A Thesis

For The Degree of Master of Veterinary Medicine

Antigenic Diversity of *Theileria* Major Piroplasm Surface Protein Gene on Korean Native Black Cattle in Jeju

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Graduate School, Cheju National University

Department of Veterinary Medicine

Myung-Soon Ko

2004. 2.

Antigenic Diversity of *Theileria* Major Piroplasm Surface Protein Gene on Korean Native Black Cattle in Jeju

Myung-Soon Ko

(Supervised by Professor Kyoung-Kap Lee)

A thesis submitted in partial fulfillment of the requirement for the degree of Master of Veterinary Medicine

2003. 12.

This thesis has been examined and approved.

____

Thesis director, Ho-Choon Woo, Prof. of Veterinary Medicine

____

Young-Min Yun, Prof. of Veterinary Medicine

____

Kyoung-Kap Lee, Prof. of Veterinary Medicine

2004. 2 .

Department of Veterinary Medicine

GRADUATE SCHOOL

CHEJU NATIONAL UNIVERSITY

Antigenic Diversity of *Theileria* Major Piroplasm Surface Protein Gene on Korean Native Black Cattle in Jeju

Myung-Soon Ko
(Supervised by Professor Kyoung-Kap Lee)

Department of Veterinary Medicine
Graduate School, Cheju National University, Jeju 690-756, Korea

Abstract

The piroplasms, *Theileria* sp. and *Babesia* sp. are both tick-transmitted intracelluar haemoprotozoan parasites and cause anorexia, fever, anemia and icterus. Bovine piropasmosis caused by *T. sergenti* and *Babesia ovata* are a cause of major economic loss in grazing cattle in Japan and Korea.

I have investigated co-infection of *Theileria* and *Babesia* sp. by PCR and PCR-RFLP and examined the antigenic diversity of MPSP gene on 35 herds of clinically healthy Korean Native Black Cattle that were born and raised in National Jeju Agricultural Experiment Station in May, 2003.

In the microscopic examination of Giemsa - stained blood smears (×1,000), intracellular parasites were rare. The hematological data were in normal range.

제주대학교 중앙도서관

Twenty (57.1%) out of 35 samples were co-infection of *Theileria* and *Babesia* sp.. All the blood samples were amplified universal MPSP gene and infected with mixed C, I, B1 and B2 of *Theileria* sp.. They were mainly C and B type. There were allelic variants in Korean Native Black Cattle in Jeju.

Key word : *Theileria, Babesia,* Major Piroplasm Surface Protein gene, Korean Native Black Cattle

CONTENTS

1. Introduction -- 1

2. Materials and Methods ------------------------------ 4

3. Results --- 10

4. Discussion -- 23

5. References --- 26

Summary (in Korean) -------------------------------- 35

I. Introduction

Theileria and *Babesia* sp. are both tick-transmitted intracellular haemoprotozoan parasites and cause anorexia, fever, anemia and icterus. There are several species of *Theileria* in cattle. *Theileria sergenti* of them is less pathogenic species and widely distributed in East Asia. But the bovine *theileriosis* caused by *T. sergenti* is a cause of major economic loss in grazing cattle in Japan and Korea.

In the Korea, bovine piroplasmosis is caused by *Babesia ovata* (Cho *et al*., 2002) and *Theileria sergenti* (Lee & Kim, 1987; Baek *et al*., 1990, 1992, 1994; Chae *et al*., 1996a,b, 1998a,b; Kang *et al*., 1997; Kang *et al*., 1999). Infected cattle suffer from chronic anemia due to intraerythrocytic piroplasms and occasionally die in severe cases. After recovery from the acute phase, the infection may assume a subclinical, chronic course and these animals could become carriers of the piroplasms. The carriers may act as a reservoirs, parasites are present in very low numbers in the blood and generally may not detected in Giemsa-stained blood smear.

Hematological data are often not reliable. Serological test lacks sensitivity and is inadequate during latent stages of the disease. Capillary tube agglutination test, immunofluorescence antibody test (IFAT) and ELISA are more sensitive but lack specificity.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

On the molecular level, Major piroplasm surface protein (MPSP), 16S and 18S rRNA genes have been extensively studied on distinction from species related to *Theileria* sp..

The MPSP is a major target antigen recognized by the host immune system and show antigenic polymorphism as an immune evasion mechanism (Zhuang *et al* ., 1994, Kim S. J *et al*., 1998). Non-pathogenic *Theileria* species are divided into at least five type, I (Ikeda), C (Chitose), B (*Buffeli*) 1, 2 and Thai type based on the allelic form of MPSP genes (Kubota *et al*., 1996, Kakuda *et al*., 1998b, Inoue *et al*., 2001, Sarataphan *et al*., 2003).

The field isolates from Japan, Korea, Australian, other Asian and European countries are reported to contain mixed populations of parasite bearing various combinations of MPSP allelic types (Kakuda *et al,* 1998b. Kubota *et al*., 1996a, Wang *et al*., 1998, Inoue *et al*., 2001, Sarataphan *et al*., 2003).

제주대학교 중앙도서관 JEJU NATIONAL UNIVERSITY LIBRARY

In Japan, *Theileria* species consist of I, C and B2 type parasites (Kubota *et al*., 1995, 1996). In Korea, I type is common and I and C type are co-infection. Some of the Korean isolates contain the parasites with allelic form of B1 type that is seen only in the *T. orientalis/buffeli.* This suggests that *T. orientalis/buffeli.* may co-exist with *T. sergenti* in Korea (Kakuda *et al*., 1998b)

Kubota *et al.* (1996b) suggested that developmental stages in tick gut and salivary gland is essential for maintenance or expansion of antigenic and genetic diversities of parasites.

