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Analysis of Immunodominant Piroplasm Surface Protein Genes of Thai *Theileria* Parasites

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ABSTRACT

The genes encoding for immunodominant piroplasm proteins 32 and 33/34 kilodalton (p32 and p33/34) were used as markers for benign *Theileria* species. These genes yield the 875 and 868 bp fragments using polymerase chain reaction (PCR) amplification. Three unclassified *Theileria* parasites, named as *Theileria* sp. (Thung Song), *Theileria* sp. (Kamphaeng Saen) and *Theileria* sp. (Ban Pong), were collected from different geographical area of Thailand. Genomic DNAs of these 3 parasites were analyzed by PCR using 2 different sets of primers which could amplify 875 bp and 868 bp fragments. The data suggested that *T.* sp. (Ban Pong) showed positive PCR fragments of 875 bp (p32) and 868 bp (p33/34). *Theileria* sp. (Thung Song) DNA was amplified by only one primer set which could amplify *T. sergenti* p32 with no detectable amplification of p33/34 gene. In contrast *T.* sp. (Kamphaeng Saen) showed no amplification fragments using either set of primers. Antigenic comparison between *T.* sp. (Ban Pong and Thung Song) and benign *Theileria* parasites (*T. sergenti* and *T. buffeli*) revealed the antigenic cross-reactivity of Thai *Theileria* parasites to *T. sergenti* and *T. buffeli*. The data indecated that benign *Theileria*, genetically and antigenically similar to *T. sergenti* and *T. buffeli*, are distributed in Thailand.

INTRODUCTION

Theileria are tick-transmitted protozoan parasites that infect both Bovidae and domestic animals throughout much of the world (Dolan, 1989). Generally, the parasites frequently cause anemia due to the presence of intraerythrocytic piroplasm in cattle which can induce abortion and mortality in the animals (Uilenberg, 1981). One group of the parasites is the benign *Theileria* which includes many species of veterinary importance. Benign *Theileria* species generally referred to as *T. sergenti, T. buffeli* and *T. orientalis* which are mainly distributed in tropical and subtropical area (Sugimoto et al, 1991; Kawazu et al, 1992; Fujisaki et al. 1994). Basically, the morphologies of benign *Theileria* parasites are indistinguishable under microscopic observation. Hence, this makes it difficult to control the parasites and assess their distributions.

At present, identification of *Theileria* species and their vectors in Thailand are still uncertain. However, it is proposed that benign *Theileria* groups are potentially abundant throughout the country. To clarify the distribution and identity of these parasites, some the preliminary experiments have been undertaken.

Recently, the genes encoding for immunodominant piroplasm surface proteins of 32 (p32) and 33/34 kDa (p33/34) have been reported to be potential markers for benign parasites group (Kawazu et al. 1992; Matsuba et al, 1993; Tanaka et al., 1993). The genes produced 875 and 868 bp fragments upon using polymerase chain reaction (PCR) amplification, respectively. Here, we reported the discrimination of *Theileria* parasites collected from different geographical area of Thailand by using p32 and p33/34 PCR fragments as markers. This paper also presents the results of antigenic comparisons among various sera from naturally *Theileria* infected cattle by indirect fluorescent antibody (IFA) test against two unclassified Thai *Theileria* parasite antigens .

MATERIALS AND METHODS

Parasite isolates

Three unclassified species of *Theileria* parasites named as *T.* sp. (Thung Song), *T.* sp. (Kamphaeng Saen) and *T.* sp. (Ban Pong) were collected from three different geographical districts in Thailand. *T.* sp. (Thung Song) infected blood was obtained from a naturally infected indigeneous cow (*Bos indicus*) at Thung Song district. The parasite was subsequently transferred by intravenously injection into splenectomized normal calves. The percent of infection was followed until the number of parasites reach the highest peak. The blood were collected and parasites were isolated as indicated below. *T.* sp. (Kamphaeng Saen) was isolated from naturally *Theileria* infected splenectomized male Holstein

Friesian calf which was purchased from Kamphaeng Saen district. The calf was splenectomized to aid parasite growth. *T.* sp. (Ban Pong) was isolated from naturally *Theileria*-infected blood collected from Holstein Friesian cow at Ban Pong district. Parasite DNA was purified as described below.

