J. Protozool. Res., 9, 103 - 112 (1999)

Susceptibility of *Theileria sergenti* Infection in Holstein Cattle Compared to Korean Native Cattle on Cheju Island

GON-HYUNG KIM[1)], KYOUNG-KAP LEE[1)] AND MISAO ONUMA[2)]

[1] *Department of Veterinary Medicine, Cheju National University, Cheju, 690-756, Korea,*
[2] *Laboratory of Infectious Diseases, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060, Japan*

Received 9 August 1998 / Accepted 26 September 1999

Key Words: *Theileria sergenti;* Holstein cow; Korean native cattle; Hematological values; Cheju.

ABSTRACT

Equal numbers of grazing Holstein, Korean native and crossbreed cattle on Cheju island (South Korea), totaling 195 were examined for *Theileria sergenti* infection and parasitemia levels by microscopic examination of Giemsa-stained blood smears and the polymerase chain reaction (PCR). Comparatively the positive rates of *T. sergenti* infection as well as the parasitemia levels were higher in Holstein cattle than both the Korean native and crossbreed cattle. Positive rates and parasitemia of Holstein cows increased continuously during the grazing period. These results suggest that Holstein cattle showed more sensitive to *T. sergenti* infection than the Korean native cattle and crossbreed cattle.

INTRODUCTION

Bovine theileriosis is an infectious disease that is transmitted principally by ticks, and characterized by severe anorexia, fever, anemia, and icterus, a consequence of piroplasms replicating in erythrocytes. Animals that survive theileriosis generally become low-level carriers of the parasite and serve as a reservoir host for transmission. There are many methods available to diagnose bovine theileriosis. The microscopic examination of Giemsa-stained blood smears for the diagnosis of theileriosis is still useful, popular and sustainable method in the field. But *Theileria* in red blood cells is detectable only during the acute stage and can not be detected during the low-level parasiting stage. Many immunodiagnostic tests, such as indirect fluorescent antibody (IFA) test and

ELISA have been developed for the detection of antibodies to *Theileria* sp. and *Babesia* sp. Additionally molecular biologic techniques such as PCR have been used recently to detect parasite DNA (Figueroa and Buening 1995; Bose et al. 1995). The PCR technique is a very powerful tool for the diagnosis of theileriosis (Kawazu et al. 1995; Bishop et al. 1992). The PCR method is sensitive enough to detect 0.5 pg of purified *T. sergenti* DNA in 10 ml, which corresponds to about 45 parasites in 10 ml of blood (Tanaka et al. 1993).

Bovine theileriosis caused by *T. sergenti* is one of the most economically important disease of cattle on Cheju island (Kim et al. 1993). Cheju island lies 96 km from the southern tip of the Korean peninsula. This island has a wet and subtropical climate. Many ticks species including *Haemaphysalis* sp., *Boophilus* sp. have been identified on Cheju island (Moon and Kim 1987), however *T. sergenti* has been shown to be predominantly transmitted by *Haemaphysalis longicornis*. In Korea, there are no paper describing the susceptibility to *T. sergenti* infection among different breeds of cattle. Recent evidences by using PCR technique showed that most field isolates consisted of mixed parasite populations (Kubota et al. 1995). To differentiate parasite populations bearing 3 allelic forms of major piroplasm surface antigen (p32/34) of *T. sergenti/buffeli*, 3 sets of oligonucleotide primers were designed to amplify each of 3 allele by PCR (Kubota et al. 1995; 1996).

The purposes of this study was to investigate the infection rates to *T. sergenti* infection in Holstein cattle, Korean native cattle and crossbreed cattle, and the changes of hematological values in Holstein cows during the grazing season.

MATERIALS AND METHODS

Experimental animals

Blood samples were collected by jugular venepuncture from equal numbers of clinically healthy Holstein cattle, Korean native cattle and crossbreed cattle, totaling 120 cattle over seven months old (Group 1, 3, 5) and 75 calves under seven months old (Group 2, 4, 6). All experimental animals were born and raised on Cheju island. In March, before grazing, *T. sergenti* infection rates of three breeds over seven months old were between 60 to 70% with Korean native cattle having the lowest rate. Those of under seven months old were from 40 to 60% with Holstein cattle having the lowest rate. After microscopic examination, blood samples were kept frozen at -20°C until DNA was extracted. In May, the early grazing season, the microscopic examination results of Giemsa-stained blood smears were compared with those obtained by the PCR method. *T. sergenti*-infected bovine blood which was used as positive control was obtained from the National Veterinary Research Institute, Korea.