The hypothesis for the presence of antigenically diversified parasites is that the parasite may increase their antigenic diversity in order to evade the immunity of an individual animal and of an animal herd (Onuma *et al*., 1998).

In this study, I have surveyed co-infection of *Theileria* and *Babesia* sp. on the Korean Native Black Cattle in Jeju and also examined the antigenic diversity of *Theileria* MPSP gene.

II. Materials and Methods

Experimental animals

Blood samples were collected in EDTA tubes on 35 herds Korean Native Black Cattle from National Jeju Agricultural Experiment Station in May, 2003 and maintained at -70℃ until DNA extraction. Thin blood film smears were made from fresh blood and stained with Giemsa by standard microscopic methods for the evaluation of intracellular parasites.

DNA Extraction

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

DNA was extracted from frozen blood samples by the following method (modified Miller's method). For each sample 500㎕ of blood was treated with two volumes of STE buffer (10mM Tris-HCl, pH 8.0, 1mM EDTA, 0.1M NaCl) and then centrifuged at 12,000×g for 5 min. The pellets were washed two or three times in STE buffer to remove the cell debris with every wash. The pellets resuspended in SDS-Proteinase K buffer (0.1mg/㎕) and then incubated at 37℃ on overnight. The DNA was extracted with phenol and precipitated with cold ethanol. The DNA was then collected by centrifugation and resuspended in 20㎕ of DW.

Amplification of DNA

Five sets of primer were used in this study. The first set (430bp) comprising of RLB-F2 and RLB-R2 was to amplify the gene encoding for *Theileria* & *Babesia* sp.. The second set (875bp) was Ts-U and Ts-R for *Theileria* Universal MPSP gene. The different sense primers, Ts-C (831bp), Ts-I (826bp), Ts-B (826bp) together with the same anti-sense primer Ts-R were to amplify the MPSP genes of two *T. sergenti* (C and I type) and *T. buffeli* (B type), respectively.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Table 1. The Oligonucleotide Primers used in PCRs and the expected Tm(℃) and Size(bp) of PCR Products.

Primer	Sequence (5′→3′)	Tm(℃)	Size(bp)
RLB-F2	GACACAGGGAGGTAGTGACAAG	52	430
RLB-R2	CTAAGAATTTCACCTCTGACAGT		
Ts-U	CACGCTATGTTG TCCAAGAG	57	875
Ts-R	TGTGAGACTCAATGCGCCTA		
Ts-C	GCGGATCCTCATCGTCTCTGCAACT		831
Ts-I	AAGGATCCGTCTCTGCTACCGCCGC		826
Ts-B	GCGGATCCGCTCTGCAACCGCAGAG		826

RLB-F2 & R2 : primer set for the *Theileria* & *Babesia sp.*
Ts-U & R : primer set for the *Theileria* Universal MPSP gene
Ts-C, I, B & Ts-R : primer set for the C, I, B type, respectively

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

The amplification mixture contained 10× PCR buffer, 20pmol each primer, one unit *Taq* polymerase (Takara, Japan), 200mM each of the dNTP, 50~100ng template DNA in final volume of 20㎕.

Amplification was carried out using an automatic DNA thermal cycler (Takara, Japan). Thermal cycling profile was followed; initial enzyme activation and hot start, 10min at 94℃; 35~40 cycles of 1min at 95℃, 30s at Tm(℃) of each primer, extension 1min at 72℃ and final extension of 7min at 72℃.

PCR products were detected and their sizes estimated by co-electrophoresis of 5㎕ of the reaction mix and standard size markers 100bp ladder, on 1.2% agarose gel (SEA KEM, FMC, USA), and visualized by UV illumination of ethidium bromide stained DNA.

PCR-RFLP

To examine the co-infection of *Theileria* and *Babesia* sp. amplified products by RLB primer set were isolated from gel and subsequently digested with *Kpn* I (Takara, Japan). Amplified products by Ts-B and Ts-R primer were analyzed based on RFLP as described previously (Kubota *et al*, 1996b; Kakuda *et al.*, 1998b) for subdivided for B1 and B2 type. PCR products were purified with Geneclean® II Kit (Q-Bio Gene, USA), followed by restriction enzyme digestion with *Bgl* I (Bioneer, USA), *Dra* I (Takara, Japan), *Eco*T14 I (Bioneer, USA), *Eco*RV (Bioneer, USA), and *Hind*III (Takara, Japan). Reaction mixture was 1㎕ PCR product, 1㎕ buffer (×10), 10～15 unit restriction enzyme, add the dH_2O to final volume 10㎕. Reaction mixture incubated at 37℃ for 2 hours. Digested PCR products were detected and their sizes estimated by co-electrophoresis of 5ul of the reaction mix and standard size marker (∅X174-*Hae*III digest), on a 2% agarose gel (SEA KEM, FMC, USA) and visualized by UV illumination of ethidium bromide stained DNA.