Isolation of Parasites and Preparation of DNA

Five hundred mls of high peak parasitemia *Theileria* infected blood from each of the infected animals were collected in sterile blood bag containing 7 mg/ml of EDTA as an anti-coagulant agent. The infected and uninfected erythrocytes were washed in phosphate-buffer saline, pH 7.0 (PBS; 0.137 M NaCl, 10 mM Na_2HPO_4, 3.2 mM KH_2PO_4) and centrifuged at 3,000 x g for 10 min. After centrifugation, the buffy coat layer was removed as fully as possible. This washing and centrifugation were repeated until no buffy coat was observed. The remained packed red blood cell were resuspended in PBS buffer to the original volume before application through CF11 column chromatography to eliminate the remaining leukocytes and blood platelets. The eluents containing RBC were collected and lysed by adding 0.25 % NaCl for 30 min on ice followed by centrifugation at 12,000 rpm for 10 min. The supernatant was discarded and the parasite-enriched pellet was washed 3 times with PBS buffer, pH 7.0 prior to digestion in TE buffer pH 8.0 in the presence of proteinase K (0.3 mg/ml) at 37°C for 12-14 hours. The lysates were further extracted with an equal volume of phenol-chloroform and chloroform. The DNA in the aqueous phase was precipitated with 2 volumes of cold absolute ethanol, then centrifuged at 10,000 x g for 10 min. The pellet was air dried and redissolved in TE buffer.

PCR amplification of p32 and p33/34 genes

PCR amplification was performed in a 50 ul reaction mixture containing 10 mM Tris-HCl, pH 8.3; 50 mM KCl; 1.5 mM $MgCl_2$; 0.001% gelatine; 200 uM each of the four dNTPs; 0.5 uM each of the oligonucleotide primers; 2.5 units of *Taq* (*Thermus aquaticus*) DNA polymerase (Perkin-Elmer Norwalk, Conn). Two sets of primers were used for PCR amplifications of the parasite DNAs. First set comprised of 5'-CACGCTATGTTGTCCAAGAG-3' and 5'-TGTGAGACTCAA TGCGCCTA-3' synthesized oligonucleotides as sense and anti-sense primers. Both were used to amplify the gene encoding for 32-kDa intraerythrocytic piroplasm surface protein (p32) of *T. sergenti* (Tanaka et al. 1993). Second set consisted of 5'-TATGTTGTCCAAGAGATCGT-3' and 5'-TGAGACTCAGTG CGCCTAGA-3' oligonucleotides. The primers amplified the genes encoding 33/34 kilodalton major piroplasm antigen (p33/34) of *T. sergenti* and *T. buffeli* (Kawazu et al. 1992). Template DNA added to the reactions were 100 ng for each reaction mixtures and PCR were proceeded in an automatic DNA thermal cycler

(Perkin-Elmer) for 30 cycles. Each cycle consisted of 1 min of denaturation at 95^{0}C, 1 min of annealing at 55^{0}C. and 1 min of polymerization at 73 ^{0}C, with an additional 3 min at 73^{0}C after the last cycle. The amplification reaction mixtures were applied to a 1.5% agarose gel and stained with ethidium bromide after electrophoretic separation.

IFA Test

The piroplasm antigens of the *Theileria* parasites for the IFA test were modified from the methods described by Burridge and Kimber (1973). One antigen was prepared from the parasitized erythrocytes of the splenectomized calves experimentally infected with the *T.* sp. (Thung Song). The other was obtained from naturally *T.* sp. (Ban Pong) infected dairy cow. Thin smear of the washed erythrocytes were made on glass slides, air-dried and fixed in cold acetone for 10 minutes. The positive control sera against *T. buffeli, T. mutans, T. parva* and *T. sergenti* were kindly provided by Dr. S. B. Morzaria, International Livestock Research Institute (ILRI), Nairobi, Kenya and Dr. T. Kamio, National Institute of Animal Health, Tsukuba, Ibaraki, Japan. The conjugate used in this test was rabbit anti-bovine IgG linked to fluorescein isothiocyanate at the dilution of 1:100 in PBS.

RESULTS

Specific amplification of p32 and p33/34 genes of three unclassified *Theileria* parasites and control parasites DNAs (*T. sergenti* and *T. buffeli*) were achieved by using PCR. Thai *Theileria* parasites showed 3 different amplified products as shown in Table 1. *T.* sp. (Ban Pong) genomic DNA was amplified with 2 sets of primers which can amplify *T. sergenti* and *T. buffeli*. *T.* sp. (Thung Song) produced only 875 bp (p32) PCR fragment but not 868 bp (p33/34) PCR fragment. In contrast, neither 875 bp (p32) nor 868 bp (p33/34) PCR fragments were observed from *T.* sp (Kamphaeng Saen).

