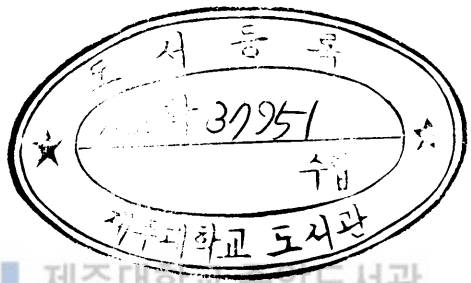
碩士學位論文

Comparison of *Theileria sergenti* Infection Rates in Cattle Breeds on Cheju Island



濟州大學校 大學院

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1998年 12月

濟州地域 소의 品種別 *Theileria sergenti* 感染率 比較

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초 록

濟州地域 소의 品種別 *Theileria sergenti* 感染率 比較

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국 문 초 록

제주지역에서 사육되는 7개월령 이상의 홀스타인, 한우, 교잡우{Charolais(Brahman×Korean native cattle)} 각 40두씩 총 120두와 7개월령 미만의 육성우 각 25두씩 총 75두를 대상으로 Giemsa 염색에 의한 광학현미경적 진단법과 중합효소연쇄반응법(Polymerase Chain Reaction, PCR)을 이용하여 소의 품종별 *Theileria sergenti* 감염률과 감염우의 혈액학치에 대한 연구를 수행하였다. 방목초기인 5월에 7개월령 이상의 소에서 중합효소연쇄반응법에 의한 *Theileria sergenti* 양성률은 홀스타인이 92.5 %로 교잡우 85 %, 한우 75 %보다 높았다. 7개월령 미만의 소에서도 홀스타인이 88 %로 교잡우 84 %, 한우 72 %보다 높았다. 소 품종간 원충 감염률은 홀스타인이 다른 품종에 비하여 높았다. 혈액검사에서는 원충에 감염된 7개월령 이상의 소에서 홀스타인의 적혈구수(RBC)와 적혈구용적(PCV)(573±88 $10^4/\mu\ell$, 31±3 %)은 한우(772±100 $10^4/\mu\ell$, 40±4 %)와 교잡우(713±130 $10^4/\mu\ell$, 36±4 %)보다 낮았다($p<0.01$). 7개월령 미만의 홀스타인의 RBC와 PCV(665±120 $10^4/\mu\ell$, 31±4 %)에서도 한우(858±167 $10^4/\mu\ell$, 41±7 %)와 교잡우(725±92 $10^4/\mu\ell$, 40±5 %)보다 낮았다($p<0.01$). 또한 원충감염이 심해질수

록 모든 품종에서 적혈구수와 적혈구용적은 유의성 있게 낮았다($p<0.05$). 홀스타인에서 방목기간인 5, 7, 9월에 *Theileria sergenti* 양성률과 원충감염률은 계속적으로 증가했으며, 적혈구수(587±99 $10^4/\mu\ell$, 538±96 $10^4/\mu\ell$, 402±82 $10^4/\mu\ell$)와 적혈구용적(31±4 %, 29±3 %, 26±4 %)은 계속적으로 낮아졌다($p<0.05$).

중심어 : 소, 타일레리아, 중합효소연쇄반응, 원충감염률, 혈액학치

Contents

I. Introduction

Bovine theileriasis is an infectious disease that is transmitted principally by ticks, and severe anorexia, fever, anemia, and icterus are caused by piroplasms in the erythrocytes (Minami et al., 1980). Animals that survive theileriasis generally become low-level carriers of the parasite and serve as a reservoir host for transmission. When theileria infected and recovered cattle are under stress, due to such things as transportation, lack of food, or suffering from other disease, they can be in serious danger and occasionally die. Although reservoir hosts are not clinically ill, milk production and weight gain are decreased.