제주대학교 중앙도서관 JEJU NATIONAL UNIVERSITY LIBRARY

Cloning and Sequencing of PCR products

PCR products were loaded on a 1.2% agarose gel and the band of the correct size was excised. Amplicons of the B type were extracted from the excised band with Geneclean® II Kit (Q-Bio Gene, USA) and were ligated into the pGEM®-T easy vector systems(Promega, USA) and transformed into DH5α One Shot *Escherichia coli*, according to the Manufacturer's instructions. A kit (*Accu Prep*® Plasmid Extraction Kit, Bioneer, Korea) was used to isolate cloned DNA. Presence of an insert was verified using an T7 primer and Ts-R primer. Three clones were chosen for sequencing.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Sequence alignment and Homology analysis

GenBank accession numbers of MPSP sequences are *T. sergenti* (D50304, D11046, E06129, AB016280) *T. buffeli* (D11047), *T. orientalis* (AB008369). Sequences listed in Table 2. Sequences alignment and homology analysis were performed by programs of CLUSTAL W (http://www.ebi.ac.uk/clustalw/).

Table 2. MPSP Sequences of *T. buffeli*-like Parasites with their Origin.

Name	Origin	GenBank acession No.
T. sergenti	Japan (Aomori)	D50304
T. sergenti	Japan (Ikeda)	D11046
T. sergenti	Japan (Chitose)	E06129
T. sergenti	Japan (Fukushima)	AB016280
T. buffeli	Australia (Warwick)	D11047
T. orientalis	England (Essex)	AB008369

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Ⅲ. Results

Hematology and Microscopy examination

The hematological values of all the samples were in the normal ranges (Table 3). The mean packed cell volume (PCV) was 40± 5.9 % in 9 parasitemic cows and was 37±5.2% in 26 non-parasitemic cows on microscopic examination.

Table 3. Hematological Values of Korean Native Black Cattle.

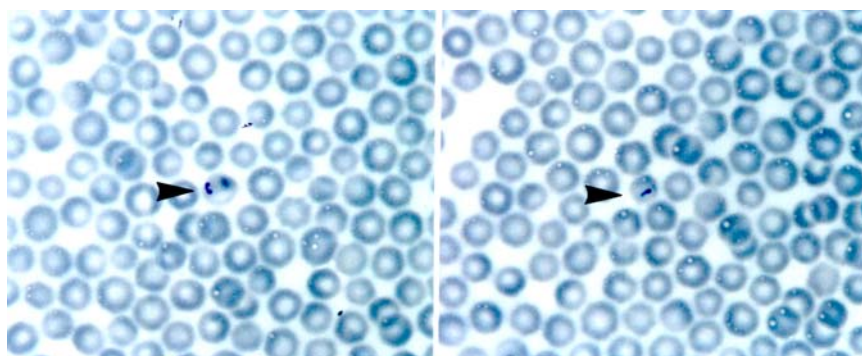
(mean ± SD)

	A	B
RBC ($10^4/\mu\ell$)	883 ± 87	846 ± 128
WBC ($\mu\ell$)	11858 ± 4775	12148 ± 3757
PCV (%)	40 ± 5.9	37 ± 5.2
Fibrinogen (mg/100㎖)	556 ± 194	592 ± 208
Total protein (g/100㎖)	7.3 ± 0.5	7.3 ± 0.6

A : Parasitemic group on microscopic examination.
B : Non-parasitemic group on microscopic examination.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

In the microscopic examination of Giemsa-stained blood smears, 9 of 35 cattle had intraerythrocytic piroplasms but infected erythrocytes were rare (Figure 1).



제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Figure 1. Intraerythrocytic forms of piroplasms. Piroplasms are indicated with arrow. Giemsa-stained blood smear, ×1000

Amplification of DNA

All the blood samples were amplified RLB primer set(430bp) and *Theileria* universal MPSP gene(875bp). The allele type of MPSP gene amplified the different sense primers, Ts-C for C type (831bp), Ts-I for I type (826bp) and Ts-B for B type (826bp) with anti-sense primer, Ts-R (Figure 2).

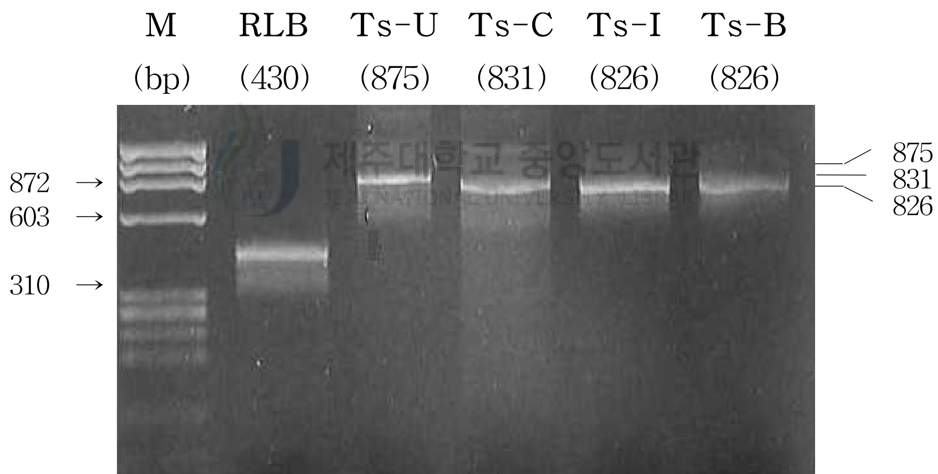


Figure 2. PCR amplification of primer sets. Primers were Ts-C, Ts-I, Ts-B used by the combination with Ts-R primer. Lane M; marker (∅X174-*Hae*Ⅲ digest).

PCR-RFLP for the Detection of *Theileria* & *Babesia* sp.

