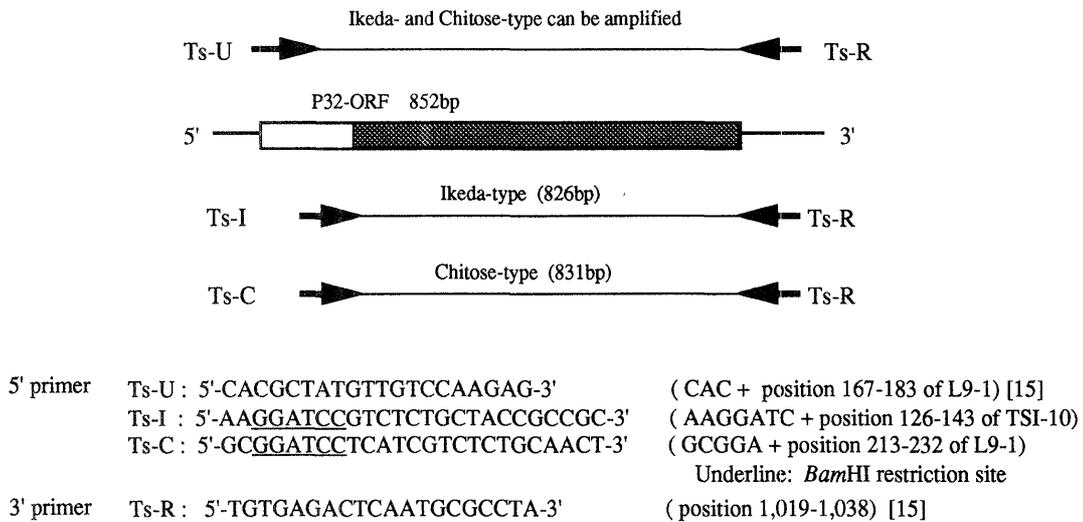


Fig. 1. Schematic representation of the genes coding for the p32 of *Theileria sergenti* piroplasm. Blanking area represents region which coding mature p32 and stippled area represents signal peptide coding region. The positions of the oligonucleotide primers used for PCR amplification are indicated by arrows.

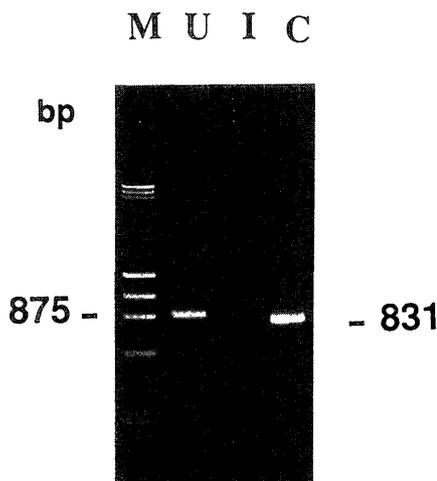


Fig. 2. PCR amplification of p33/32 cDNA cloned from *Theileria sergenti* Chitose stock (Clone L9-1 [8]). A total of 0.1 ng DNA was used for each PCR amplification using three different combinations of primers (lane U, Ts-universal primer; lane I, Ikeda-specific primer; lane C, Chitose-specific primer) as a forward primer, respectively. The products (5  $\mu$ l each) were electrophoresed on a 2% agarose gel and stained with ethidium bromide. Lane M, molecular mass markers (Marker 1 + Marker 4: Nippon Gene, Tokyo, Japan)

preparation. Specificity of the PCR was confirmed by Southern hybridization of the products with L9-1 cDNA probe (data not shown). When these amplified DNAs were subjected to restriction enzyme digestion with *Bgl* I, *Dra* I, *Eco*T14I and *Hind* III, restriction patterns expected from Ikeda or Chitose cDNA sequences were obtained as

Table 1. Analysis of parasite populations of Japanese *T. sergenti* isolates by allele-specific PCR