There are many methods available to diagnosis bovine theileriasis. The microscopic examination of Giemsa-stained blood smears for the diagnosis of theileriasis is still useful and popular method in the field. But *T. sergenti* piroplasms in red blood cells are detectable only during the acute stage and can not be detected during the low-level parasiting stage. Many immunodiagnostic tests, such as IFA test and ELISA have been developed for the detection of antibodies to *Theileria sp.* and *Babesia sp.*, and molecular biologic techniques have been used recently (Figueroa and Buening, 1995; Bose R et al., 1995). For the later, the polymerase chain reaction (PCR) technique is a very powerful tool for the diagnosis of theileriasis (Kawazu et al., 1992; Bishop et al., 1992). The PCR method is sensitive enough to detect 0.5pg of purified *T. sergenti* DNA in 10$\mu\ell$, which corresponds to about 45 parasites in 10$\mu\ell$ of blood (Tanaka et al., 1993). In Japan, recent evidences 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 (for I, C and B type) were designed to amplify each of 3 allele by PCR (Kubota et al., 1995; 1996)

In Korea, bovine theileriasis is caused by *T. sergenti* and it is one of the most economically important diseases of cattle on Cheju Island (Kim et al., 1993). This island has a wet and subtropical climate. As such there are many ticks, especially *Haemaphysalis sp.* which causes *T. sergenti* infection (Moon and Kim, 1987). However, though many papers deal with theileriasis of Korean native cows and imported cows, there are few papers about theileriasis of Holstein cattle and other cattle, which have lived in Cheju for many years.

In this paper *T. sergenti* infection rates were investigated by the microscopic examination and the PCR method in Holstein cattle, Korean native cattle. Also changes of hematological data were examined during pasture season in Holstein cows.

II. Materials and Methods

1. Experimental animals

Blood samples were collected by jugular venipucture from equal numbers of clinically healthy Holstein cattle, Korean native cattle (KNC) and crossbreed cattle {Charolais(Brahman × Korean native cattle)}, totaling 120 cows over seven months old (Group 1, 3, 5) and 75 calves under seven months old (Group 2, 4, 6). All of the experimental animals were born and raised on Cheju Island. After the microscopic examination, blood samples were kept frozen at -20℃ until they were used for the PCR method. In May, the early pasture season, the microscopic examination results of Giemsa-stained blood smears were compared with those obtained by the PCR method. *T. sergenti* infected blood as a positive control was obtained from an experimentally infected calf housed in the Department of Infectious Disease, National Veterinary Research and Quarantine Service, Ministry of Agriculture and Forestry.

2. Blood examination

White blood cell (WBC) counts, RBC counts and PCV were determined by an automatic blood cell counter (Coulter Electric co., USA). Fibrinogen and total protein were measured by refractometer (AO spencer, USA). The rate of *T. sergenti* parasitized erythrocytes (‰, permillage) were checked by finding the parasites in 1,000 erythrocytes under the microscopic examination of Giemsa-stained blood smears (× 1,000).

3. DNA isolation

Blood samples of 1㎖ were frozen at -20℃ and were thawed twice at room temperature before centrifuging 12,000 rpm for 10 min at 4℃ (Figueroa et al., 1992; Calder et al., 1996). The pellets were resuspended in 1㎖ of TE buffer (0.1M Tris-HCl[pH 8.0], 10㎜ EDTA), and centrifuged. The pellets were resuspended in 0.47㎖ of 1×SSCE (0.15M NaCl, 15㎜ Trisodium citrate, 1㎜ EDTA), 30㎕ of 20% sodium dodecyl sulfate and 10㎕ of proteinase K solution. After overnight incubation at 37℃, *T. sergenti* DNA was purified by standard phenol-chloroform extraction and ethanol precipitation.

4. DNA amplification by PCR

In the initial reactions, final concentrations of each component in a 25㎕ reaction mixture was 10ng of templated genomic DNA, 10㎜ Tris HCl (pH8.3), 50㎜ KCl, 1.5㎜ $MgCl_2$, 0.001% gelatin, 200㎛ each of the four dNTPs, 1 unit of Taq DNA polymerase and 0.5㎛ 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). The reactions proceeded in an automatic DNA thermal cycler (Perkin-Elmer co., USA) for 30 cycles. Each cycle consisted of 20s of denaturation at 94℃, 20s of annealing at 58℃, and 80s of polymerization at 72℃, with an additional 10min at 72℃ after the last cycle. Amplification products were analyzed by electrophoresis in 1.5% agar gels and detected by UV illuminator, after ethidium bromide staining.

5. Statistics

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

III. Results

In the microscopic examination of Giemsa-stained blood smears, the diagnosis of theileriasis was confirmed by finding the parasites in erythrocytes. Positive results and negative results are shown in Figure 1.