In PCR-RFLP, the PCR amplification of RLB-F2/R2 with *Kpn* I showed as figure 3. When co-infection of *Theileria* and *Babesia* sp. showed two band (digested and non-digested band) on agarose gel electrophoresis (Figure 3). The *Theileria* infection showed one band (digested). Twenty (57.1%) of 35 samples were co-infection of *Theileria* and *Babesia* sp..

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Figure 3. PCR amplification of primer set RLB-F2/R2 (U) and restriction pattern with *Kpn* I (K) on a 2.0% agarose gel electrophoresis. Lane M; marker (100bp ladder)
Co-infection : *Theileria* & *Babesia* sp. co-infection
Ts-infection : *Theileria* infection

Analysis of Allele type of *Theileria* MPSP gene

The result of allele-specific PCR were mixed infection with C, I, B type. The twenty of 35 blood samples were C type and B type were 17. The eleven samples were unknown type (Table 4).

Table 4. Analysis of *Theileria* parasite isolates by allele-specific PCR.

No. of isolates	MPSP allele type			
	C type	B type	I type	Unknown type
5/35	+	−	−	−
9/35	+	+	−	−
4/35	+	+	+	−
2/35	+	−	+	−
4/35	−	+	−	−
11/35	−	−	−	+
	20/35	17/35	6/35	11/35

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

PCR-RFLP of B type of MPSP gene

When amplified products by Ts-B and Ts-R primer were analyzed based on RFLP, the eleven of 17 amplification of B type showed as Figure 4A. Three restriction enzyme, *Bgl* I , *Dra* I and *Eco*T14 I , had not enzyme site in amplificons. But *Eco*RV and *Hind* Ⅲ digested the PCR products and produced three band with *Eco*RV and four band with *Hind*Ⅲ. The five of 17 were similar to pattern of Figure 4A with *Dra* I , *Eco*T14 I , *Eco*RV and *Hind*Ⅲ. But digested with *Bgl* I and produced two band as Figure 4B.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

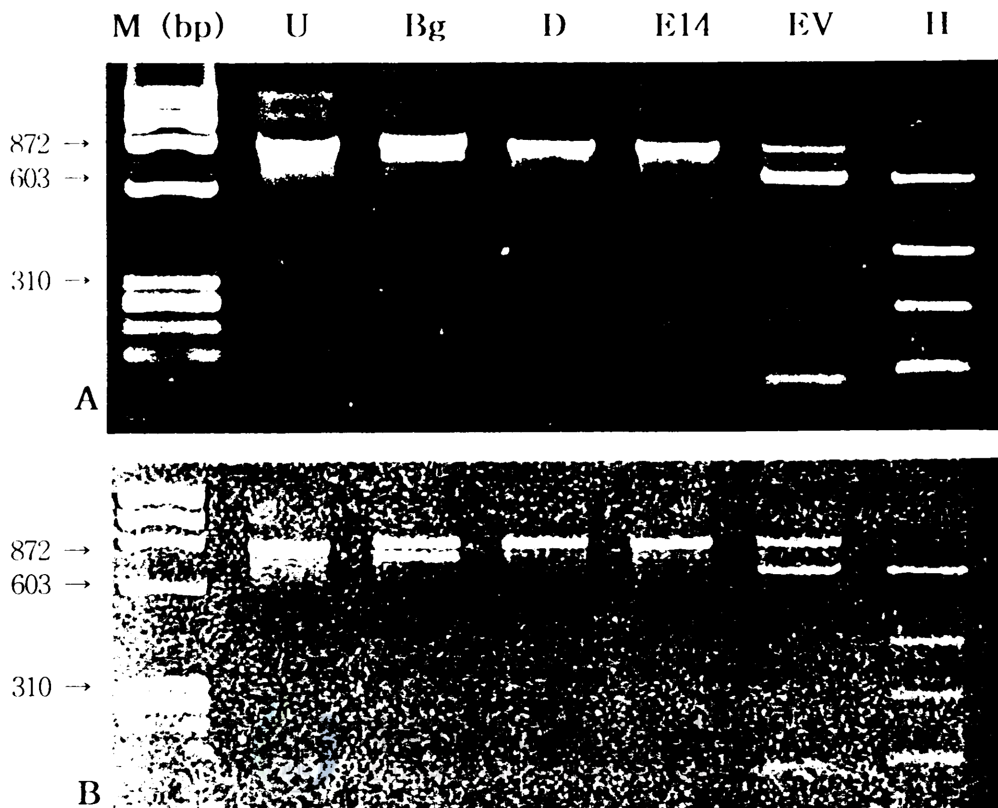
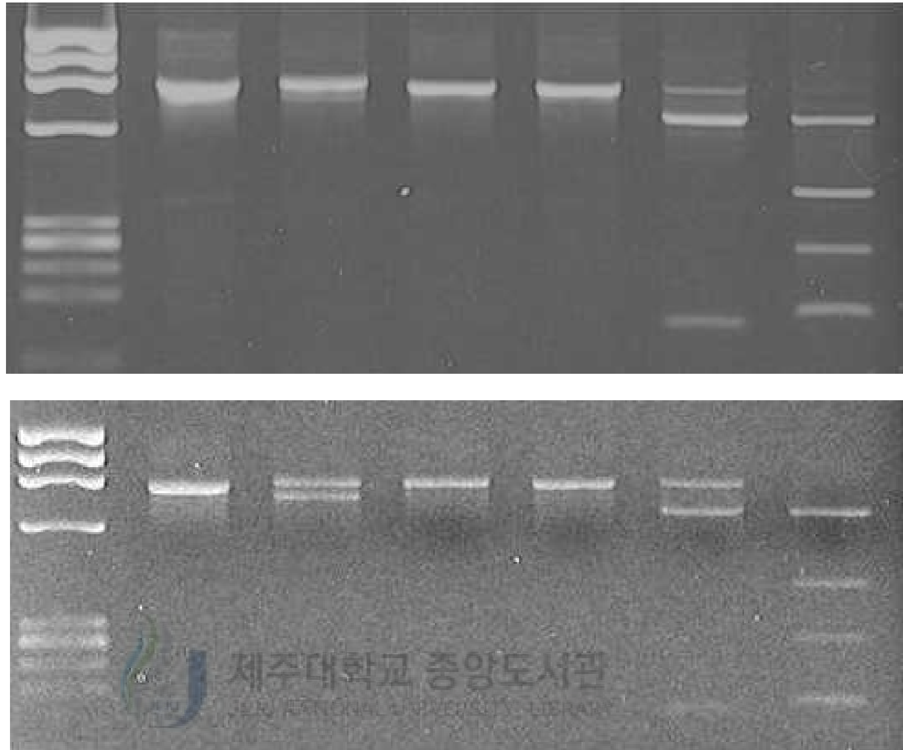
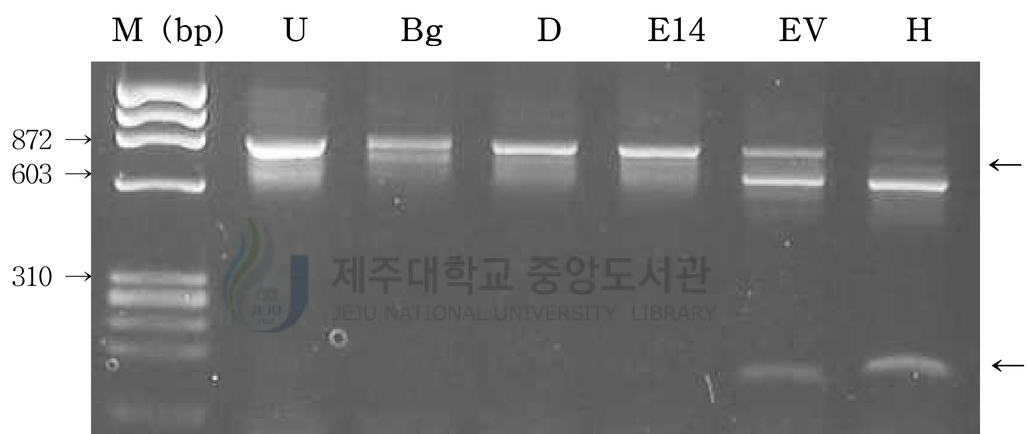