Isolate	5'-primer		
	Universal	Ikeda	Chitose
Hokkaido			
Abashiri 1	+	+	-
Shintoku <sup>a)</sup>	+	+	+
Chitose <sup>a)</sup>	+	+	+
Oshima	+	+	+
Hiyama 116	+	+	+
Aomori 15	+	+	+
Miyagi 9	+	+	+
Fukushima <sup>a)</sup>	+	-	+
Tochigi			
Ikeda <sup>a)</sup>	+	+	-
Takahara <sup>a)</sup>	+	+	-
Niigata 2	+	+	+
Nagano	+	+	-
Shizuoka 15	+	+	-
Okayama 43	+	-	+
Okayama 621	+	+	+
Tokushima 18	+	+	+
Tokushima 22	+	+	+
Fukuoka 50	+	+	+
Kumamoto 1	+	+	+
Kagoshima 33	+	+	+

a) Laboratory stocks.

shown in Fig. 3a. These results indicated that universal and allele-specific amplification of p33/32 gene was possible by using suitable combination of primers.

*Analysis of field isolates by allele-specific PCR:* To determine the distribution of I- and C-type parasite within isolates, we analyzed field isolates collected from different geographical regions in Japan. DNAs of 5 stocks and 15 isolates were analyzed by PCR using Ts-U, -I, and -C