Blood examination

The parameters which were included white blood cell (WBC) counts, red blood cell (RBC) counts and packed cell volume (PCV) were determined by an automatic blood cell counter (Coulter Electric Co., USA). Fibrinogen and total protein were measured by refractometer (AO spencer, USA). Parasitemia was determined by counting infected erythrocyte per 1,000 RBCs under microscopic examination of Giemsa-stained blood smears (×1,000).

DNA isolation

DNA extraction from frozen blood samples was done as described by Figueroa et al. (1992) and Calder et al. (1996). Briefly, 1 ml of frozen blood samples were thawed at room temperature before centrifuging 12,000 rpm for 10 min at 4°C. The pellet was resuspended in 1 ml of TE buffer (0.1 M Tris-HCl [pH 8.0], 10 mM EDTA), and centrifuged. Subsequently the pellet was resuspended in 0.47 ml of 1×SSCE (0.15 M NaCl, 15 mM Trisodium citrate, 1 mM EDTA), and 30 ml of 20% sodium dodecyl sulfate plus 10 ml of proteinase K solution were added. After overnight incubation at 37°C, *T. sergenti* DNA was purified by standard phenol-chloroform extraction and ethanol precipitation.

DNA amplification by PCR

PCR were carried out in a 25 ml reaction mixture containing 10 ng of templated genomic DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 200 mM each of the four dNTPs, one unit of Taq DNA polymerase and 0.5 mM each of the oligonucleotide primers, forward primer; 5'-TAT GTT GTC CAA GAG ATC GT-3' and reverse primer; 5'-TGA GAC TCA GTG CGC CTA GA-3' (Kawazu et al. 1992). For allele-specific PCR, three sets of oligonucleotide primers were designed to amplify each of three alleles (C, I and B types) (Kubota et al. 1995; 1996). PCR was carried out using an automatic DNA thermal cycler (Perkin-Elmer Co., USA) for 30 cycles. Each cycle consisted of 20 sec of denaturation at 94°C, 20 sec of annealing at 58°C, and 80 sec of polymerization at 72°C, with an additional 10 min at 72°C after the last cycle. Amplification products were analyzed by electrophoresis in 1.5% agar gels and detected by UV illuminator, after ethidium bromide staining.

Statistic analysis

Data was analyzed statistically by paired and unpaired *t*-test.

RESULTS

In May, the early grazing period, the infection rate of *Theileria* parasites in Holstein, Korean native and crossbreed cattle were examined by both PCR and microscopic examination. By PCR, an expected 870 bp fragment was observed in

the positive samples (Fig. 1). In the microscopic examination of Giemsa-stained blood smears, Theileria parasites were detected in erythrocytes (Fig. 2).

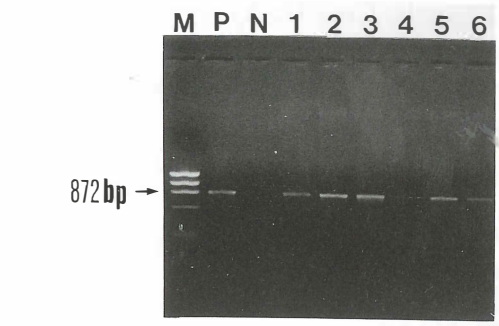

Figure 1. Agarose gel electrophoretic patterns of PCR amplification from blood samples. M; size marker, P; positive control, N; negative control, Lanes 1-6; Holstein cows samples.

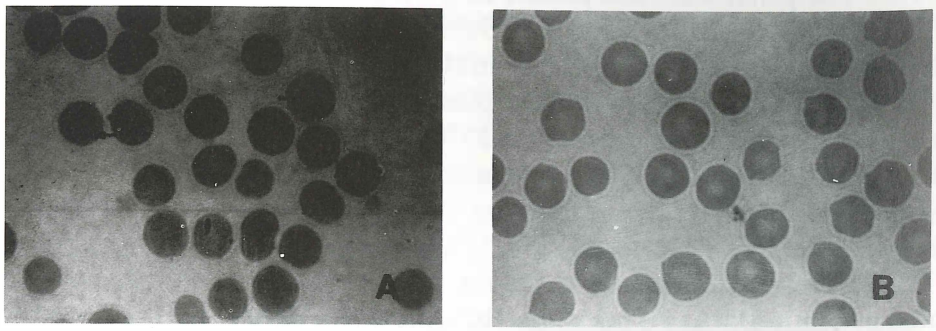

Figure 2. Intraerythrocytic forms of *T. sergenti*. Giemsa-stained blood smear. A; positive result, B; negative result.