Figure 4. Restriction pattern of the PCR product that amplified by Ts-B and Ts-R primer set. The PCR product was digested with the restriction enzyme, *Bgl* I (Bg), *Dra* I (D), *Eco*T14 I (E14), *Eco*RV (EV) and *Hind*Ⅲ (H), eletrophoresed on a 2.0% agarose gel and stained with ethidium bromide. Lane M; marker (∅X174-*Hae*Ⅲ digest), U(un cut); PCR product.



제주대학교 중앙도서관

Figure 4. Restriction pattern of the PCR product that amplified by Ts-B and Ts-R primer set. The PCR product was digested with the restriction enzyme, *Bgl* I (Bg), *Dra* I (D), *Eco*T14 I (E14), *Eco*RV (EV) and *Hind*Ⅲ (H), eletrophoresed on a 2.0% agarose gel and stained with ethidium bromide. Lane M; marker (∅X174-*Hae*Ⅲ digest), U(un cut); PCR product.



제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Comparison of nucleotide sequences

Comparative sequence analysis was carried out with the 3 sequences obtained in the present study, together with 6 sequences reported previously of MPSP gene of *Theileria* sp. The results shown Figure 6 and the homology value were Table 5. The homology of these three sequence of *Theileria* sp. isolates from Korean Native Black Cattle showed 91% (Clone 2), 87% (Clone 4) and 93% (Clone 5) with B2 type (D50304) and they were 91% (Clone 2), 96% (Clone 4) and 91% (Clone 5) with B1 type (D11047) respectively.

```
D11047       1: GCTCTGCAACCGCAGAGGAGAAAAAAGAACCAGCAAAGGCTGAAGAGAAGAAAGATTTAG : 60
AB008369     1: G******AA*CGCA*AGGAGA*****G**CCA*****G*****A********A**T***G : 60
D50304       1: G******AA*CGCA*AG---A*****G**GCA*****A*****T********G**T***G : 57
AB016280     1: T******AA*TGCA*AAGAGA*****G**GCT*****G*****T********G**T***G : 60
D11046       1: T******TA*CGCC*CAGAGG*****A**GAT*****G*****A********G**C***A : 60
E06129       1: T******TA*CGCC*CAGAGG*****A**GAT*****G*****A********G**C***A : 60
Clone 2      1: G******AA*C-CA*AGGAGA*****G**CCA*****G*****A********A**T***G : 59
Clone 4      1: G******AC*---A*AGGAGA*****G**CCA*****G*****A********A**T***G : 57
Clone 5      1: G******AA*CGCA*AGGAGA*****G**CCA*****G*****A********A**T***G : 60

D11047      61: CTCTGGAAGTTAACGCCACCCAGGGTGAAAATTTTACAGTCAATGCAACCAATGCCAACG : 120
AB008369    61: ****G*****T*********CAG*GT***A*****AC****A*T**AA*CA*T******* : 120
D50304      58: ****T*****T*********CAG*CT***A*****AC****A*T**AA*CA*C******* : 117
AB016280    61: ****T*****A*********CAG*CT***A*****AC****A*T**AA*CA*T******* : 120
D11046      61: ****C*****T*********GCA*CC***C*****AA****G*C**CT*AA*C******* : 120
E06129      61: ****C*****T*********GCA*CC***C*****AA****G*C**CT*AA*C******* : 120
Clone 2     60: ****T*****A*********CAG*CT***A*****AC****A*T**AA*CG*C******* : 119
Clone 4     58: ****G*****T*********CAG*GT***A*****GC****A*T**AA*CA*T******* : 117
Clone 5     60: ****T*****A*********CAG*CT***A*****AC****A*T**AA*CG*C******* : 120

D11047     121: ACGTCGTTTTTACTGCCTCGGATGGATATCGTTTCAAGACTCTTAAGGTTGGAGATAAAA : 180
AB008369   121: *****************TCG**T****AT**TT*******TC*T*****T********A* : 180
D50304     118: *****************AAT**G****TT**CA*******AG*T*****T********A* : 177
AB016280   121: *****************AAT**G****AC**TA*******AC*T*****T********A* : 180
D11046     121: *****************GAA**G****AC**CA*******AC*C*****C********G* : 180
E06129     121: *****************GAA**G****AC**CA*******AC*C*****C********G* : 180
Clone 2    120: *****************AAT**G****TT**CG*******AC*T*****T********A* : 179
Clone 4    118: *****************TCG**G****AT**TT*******TC*T*****T********A* : 177
Clone 5    121: *****************AAT**G****TT**CG*******AC*T*****T********A* : 180
```

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

to be continued

D11047	181:	CTTTGTATACCGTTGATACATCCAAATTCACTCCAACCGTTGCCCACAGAATTAAGCATG	: 240
AB008369	181:	CTT*******C**T*****AT****A*****T*****C**T**C******A*T*******	: 240
D50304	178:	CAT*******C**T*****AG****A*****T*****A**C**C******C*C*******	: 237
AB016280	181:	CTT*******T**A*****AT****A*****T*****A**C**C******C*G*******	: 240
D11046	181:	ACC*******C**A*****TT****G*****C*****T**C**C******C*G*******	: 240
E06129	181:	ACC*******C**A*****TT****G*****C*****T**C**C******C*G*******	: 240
Clone 2	180:	CAT*******C**T*****AT****A*****T*****A**C**T******C*T*******	: 239
Clone 4	178:	CAT*******C**T*****AT****A*****T*****A**C**C******A*T*******	: 237
Clone 5	181:	CAT*******C**T*****AT****A*****T*****A**C**T******C*T*******	: 240

D11047	241:	GTGATGCCTTGTTCTTCAAGCTTGACCTTTCCCATGCCAAGCCAC*CTTGTTCAAGAAGA	: 300
AB008369	241:	GT**TGC*T*******C*****TGA***T*****T**C**G***C*CT*G**********	: 300
D50304	238:	GA**TAA*T*******C*****TGA***T*****T**T**G***C*TT*A**********	: 297
AB016280	241:	CT**AGA*C*******A*****CGA***T*****T**A**A***C*TT*G**********	: 300
D11046	241:	CT**CGA*C*******C*****CAA***G*****C**A**G***T*GC*G**********	: 300
E06129	241:	CT**CGA*C*******C*****CAA***G*****C**A**G***T*GC*G**********	: 300
Clone 2	240:	GT**TGC*T*******C*****CGA***T*****T**C**G***C*CC*G**********	: 299
Clone 4	238:	GT**ATG*C*******C*****TGG***T*****T**C**G***C*CT*G**********	: 297
Clone 5	241:	GT**TGC*T*******C*****CGA***T*****T**C**G***C*CC*G**********	: 300

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

D11047	301:	AGACTGACAAGGATTGGGTTCAGTTTAACTTTGGCCAGTACCTTGACGAATTTGTATGGA	: 360
AB008369	301:	***CT********T*****T**G**T*A***T*G*********T**C***T*TG*A****	: 360
D50304	298:	***CC********T*****T**G**T*G***C*C*********C**T***G*AG*C****	: 357
AB016280	301:	***GC********A*****A**G**C*G***C*C*********C**T***G*TC*C****	: 360
D11046	301:	***CT********T*****T**A**C*G***C*C*********C**T***G*TG*A****	: 360
E06129	301:	***CT********T*****T**A**C*G***C*C*********C**T***G*TG*A****	: 360
Clone 2	300:	***CC********T*****T**G**T*G***C*C*********T**G***G*AG*C****	: 359
Clone 4	298:	***CT********T*****T**G**T*A***T*G*********T**T***T*TG*A****	: 357
clone 5	301:	***CC********T*****T**G**T*G***C*C*********T**G***G*AG*C****	: 360