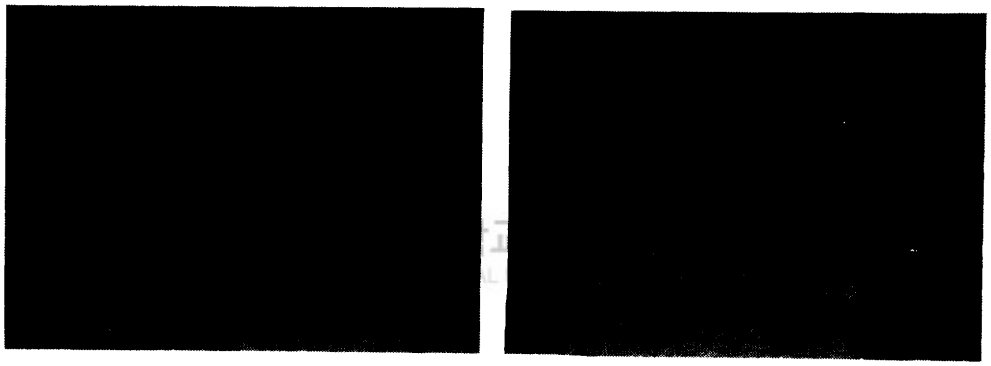

Figure 1. Intraerythrocytic forms of *T. sergenti*. Giemsa-stained blood smear, ×1,000

A; positive result, B; negative result

The ethidium bromide-stained 1.5% agarose gel indicated that the 870-bp band was the only detectable product by the PCR method as in Figure 2.

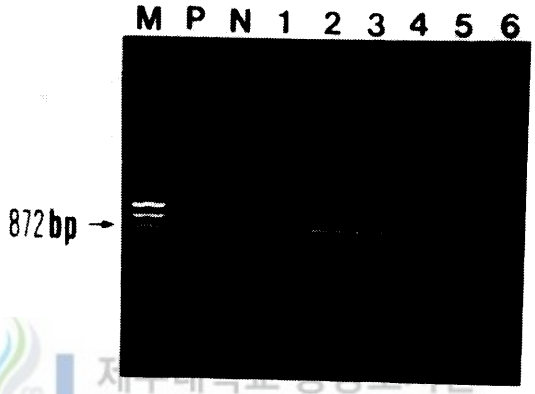

Figure 2. Agarose gel electrophoretic patterns of PCR amplification from blood samples

M; size marker ($\Phi \times 174$ Hae Ⅲ digest), P; positive control,

N; negative control, Line 1~6; Holstein cows samples

Positive rates of *T. sergenti* by the microscopic examination and the PCR method are summarized in Table 1. By the PCR method, Holstein cows tested positive at a higher rate (92.5 %) than did Korean native cows (75.0 %) and crossbreed cows (85.0 %) over 7 months old. Also, Holstein calves tested positive at a higher rate (88.0 %) than did Korean native calves (72.0 %) and crossbreed calves (84.0 %) under 7 months old. The PCR method was more sensitive than the microscopic examination.

Table 1. Comparison of *T. sergenti* Infection Rates in Cattle by the Microscopic Examination and the PCR Method.

Breeds	Number of head	Positive rates (%)	
		Microscopy	PCR
Holstein	65		
Group 1	40	87.5	92.5
Group 2	25	84.0	88.0
KNC	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

Group 1, 3, 5; cows over seven months old

Group 2, 4, 6; calves under seven months old

The rates of *T. sergenti* parasitized erythrocytes in Holstein cows were higher than in other breeds. The number of Holstein cows (23) over 1 ‰ parasitemia was higher than Korean native cows (11) and crossbreed cows (13) over 7 months old. Under 7 months old, the number of Holstein calves (10) was higher than Korean native calves (5) and crossbreed calves (8), and 4 Holstein calves tested at over 10 ‰ parasitemia (Table 2). In all groups, RBC counts and PCV decreased significantly as parasitemia progressed ($p<0.05$).

Table 2. Changes of RBC and PCV according to Rates of *T. sergenti* Parasitized Erythrocytes.

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

[a]; mean±SD

RBC counts and PCV in all groups decreased significantly as parasitemia progressed ($p<0.05$)

In all groups, RBC counts and PCV of *T. sergenti* infected cows were significantly lower than those of noninfected cattle ($p<0.05$) and WBC counts, fibrinogen and total protein were not changed significantly (Table 3). RBC counts and PCV of *T. sergenti* infected Holstein cattle were significantly lower than those of other breeds ($p<0.01$).