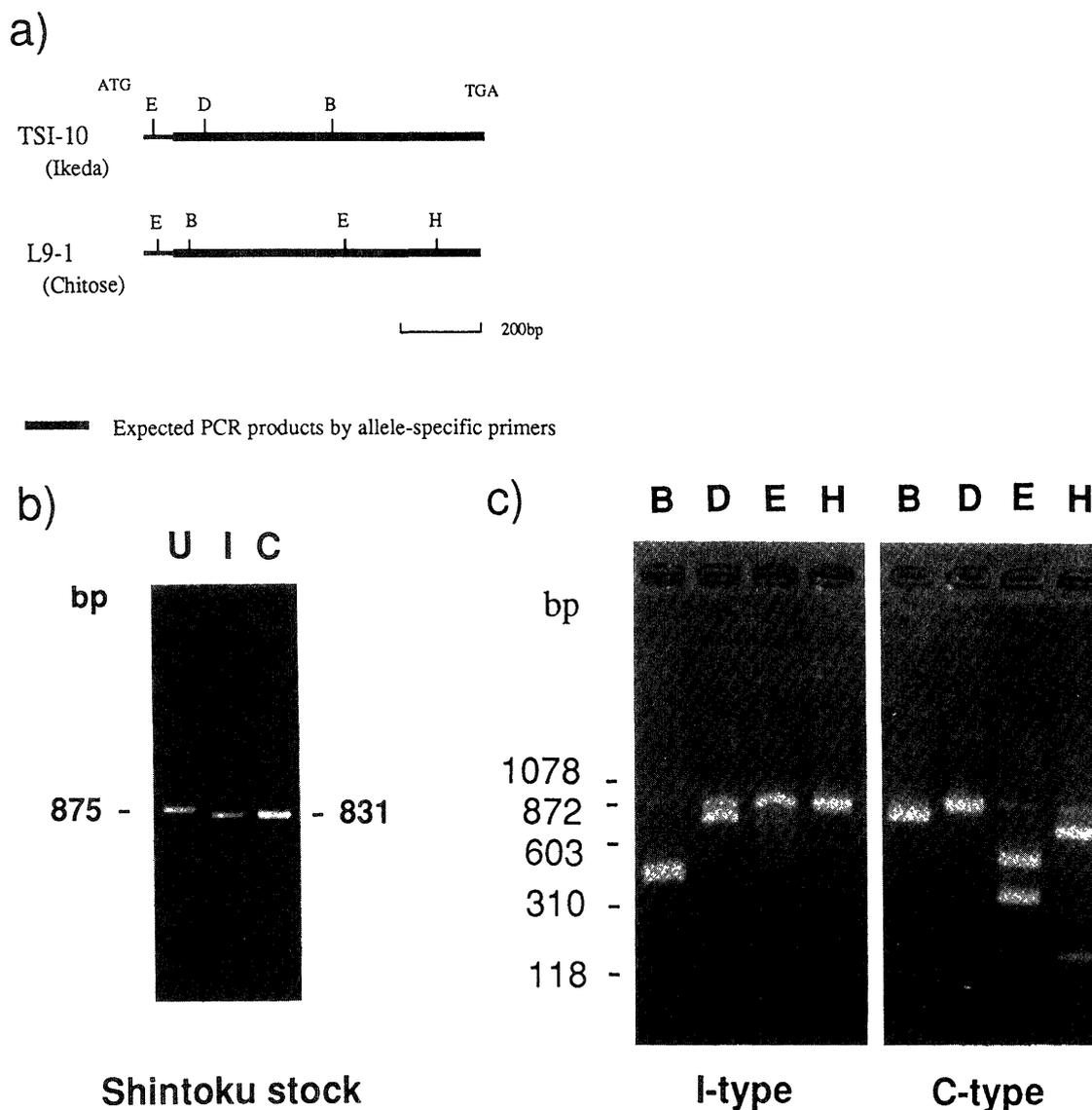


Fig. 3. PCR amplification of p33/32 gene from *Theileria sergenti* Shintoku stock. (a) Restriction maps of p33 cDNA of Ikeda-type (TSI 10) [4] and p32 cDNA of Chitose-type (L9-1) [8]. Restriction enzymes sites are indicated as follows: *Bgl*I, B; *Dra*I, D; *Eco*T14 I, E; *Hind* III, H. The PCR products are denoted by thick lines in the maps. (b) Results of PCR of Shintoku stock. A total of 100 ng of DNA were used as template for each reaction. Ts-universal (lane U), Ikeda-specific (lane I) or Chitose-specific (lane C) primer was used. (c) Restriction patterns of the PCR products. The PCR products amplified by Ikeda-specific or Chitose-specific primer were digested with the restriction enzymes, electrophoresed on a 2.0% agarose gel and stained with ethidium bromide [4]. The restriction enzymes used were *Bgl*I (B), *Dra* I (D), *Eco*T14 I (E) and *Hind* III (H).

primer as 5'-primer in combination with Ts-R primer as 3'-primer (Table 1). In our previous study, all *T. sergenti* isolates which were collected from Japan [17], *Theileria* species from Korea (Korean Chonju stock; referred to as *T. sergenti* [1, 2]), *T. buffeli* Warwick stock [13] and *Theileria* species detected in calves imported from Australia [15] were positive in PCR by combination of Ts-U and Ts-R primers [15]. Therefore, we used these primers for universal amplification of *T. sergenti* p33/32 gene. Out of 20 samples which were positive with the universal primer, 13 showed positive reaction with both of Ts-I and Ts-C

primers. Other 7 showed positive reaction with either of the primers. The PCR products from 5 laboratory stocks and 4 field isolates (Aomori 15, Miyagi 9, Tokushima 18 and Kagoshima 33) by Ts-I or Ts-C primers, were analyzed by restriction enzyme digestion using *Bgl* I, *Dra* I, *Eco* T14 I and *Hind* III. The restriction patterns of them were identical to those shown in Fig. 3c, as expected from restriction sites in I- or C-type (data not shown).

## DISCUSSION

In the present study, we developed PCR specific to two allelic forms of p33/32 of *T. sergenti* and demonstrated mixed parasite populations within field isolates. The majority of the field isolates (11/15) tested in this study contained parasites with two major allelic forms of p33/32, namely I- and C-types. Two parasite stocks, Shintoku and Chitose stocks, which had been passaged in experimental calves several times after isolation also contained mixed parasite populations with different allelic forms of p33/32. By present, two additional allelic variants which contained a few nucleotides different from the C-type allele have been reported by Matsuba *et al.* [8]. P34 of *T. buffeli*, a parasite closely related to *T. sergenti* and *T. orientalis* [4, 5], might be an additional allelic forms of p33/32, but parasites with this allelic form were not demonstrated in Japanese isolates in this study.

The presence of mixed parasite population of *T. sergenti* and population changes during persistent infection in cattle have been demonstrated by Southern blot analysis by using cDNA encoding the immunodominant piroplasm surface protein as probes [8]. The allele-specific PCR developed in the present study may be a powerful tool not only for epidemiological studies of the parasite but also for analyzing biological relevance of p33/32 diversity in persistent infection.

Infection with *T. sergenti* persists in cattle, but mechanisms involved in parasite evasion from host immune systems during the persistent infection have not been clarified. As p33/32 is a major surface protein of piroplasm stage of this parasite [12, 14, 16], and recognized predominantly by host antibodies [6, 11], the presence of multiple allelic forms of this molecule may benefit parasites to persist in mammalian hosts. From this point of view, we are analyzing antigenic differences between two major allelic forms of p33/32 and other allelic variants, which is essential for developing control methods of bovine theileriosis in Japan.