to be continued

```
D11047      361: AGGAAAAGAAGGAACTCAAGGATATAGATGCATCCAAGTTTGCAGAGGCAGGTCTTTTTG : 420
AB008369    361: *G**A********ACTC**G**TC*A**T**AT*******TG****GA*A********T* : 420
D50304      358: *G**G********TGTG**A**CC*C**T**CT*******TG****CG*T********C* : 417
AB016280    361: *A**A********ATCC**A**CC*C**T**CT*******TG****AG*T********T* : 420
D11046      361: *G**G********AGTA**A**CC*C**C**AT*******CG****CG*A********C* : 420
E06129      361: *G**G********AGTA**A**CC*C**C**AT*******CG****CG*A********C* : 420
Clone 2     360: *A**A********ACTC**G**CC*C**T**AG*******TG****GG*A********T* : 419
Clone 4     358: *A**A********ACTC**G**TC*C**T**AT*******TA****GG*A********T* : 417
Clone 5     361: *A**A********ACTC**G**CC*C**T**AG*******TG****GG*A********T* : 420

D11047      421: CAGCTGATACATTCGGTACTGGTAAGGTTTATGACTTT                       : 458
AB008369    421: *AG*T**TA*A********T**T***G*T**TG****T                       : 458
D50304      418: *CG*T**TG*A********T**T***G*C**CG****C                       : 455
AB016280    421: *CC*T**TG*A********C**A***G*T**CG****C                       : 458
D11046      421: *CG*T**GG*T********C**A***C*G**CA****C                       : 458
E06129      421: *CG*T**GG*T********C**A***C*G**CA****C                       : 458
Clone 2     420: *CG*T**TG*A********C**A***G*T**CG****C                       : 457
Clone 4     418: *AG*A**TA*A********C**T***G*A**TG****C                       : 455
Clone 5     421: *CG*T**TG*A********C**A***G*T**CG****C                       : 458
```

제주대학교 중앙도서관
UNIVERSITY LIBRARY

Figure 6. Comparison of partial nucleotide sequence from PCR product (Clone 2, 4, 5) and MPSP genes of other *Theileria* sp. from the GenBank database. Gaps (-) represent space introduced into the aligned sequences by the Multiple Alignment program in the CLUSTAL W program. An asterisk marks represent the nucleotide identity.

Table 5. Nucleotide sequence comparison of MPSP gene of other *Theileria* sp.

Origin	Homology of nucleotide sequence		
	Clone 2	Clone 4	Clone 5
Japan (Aomori, D50304)	91	87	92
Japan (Ikeda, D11046)	83	80	83
Japan (Chitose, E06129)	83	80	83
Japan (Fukushima, AB016280)	90	87	90
Australia (Warwick, D11047)	91	96	91
England (Essex, AB008369)	91	96	91

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Ⅳ. Discussion

The major clinical sign of bovine piroplasmosis is a hemolytic anemia, but this is not clearly established in subclinical stage of herd investigation (Stockham *et al.*, 2000). A combination of predisposing factors influences the course of clinical illness. In this study, although I found piroplasms in 9 cows in microscopic examination, all the samples were positive for *Theileria* sp. by PCR and all the cows were in subclinical stage.

Both *Theileria segenti* and *Babesia ovata* are often collectively referred as bovine piroplasmosis in Korea. The co-infection rate of *Theileria sergenti* and *Babesia ovata* was 40% by serological test (Jeon *et al.*, 1978) and 33% in Japan by microscopic examination (Arai *et al.*, 1998). In the present study, the result showed 57.1% co-infection rate higher than that previously studied. The prevalence of *Theileria sergenti* infection in Jeju (Kim G.H *et al.*, 1999) was higher than that in other province (Song *et al.*, 2003) and the biological vector was identical as *Haemaphysalis longicornis* which of *Theileria sergenti* (Kawazu *et al.*, 1995), *Babesia ovata* (Cho *et al.*, 2002) and *B. caballi* (Bautista *et al.*, 2003). Kubota *et al.* (1996b) and Onuma *et al.* (1998) suggested that the presence of multiple parasite clone in a vector is essential to cross-fertilization which will result in further genetic diversity.

제주대학교 중앙도서관 JEJU NATIONAL UNIVERSITY LIBRARY

Majority of *T. sergenti*-infected cattle in Japan presented mixed parasite population bearing I and C type parasites. *T. buffeli* is distributed mainly in Australian and in adjacent areas in Asia (Kubota *et al*., 1996b, Kakuda *et al*., 1998b, Wang *et al*., 1998). In Taiwan and other East Asia, I type parasite could not be identified (Wang *et al*., 1998, Inoue *et al*., 2001, Sarataphan *et al*., 2003), while I type is major parasite distributed in Japan and Korea (Kakuda *et al*., 1998b; Kubota *et al*., 1996b; Kim G.H *et al*., 1999). There is no report concerning the relation between the allelic form and virulence of *T. sergenti/buffeli*. However, there are several suggestive evidence that I type is more pathogenic than C and B type. Ikeda (I type) stock is more pathogenic than fukushima (C type) stock and all *Theileria* isolates contained I type parasite and showed severe symptoms in Korea (Kakuda *et al*., 1998b). In this study, they were mainly C type (20 of 35) and B type (17 of 35). I type were rare (6 of 35) and all cattle were normal in clinical signs and hematological examination.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

In this study, I tried PCR-RFLP for subdivided of B type as described previously (Kubota *et al*., 1996b; Kakuda *et al*., 1998b). The major pattern was identified to B1 type (11 of 17) and the five of 17 were mixed form of B1 and B2 type. But one sample was new pattern that showed variation of HindⅢ enzyme site. The similarity of MPSP gene of B type was confirmed by sequence analysis. The results of sequence analysis suggest that the PCR

product of *Theileria* isolates from Korean Native Black Cattle may be closely related to *T. sergenti* (B2 type) and *T. buffeli* (B1 type).