Table 3. Hematological Values of Cattle according to Breeds.

Breeds		Number of head	RBC ($10^4/\mu\ell$)	WBC ($/\mu\ell$)	PCV (%)	Fib (mg/dℓ)	TP (g/dℓ)
Holstein		65					
Group 1	P[F]	37	573 ± 88^{b}	10014 ± 2585^{a}	31 ± 3^{d}	432 ± 186	7.1 ± 0.6
	N[f]	3	760 ± 55	9267 ± 902	39 ± 2	267 ± 115	7.4 ± 0.4
Group 2	P	22	665 ± 120^{c}	9501 ± 3287	31 ± 4^{e}	391 ± 144	6.3 ± 0.8
	N	3	822 ± 44	10233 ± 802	36 ± 1	400 ± 200	7.0 ± 0.2
KNC		65					
Group 3	P	30	772 ± 100^{B}	10750 ± 3255	40 ± 4^{D}	470 ± 176	7.1 ± 0.5
	N	10	871 ± 84	10870 ± 2953	42 ± 2	520 ± 140	6.9 ± 0.3
Group 4	P	18	858 ± 167^{C}	8338 ± 1728	41 ± 7^{E}	389 ± 175	6.6 ± 0.5
	N	7	1045 ± 108	7270 ± 2358	47 ± 5	360 ± 126	6.5 ± 0.4
Crossbreed		65					
Group 5	P	34	713 ± 130^{B}	13623 ± 3024	36 ± 4^{D}	450 ± 148	6.6 ± 0.6
	N	6	783 ± 108	13907 ± 3429	37 ± 6	500 ± 109	6.3 ± 0.2
Group 6	P	21	725 ± 92^{C}	9392 ± 2356	40 ± 5^{E}	431 ± 138	6.1 ± 0.6
	N	4	909 ± 19	8900 ± 1293	47 ± 3	350 ± 100	6.4 ± 0.6

[a]; mean ± SD, P; positive result, N; negative result

[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 were significantly lower than those of noninfected cattle in all gruops ($p<0.05$)

During pasture period, parasite detection and hematological tests were performed on Holstein cattle (Group 1) on July and September, and compared with the results of May (Table 4 & 5). The number of positive cattle and rates of parasitized erythrocytes were continuously increased as shown in Table 4. RBC counts and PCV decreased significantly as parasitemia progressed ($p<0.05$).

Table 4. Changes of RBC and PCV according to Rates of *T. sergenti* Parasitized Erythrocytes in Holstein Cattle (Group 1) during Pasture Period.

Months	No. of Positive	‰ of parasitized RBC			
		> 10 ‰	9 ~ 6 ‰	5 ~ 1 ‰	< 1 ‰
May	37	0	5	18	14
RBC ($10^4/\mu\ell$)			498±55[a]	568±76	616±78
PCV (%)			27±4	30±2	33±3
July	38	1	7	17	13
RBC ($10^4/\mu\ell$)		405	443±55	546±76	557±71
PCV (%)		24	27±2	28±3	32±3
September	40	6	14	19	1
RBC ($10^4/\mu\ell$)		328±51	375±68	443±78	458
PCV (%)		23±3	26±4	28±4	32

[a]; mean±SD

RBC counts and PCV in all groups decreased significantly as parasitemia progressed ($p<0.05$)

From May to September, hematological values of Holstein cattle (Group 1) changed as shown in Table 5. RBC counts (587 ± 99 $10^4/\mu\ell$, 538 ± 96 $10^4/\mu\ell$, 402 ± 82 $10^4/\mu\ell$) and PCV (31 ± 4 %, 29 ± 3 %, 26 ± 4 %) continued to decrease significantly ($p<0.05$) and WBC counts, fibrinogen and total protein were not significant changed.

Table 5. Hematological Values of Holstein Cattle (Group 1) during Pasture Period.