Table 1. Comparison of *T. sergenti* infection rates in different cattle breeds by the PCR method and the microscopic examination.

Breeds	Number of Head	Positive rates (%)	
		Microscopy	PCR
Holstein	65		
Group 1	40	87.5	92.5
Group 2	25	84.0	88.0
Korean native	65		
Group 3	40	67.5	75.0
Group 4	25	64.0	72.0
Crossbreed	65		
Group 5	40	80.0	85.0
Group 6	25	76.0	84.0

Groups 1, 3, 5; cattle over seven months old, Groups 2, 4, 6; calves under seven months old.

Table 2. Changes of RBC and PCV according to rates of *T. sergenti* parasitized erythrocytes.

Breeds	No. of positive	% of parasitized RBC			
		>1%	0.9-0.6%	0.5-0.1%	<0.1%
Holstein					
Group 1	37	0	5	18	14
RBC (10^4/ml)		-	498±55[a]	568±76	616±78
PCV (%)		-	27±4	30±2	33±3
Group 2	22	4	4	2	12
RBC (10^4/ml)		513±29	568±41	660±25	736±87
PCV (%)		25±2	28±2	31±1	33±4
Korean native					
Group 3	30	0	2	9	19
RBC (10^4/ml)		-	630±95	744±78	799±98
PCV (%)		-	34±1	38±2	41±3
Group 4	18	0	1	4	13
RBC (10^4/ml)		-	605	752±202	905±133
PCV (%)		-	26	37±7	43±5
Crossbreed					
Group 5	34	0	3	10	21
RBC (10^4/ml)		-	551±62	683±109	755±108
PCV (%)		-	29±3	34±3	37±4
Group 6	21	1	2	5	13
RBC (104/ml)		613	692±26	738±34	797±60
PCV (%)		31	35±0	38±2	41±4

RBC counts and PCV in all groups decreased significantly as parasitemia progressed ($p<0.05$). [a] mean±SD.

As shown in Table 1, both PCR and microscopic examination showed that Holstein cattle had the highest infection rate when compared to both Korean native or crossbreed cattle. To determine the allelic form of p32/34 of *Theileria* parasites, allele-specific PCR was performed. All the positive samples tested contained mixed parasite populations of *T. sergenti* C and I types.

Table 3. Hematological values of cattle according to breeds.

Breeds		No. of head	RBC (10^4/ml)	WBC (ml)	PCV (%)	Fibrinogen (mg/dl)	Total protein (g/dl)
Holstein		65					
Group 1	P^F	37	573 ± 88^b	$10{,}014\pm2{,}585^a$	31 ± 3^d	432 ± 186	7.1 ± 0.6
	N^f	3	760 ± 55	$9{,}267\pm902$	39 ± 2	267 ± 115	7.4 ± 0.4
Group 2	P	22	665 ± 120^c	$9{,}501\pm3{,}287$	31 ± 4^e	391 ± 144	6.3 ± 0.8
	N	3	822 ± 44	$10{,}233\pm802$	36 ± 1	400 ± 200	7.0 ± 0.2
Korean native		65					
Group 3	P	30	772 ± 100^B	$10{,}750\pm3{,}255$	40 ± 4^D	470 ± 176	7.1 ± 0.5
	N	10	871 ± 84	$10{,}870\pm2{,}953$	42 ± 2	520 ± 140	6.9 ± 0.3
Group 4	P	18	858 ± 167^C	$8{,}338\pm1{,}728$	41 ± 7^E	389 ± 175	6.6 ± 0.5
	N	7	$1{,}045\pm108$	$7{,}270\pm2{,}358$	47 ± 5	360 ± 126	6.5 ± 0.4
Crossbreed		65					
Group 5	P	34	713 ± 130^B	$13{,}623\pm3{,}024$	36 ± 4^D	450 ± 148	6.6 ± 0.6
	N	6	783 ± 108	$13{,}907\pm3{,}429$	37 ± 6	500 ± 109	6.3 ± 0.2
Group 6	P	21	725 ± 92^C	$9{,}392\pm2{,}356$	40 ± 5^E	431 ± 138	6.1 ± 0.6
	N	4	909 ± 19	$8{,}900\pm1{,}293$	47 ± 3	350 ± 100	6.4 ± 0.6

B:b, C:c, D:d, E:e RBC counts and PCV of *T. sergenti* infected Holstein cattle were significantly lower than those of other breeds ($p<0.01$). F:f RBC counts and PCV of *T. sergenti* infected cows (P) were significantly lower than those of non-infected cattle (N) in all groups ($p<0.05$). P; positive, N; negative. All values represent mean±SD.