The relative specificity of *T.* sp. (Thung Song) and *T.* sp. (Ban Pong) piroplasm antigens were tested against other *Theileria* parasites by using IFA test (Table 2). The results showed cross-reactivity of the piroplasm antigens of *T.* sp. (Thung Song) and *T.* sp. (Ban Pong) with sera to *T. sergenti* and *T. buffeli* but negative to *T. mutans* and *T. parva*. However, the piroplasm antigen of *T.* sp. (Thung Song) could not detect serum antibody against *T. buffeli* at higher dilution (>1: 64).

The two antigens (Thung Song & Ban Pong) were also tested by IFA test against 61 of *Theileria*-positive-blood smear samples. The data revealed that 45.9 and 80.3 % of samples showed positive at 1:16 or higher dilution of IFA titer to the piroplasm antigens of *T.* sp. (Thung Song) and *T.* sp. (Ban Pong), respectively.

Table 1. Amplification of immunodominant piroplasm protein (p32, p33/34) genes of Thai *Theileria* parasites by PCR

Theileria parasites	Genes of	
	p32	p33/34
T. sp. (Ban Pong)	+	+
T. sp. (Thung Song)	+	-
T. sp. (Kamphaeng Saen)	-	-
T. sergenti (Chitose)	+	+
T. buffeli (Warwick)	+	+

+ : Amplified products of either 875 bp for p32 or 868 bp for p33/34 or both could be detected.

Table 2. Cross reactivity of Thai *Theileria* parasites to *T. sergenti, T. buffeli, T. mutans* and *T. parva* antisera

	Antisera against							
	T. sergenti			*T. buffeli*			*T. mutans*	*T. parva*
	1:16	1:32	1:64	1:16	1:32	1:64	1:16	1:16
T. sp. (Thung Song)	++	++	++	+	±	-	-	-
T. sp. (Ban Pong)	++++	+++	++	++++	++++	+++	-	-

Piroplasm antigens of Thai *Theileria* parasites were reacted with a 1:16 to 1:64 dilution of antisera against different species of *Theileria parasites.*
++++ to + : Strength of reactivity

DISCUSSION

In this study, we demonstrated the differences among three unclassified Thai *Theileria* species and other *Theileria* species based on the p32 and the p33/34 marker genes and the antigenic specificity. Both PCR and IFA test were correlated with each other such that *T.* sp. (Ban Pong), *T.* sp. (Thung Song) consisted of different immunodominant piroplasm surface proteins. According to the data, it is possible that *T.* sp.(Ban Pong) may contain two species of parasites, *T.sergenti* and *T. buffeli* whereas *T.* sp. (Thung Song) has only one species of parasite, *T. sergenti*.

In order to differentiate mixed *Theileria* parasite populations, we developed the allele-specific PCR method (Kubota et al, 1995,1996). Using this PCR method,

we tested the distribution of benign *Theileria* parasites in eastern Asian countries. Most of the isolates were mixed parasites with different allelic form of parasites. (Wang et al, 1997; Kakuda et al, 1997; Sugimoto et al, 1997). Some *Theileria* parasite samples in Thailand have also been preliminary tested by allele-specific PCR and revealed that parasites in Thailand contained C type of *T. sergenti, T. buffeli* and also *T.* sp (Kamphaeng Saen) type.

From the result of negative PCR amplification of *T.* sp. (Kamphaeng Saen), this parasite may not contain the p32 and p33/34 as the major immunodominant piroplasm surface proteins. In our preliminary experiments, we can amplify *T.* sp.(Kamphaeng Saen) by using a special primer set which cannot amplify both *T. sergenti* and *T. buffeli*. Thus, this *Theileria* parasite may differ from other benign *Theileria* parasites. Comparison of nucleotide sequences and distribution of this type of parasite are now in progress.

In summary, it could be concluded that the mixed populations of *Theileria* parasites occur in Thailand. Hence, the only PCR detection using specific primers for p32 and p33/34 genes and IFA test may not be sufficient in identification of *Theileria* infection in this country. The determination of small and large subunit ribosomal DNA, the house-keeping genes as well as spacer sequences should be the alternative targets for studying and designing the potential primers for detection of mixed populations of *Theileria* parasites.

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