Month	RBC ($10^4/\mu\ell$)	WBC ($/\mu\ell$)	PCV (%)	Fib (mg/dℓ)	TP (g/dℓ)
May	587 ± 99^{BC}	9958 ± 2499^{a}	31 ± 4^{DE}	420 ± 186	7.1 ± 0.6
July	538 ± 96^{Bc}	10993 ± 2529	29 ± 3^{De}	463 ± 168	7.2 ± 0.6
September	402 ± 82^{b}	12278 ± 3848	26 ± 4^{d}	328 ± 130	7.2 ± 0.7

[a]; mean $\pm$ SD

[B:b, C:c, D:d, E:e]; RBC counts and PCV significantly decreased from May to September ($p<0.05$)

IV. Discussion

Ticks on Cheju Island are primarily *Heamaphysalis sp.* and there are a few *Boophilus sp.* (Moon and Kim, 1987). Therefore, dogs are infected with babesia (Rhee et al., 1984; Ihn et al., 1991) or hemobartonella. Hemoparasitized horses have not been reported, however some cases with similar signs are reported. Babesiosis, theileriasis and anaplasmosis are reported in cattle on Cheju Island (Suh et al., 1982).

All over the world, bovine theileriasis is caused by *T. parva, T. annulata, T. mutants, T. turotragi, T. velifera, T. buffeli* and *T. sergenti*, while bovine babesiosis is caused by *B. occultans, B. bovis, B. divergens* as small babesia, and *B. bigemina, B. jakimove, B. major, B. ovata* as large babesia (Soulsby, 1982). Theileriasis and babesiosis are infectious diseases that are transmitted by ticks. Severe anorexia, fever, anemia, icterus are caused by the protozoa of tick parasites in the erythrocytes. Susceptibilities to theileriasis and babesiosis are different according to breeds. Terada et al. (1995) reported that Japanese black cattle shows solider resistance to *T. sergenti* infection than the Holstein breed does under experimentally controlled condition without external factors.

In Korea, bovine theileriasis, which is caused by *T. sergenti* and babesiosis which is caused by *B. ovata* have been reported. Studies of morphology (Baek et al., 1990), diagnosis methods, infection rate and treatment and prevention of *T. sergenti* are reported. In studies of *T. sergenti* infection rates, Suh et al. (1982) reported that infection rate of *T. sergenti* in imported cattle is 100% on Cheju Island in July. Kim et al. (1993) reported that the resistance to *T. sergenti* in imported cattle was weak. In studies of diagnosis methods of *T. sergenti*, many available methods are reported such as microscopic

examination of Giemsa-stained blood smears, IFA test and ELISA. For the later, the PCR technique is a very powerful tool for diagnosis of theileriasis. Chae et al. (1996; 1996), Kang et al. (1997) and Choi et al. (1997) reported the results of PCR diagnosis in Korean native cows.

Majority of *T. sergenti* infected calves in Japan and Korea presented mixed parasite populations bearing I and C type parasites (Kubota et al., 1995; Kakuda et al., 1998). Kakuda et al. (1998) reported that the typing of Cheju isolates by using 3 sets of primers (for I, C and B type) showed mixed populations of I and C type parasites. It has been believed that I type parasite is more pathogenic than C and B type parasites (Onuma et al., 1998).

The microscopic examination and the PCR method were used for this study. In the former examination, top quality staining and observation techniques are necessary, and in the later method for the diagnosis of theileriasis, phenol-chloroform extraction should be repeated several times due to the sensitive nature of this study.

When *T. sergenti* infected cattle are under stress or suffering from other disease, they can be in a critical condition. Furthermore, their conditions differ in degree according to breed and age. Holstein cows are usually under stress during milking period and pregnancy. And *T. sergenti* infection rates of Holstein cattle are higher than those of other breeds. Therefore, it was thought that Holstein cattle would have a lower resistance to *T. sergenti* infection than other breeds. Baek et al. (1993) reported that *T. sergenti* infection rates in neonatal calves were about 40 %. Therefore, to clarify the cause of death in calves during the suckling period, it will be necessary to investigate complications with *T. sergenti* infection on Cheju Island.

In studies about the hematological value of Holstein cows, Son et al. (1995) reported that the normal value of the number of erythrocytes and PCV are

572±273 $10^4/\mu\ell$, 31±1 %, and Moon et al. (1974) reported them to be 583±12 $10^4/\mu\ell$, 30±1 %. In May, we reported similar results (587±99 $10^4/\mu\ell$, 31±4 %) in May. In July and September, we reported lower values (538±96 $10^4/\mu\ell$ 29±3 %, 402±82 $10^4/\mu\ell$ 26±4 %) than their data.