All the parasites infected cattle were classified according to the rates of parasitized erythrocytes and compared the total number of RBC and PCV among 3 breeds (Table 2). The number of positive cattle among Holstein, Korean native and crossbreed cattle were 37, 30 and 34, respectively. RBC counts and PCV in all groups decreased significantly as parasitemia progressed ($p<0.05$).

The RBC counts and PCV in parasite infected Holstein were lower when compared to those of the other 2 breeds ($p<0.01$). In all 6 groups, RBC counts and PCV were significantly lower in the infected cattle ($p<0.05$), however WBC counts, fibrinogen and total proteins were not significantly different between infected and non-infected cattle (Table 3).

Table 4. Hematological values of Holstein cattle (Group 1) during grazing period.

Months	RBC (10^4/ml)	WBC (ml)	PCV (%)	Fibrinogen (mg/dl)	Total protein (g/dl)
May	587±99	9,958±2,499	31±4	420±186	7.1±0.6
July	538±96	10,993±2,529	29±3	463±168	7.2±0.6
September	402±82	12,278±3,848	26±4	328±130	7.2±0.7

RBC counts and PCV significantly decreased from May to September ($p<0.05$). All values represent mean±SD.

Table 5. Changes of RBC and PCV according to rates of *T. sergenti* parasitized erythrocytes in Holstein cattle (Group 1) during grazing period.

Months	No. of positive	% of parasitized RBC			
		>1%	0.9-0.6%	0.5-0.1%	<0.1%
May	37	0	5	18	14
RBC (10^4/ml)		-	498±55	568±76	616±78
PCV (%)		-	27±4	30±2	33±3
July	38	1	7	17	13
RBC (10^4/ml)		405	443±55	546±76	557±71
PCV (%)		24	27±2	28±3	32±3
September	40	6	14	19	1
RBC (10^4/ml)		328±51	375±68	443±78	458
PCV (%)		23±3	26±4	28±4	32

RBC counts and PCV decreased significantly as parasitemia progressed ($p<0.05$). All values represent mean±SD.

During the grazing period between July to September, Group 1 of Holstein cattle were followed parasitemia levels and hematological values and compared with the results of May (Table 4 and 5). From May to September, hematological values such as RBC counts and PCV continued to decrease in Group 1 Holstein cattle, however WBC counts, fibrinogen and total protein were not significant changed (Table 4). The number of infected cattle and percentage of parasitized RBC continuously increased during the grazing period as shown in Table 5.

DISCUSSION

Cattle susceptibilities to theileriosis and babesiosis are thought to be different according to breeds. Terada et al. (1995) reported the susceptibility of the two breeds under experimentally controlled condition without external factor that Japanese black cattle showed a more solid resistance to *T. sergenti* infection than that of Holstein cattle. Under natural condition in the present experimental, we found the similar results that Korean native cattle was more resistance to *T. sergenti* infection when compare to Holstein cattle.

In the present experiment, most cattle on Cheju island were infected with *T. sergenti,* however severe clinical conditions caused by *T. sergenti* were rare. Holstein cattle showed higher parasitemia and lower hematological value than the other breeds. These results suggest that Holstein cattle showed more sensitivity to *T. sergenti* infection than the other breeds. It has been previously documented that milk production of Holstein cows continuously decreased from June to October (Yang et al. 1989). Moreover, Kim et al. (1983; 1984) showed that Holstein cows infected with *T. sergenti* produced lower milk production. It is therefore thought that the decrease in the milk production of the Holstein cows in Cheju island may be closely associated with high infection rate of *T. sergenti.*

In majority of *T. sergenti* infected calves in Japan and Korea presented in mixed parasite populations bearing I and C type parasites (Kubota et al. 1995; Kakuda et al. 1998). In the present experiment, we tested the typing of Cheju isolates by using 3 sets of primers (for I, C and B types) and found that all isolates tested showed mixed populations of I and C type parasites, similar to that of previous report (Kakuda et al. 1998). It has been believed that I type parasite is more pathogenic than C and B type parasites (Onuma et al. 1998). The most effective way to control theileriosis is through the elimination of ticks, but tick eradication is impractical in most areas. Therefore, the best approach is to raise Holstein cows with better nutrition and in a better environment as well as vaccination using cocktail of I and C type parasites (Onuma et al. 1998).