**ACKNOWLEDGEMENTS.** The authors thank Dr. K. Fujisaki, National Institute of Animal Health, Tsukuba, Japan, for providing Fukushima stock and DNA from Ikeda stock, and Dr. M. Tanaka, Kyoto Biken Laboratories, Uji, Kyoto, for Ts-U and Ts-R primers. This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan, and by a grant for scientific research from Bayer Japan Ltd..

## REFERENCES

1. Beak, B. K., Kim, B. S., and 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).
2. Beak, B. K., Song, H. J., Kim, B. S., and 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).
3. Fujisaki, K., Ito, Y., Kamio, T., and Kitaoka, S. 1985. The presence of *Theileria sergenti* in *Haemaphysalis longicornis* overwintering in pasture in Japan. *Ann. Trop. Med. Parasitol.* 79: 519-524.
4. Kawazu, S., Sugimoto, C., Kamio, T., and Fujisaki, K. 1992. 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.
5. Kawazu, S., Sugimoto, C., Kamio, T., and Fujisaki, K. 1992. 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.
6. Kobayashi, N., Onuma, M., Kirisawa, R., Ohgitani, T., Takahashi, K., Sasaki, N., and Kawakami, Y. 1987. Monoclonal antibodies against intraerythrocytic merozoites (piroplasma) of *Theileria sergenti*. *Jpn. J. Vet. Sci.* 49: 697-702.
7. Matsuba, T., Kawakami, Y., Iwai, H., and Onuma, M. 1992. Genomic analysis of *Theileria sergenti* stocks in Japan with DNA probes. *Vet. Parasitol.* 41: 35-43.
8. Matsuba, T., Kubota, H., Tanaka, M., Hattori, M., Murata, M., Sugimoto, C., and Onuma, M. 1993a. Analysis of mixed parasite populations of *Theileria sergenti* using cDNA probes encoding a major piroplasm surface protein. *Parasitology* 107: 369-377.
9. Matsuba, T., Sugimoto, C., Onoe, S., Kawakami, Y., Iwai, H., and Onuma, M. 1993b. Changes in the hybridization patterns of populations of *Theileria sergenti* during infection. *Vet. Parasitol.* 47: 215-223.
10. Minami, T., Fujinaga, T., Furuya, K., and 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.
11. Ohgitani, T., Okabe, T., and Sasaki, N. 1987. Antigenic properties of *Theileria sergenti* in ELISA serodiagnosis. *Jpn. J. Vet. Sci.* 49: 531-534.
12. Shirakata, S., Onuma, M., Kirisawa, R., Takahashi, K., and Kawakami, Y. 1989. Localization of surface antigens on *Theileria sergenti* merozoite by monoclonal antibodies. *Jpn. J. Vet. Sci.* 51: 831-833.
13. Stewart, N. P., DeVos, A. J., Shiels, I., and McGregor, W. 1987. The experimental transmission of *Theileria buffeli* of cattle in Australia by *Haemaphysalis humerosa*. *Aust. Vet. J.* 64: 81-83.
14. Tanaka, M., Ohgitani, T., Okabe, T., Kawamoto, S., Takahashi, K., Onuma, M., Kawakami, Y., and Sasaki, N. 1990. Protective effects against intraerythrocytic merozoites of *Theileria sergenti* infection in calves by passive transfer of monoclonal antibodies. *Jpn. J. Vet. Sci.* 52: 643-645.
15. Tanaka, M., Onoe, S., Matsuba, T., Katayama, S., Yamanaka, M., Yonemichi, H., Hiramatsu, K., Beak, B. K., Sugimoto, C., and 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.
16. Zhuang, W. Z., Kubota, S., Sugimoto, C., and Onuma, M. 1993. Characterization of epitopes on a 32 kDa merozoite surface protein of *Theileria sergenti*. *Parasite Immunol.* 15: 113-119.
17. Zhuang, W. Z., Sugimoto, C., Matsuba, T., Niinuma, S., Murata, M., and Onuma, M. 1994. Analysis of antigenic and genetic diversities of *Theileria sergenti* piroplasm surface proteins. *J. Vet. Med. Sci.* 56: 469-473.