During the period of this study, critical conditions caused by only theileriasis were rare in all cattle. But it was thought that Holstein cattle are greater danger than other breeds due to their high parasitemia and lower hematological value. Kim et al. (1983; 1984) reported that Holstein cows infected with *T. sergenti* showed lower milk production. And it is economically significant that in Cheju, milk production decreased continuously from June to October in Holstein cows (Yang et al., 1989).

The reduction rates of RBC counts in Holstein cows were lower than other breeds in spite of higher parasitemia rates. It is likely that this result is caused by the osmotic fragility of erythrocytes. Osmotic fragility of erythrocytes increases in *T. sergenti* infection (Yagi et al., 1989), but that of Holstein cows is lower than that of Korean native cows (Min and Lee, 1990). Lee at al. (1994) reported that the serum vitamin E level of calves with theileriasis was lower than those of healthy calves. Therefore, it is necessary to investigate the relationship between theileriasis and vitamin E.

V. Conclusion

Bovine theileriasis is caused by *T. sergenti* and it is one of the most economically important diseases of cattle on Cheju Island. However, though many papers deal with theileriasis of Korean native cows and imported cows, there are few papers about theileriasis of Holstein cattle and other cattle which have lived in Cheju for many years. Therefore, in this paper *T. sergenti* infection rates were investigated by the microscopic examination and the PCR method in Holstein cattle, Korean native cattle. Also changes of hematological data were examined during pasture season in Holstein cows. In May, the early pasture season, positive results (92.5 %) and parasitemia of Holstein cattle were higher than those (75 %, 85 %) of Korean native cattle and crossbreed cattle. Also, red blood cell (RBC) counts and the packed cell volume (PCV) of Holstein cattle were lower than those of other breeds ($p<0.01$). As parasitemia progresses, RBC counts and PCV decreased significantly in all breeds ($p<0.05$). During pasture period, positive results and parasitemia of Holstein cows increased and RBC counts and PCV decreased continuously ($p<0.05$). We concluded that Holstein cattle are in greater danger than other breeds due to their high parasitemia and lower hematological values.

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Comparison of *Theileria sergenti* Infection Rates in Cattle Breeds on Cheju Island

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Abstract

To study about *Theileria sergenti* infection rates and hematological values in cattle breeds on Cheju Island, equal numbers of Holstein cattle, Korean native cattle and crossbreed cattle {Charolais(Brahman×Korean native cattle)}, totaling 120 cows over seven months old and 75 calves under seven months old, were examined by the microscopic examination of Giemsa-stained blood smears and the polymerase chain reaction (PCR) method. In May, the early pasture season, the positive rate of parasites (92.5 %) in Holstein cattle was higher than those (75 %, 85 %) of Korean native cattle and crossbreed cattle over seven months old. And positive rate (88 %) of Holstein cattle was higher than those (72 %, 84 %) of Korean native cattle and crossbreed cattle under seven months old. In comparison of parasitemia, the result in Holstein cattle was higher than in other breeds. In *T. sergenti* infected cattle, red blood cell

(RBC) counts and the packed cell volume (PCV)(573±88 $10^4/\mu\ell$, 31±3 %) of Holstein cattle were lower than those of Korean native cattle (772±100 $10^4/\mu\ell$, 40±4 %) and crossbreed cattle (713±130 $10^4/\mu\ell$, 36±4 %) over seven months old ($p<0.01$). Also, RBC counts and PCV (665±120 $10^4/\mu\ell$, 31±4 %) of Holstein cattle were lower than those of Korean native cattle (858±167 $10^4/\mu\ell$, 41±7 %) and crossbreed cattle (725±92 $10^4/\mu\ell$, 40±5 %) under seven months old ($p<0.01$). As parasitemia progresses, RBC counts and PCV decreased significantly in all breeds ($p<0.05$). In the other study of hematological values in Holstein cattle during pasture period (May, July and September), positive rate and parasitemia in Holstein cows increased, and RBC counts (587±99 $10^4/\mu\ell$, 538±96 $10^4/\mu\ell$, 402±82 $10^4/\mu\ell$) and PCV (31±4 %, 29±3 %, 26±4 %) decreased continuously ($p<0.05$).

Key Words : Cattle, *Theileria sergenti*, PCR, Parasitemia, Hematological values

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마지막으로 재미없는 신혼기간을 보내면서도 많은 도움을 준 사랑하는 아내 경미에게 이 논문이 조그만 선물이 되었으면 합니다.