REFERENCES

Bishop, R., Sohanpal, B., Kariuki, D. P., Young, A. S., Nene, V., Baylis, H., Allsopp, B. A., Spooner, P. R., Dolan, T. T. & Morzaria, S. P. 1992. Detection of a carrier state in *Theileria parva*-infected cattle by the polymerase chain reaction. *Parasitology 104:* 215-232.

Bose, R., Jorgensen, W. K., Dalgliesh, R. J., Friedhoff, K. T. & de Vos, A. J. 1995. Current state and future trends in the diagnosis of babesiosis. *Vet. Parasitol. 57:* 61-74.

Calder, J. A. M., Reddy, G. R., Chieves, L., Courtney, C. H., Littell, R., Livengood, J. R., Norval, R. A. I., Smith, C. & Dame, J. B. 1996. Monitoring *Babesia bovis* infections in cattle by using PCR-based tests. *J. Clin. Microbiol. 34:* 2748-2755.

Figueroa, J. V., Chieves, L. P., Johnson, G. S. & Buening, G. M. 1992. Detection of *Babesia bigemina*-infected carriers by polymerase chain reaction amplification. *J. Clin. Microbiol. 30:* 2576-2582.

Figueroa, J. V. & Buening, G. M. 1995. Nucleic acid probes as a diagnostic method for tick-borne hemoparasites of veterinary importance. *Vet. Parasitol. 57:* 75-92.

Kakuda, T., Kubota, S., Sugimoto, C., Baek, B. K. & Yin, H. 1998. Analysis of immunodominant piroplasm surface protein genes of benign *Theileria* parasites distributed in China and Korea by allele-specific polymerase chain reaction. *J. Vet. Med. Sci. 60:* 237-239.

Kawazu, S., Sugimoto, C., Kamio, T. & 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:* 169-175.

Kim, D. C., Oh, W. Y., Kim, H. S., Hyun, K. J. & Kang, Y. B. 1993. Studies on the adaptability of Korean native cattle in Cheju island. *R. I. Anim. Sci. 8:* 29-35.

Kim, I. C. & Son, J. Y. 1984. Studies on the change of blood values and daily milk yield after parturition on Holstein cows infected with *Theileria sergenti* in a farm where tick population is sparse. *Korean J. Anim. Sci. 25:* 464-469.

Kim, I. C. & Son, J. Y. 1984. Studies on the change of blood values and daily milk yield after parturition on Holstein cows infected with *Theileria sergenti* in a farm where tick population is dense. *Korean J. Anim. Sci. 26:* 137-144.

Kubota, S., Sugimoto, C. & Onuma, M. 1995. A genetic analysis of mixed population in *Theileria sergenti* stocks and isolates using allele-specific polymerase chain reaction. *J. Vet. Med. Sci. 57:* 279-282.

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

Moon, C. R. & Kim, S. H. 1987. Studies on the control of ticks and tick-borne diseases in exotic cattle in Cheju-Do; 1) Survey on the monthly fluctuation and population density of ticks on pasture in Cheju-Do. *R. I. Animal Sci. 1:* 31-35.

Onuma, M., Kakuda, T. & Sugimoto, C. 1998. *Theileria* parasite infection in East Asia and control of the disease. *Comp. Immunol. Microbiol. Dis. 21:* 165-

177.

Tanaka, M., Onoe, S., Matsuba, T., Katayama, S., Yamanaka, M., Yonemichi, H., Hiramatsu, K., Baek, 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.

Terada, Y., Ischida, M. & Yamanaka, H. 1995. Resistibility to *Theileria sergenti* infection in Holstein and Japanese black cattle. *J. Vet. Med. Sci. 57:* 1003-1006.

Yang, S. J., Lee, H. J., Park, H. S. & Yun, Y. B. 1989. A study on the milk yield and raw milk quality of the Holstein cows in Cheju-Do area. II. Effect of parity, stage of lactation, conditions of feeding and management, calving season and milking season on the milk yield and compositions of raw milk. *Korean J. Anim. Sci. 31:* 114-131.