Kubota *et al.* (1996b) demonstrated that parasite population ratio changes between I and C type parasites occurred during persistent infection in cattle and Iwasaki *et al* (1998) provided further evidence that population shift from parasite expressing one allelic type of MPSP to those expressing another type result in apparent antigenic change of the parasites.

Many studies reported that susceptibilities to piroplasmosis are different according to breed. Kim *et al* (1999) reported that Korean native cattle show solider resistance to *T. sergenti* infection than the Holstein in Jeju. Results in this study suggested that the different of resistance according to breed and host immuno response. So, it is necessary more investigation about the resistance and adaptation of Korean Native Black Cattle in Jeju conditions compare with another breed.

In this study demonstrates that the co-infection of *Theileria* and *Babesia* sp. is high and infects with mixed allele types of MPSP gene of *Theileria* sp.. Also, there are allelic variants in Jeju. Therefore, further studies on the tick, analysis of antigenic difference between variant of each type and the seasonal variation of allele type are essential for the developing optimal treatment and control methods.

제주대학교 중앙도서관 JEJU NATIONAL UNIVERSITY LIBRARY

V. References

Arai, S., Tsuji, M., Kim, S.J., Nakada, K., Kirisawa, R., Ohta, M., Ishihara, C., 1998. Antigentic and Genetic Diversities of *Babesia ovata* in Persistently Infected Cattle. *J. Vet. Med. Sci*. 60: 1321-1327.

Baek, B.K., Kim, B.S., Lee H.I., 1990. Fine structure of *Theileria sergenti* merozoite in Korean native cattle. *Korean J. Vet. Sci.* 30: 465-471.

Baek, B.K., Choi, B.S., Hansen, R., Kakoma, I., 1992. Immunogenicity and Protective Efficacy of Solubilzed Merozoite- enriched *Theileria sergenti* Immunogens: Ⅰ. protection against Homologous Challenge. *Korean J. Parasitol.* 30: 133-140.

Baek, B.K., Kim, B.S., Whim, B.M., Lee, H.I., Park, Y.H., Kakoma, I., 1994. Immunogenicity and protective effecacy solubilized merozoite-enriched *Theileria sergent*i Immunogens: Ⅲ. Characterization of immunodominant peptides. *Korean J. Parasitol*. 32: 111-116.

Bautista, J.L.R., Ikadai, H., You, M.J., Battsetseg, B., Igarashi, I., Nagasawa, H., Fujisaki, K., 2001. Molecular evidence of *Babesia*

제주대학교 중앙도서관 JEJU NATIONAL UNIVERSITY LIBRARY

caballi (Nuttall and Strickland, 1910) parasite transmission from experimentally-infected SCID mice to the ixodid tick, *Haemaphysalis longicornis* (Neuman, 1901). *Vet. Parasitol.* 102: 185-191.

Chae, J.S., Lee, J.M., Kwon, O.D., Lee, S.O., Chae, K.S., Onuma, M., 1996a. Comparative analyses of *Theileria sergenti* isolated from Korea and Japan by Southern hybridization and polymerases chain reaction. *Korean J. Vet. Res.* 36: 187-193.

Chae, J.S., Lee, J.M., Kwon, O.D., Park, J.H., Chae, K.S., 1996b. Rapid detection of *Theileria sergenti* by the polymerase chain reaction in Korea cattle. *Korean J. Vet. Res.* 36: 195-207.

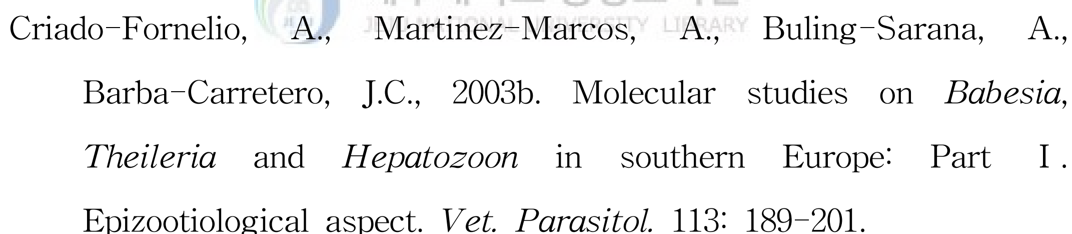
Chae, J.S., Kwon, O.D., Holman, P.J., Waghela, S.D., Waner, G.G., Lee, J.M., 1998a. Identical small subunit ribosomal nucleotide sequence of bovine *Theileria* isolates (Korea and Japan) and *Theileria buffeli*(marula, Kenya). *Kor. J. Parasitol.* 36: 47-53.

Chae, J. S., Lee, J.M., Kwon, O.D., Holman, P.J., Waghela, S.D., Wagner, G.G., 1998b. nucleotide sequence heterogeneity in the small subunit ribosomal RNA gene variable(V4) region among and within geographic isolates of *Theileria* from cattle, elk and white-tailed deer. *Vet. Parasitol.* 75: 41-52.

제주대학교 중앙도서관 JEJU NATIONAL UNIVERSITY LIBRARY

Cho, S.H., Kim, T.S., Lee, H.W., Tsuji, M., Ishihara, C., Kim, J.T., Wee, S.H., Lee, C.G., 2002. Identification of newly isolated *Babesia* parasites from cattle in Korea by using the Bo-RBC-SCID mice. *Kor. J. Parasitol.* 40: 33-40.

Criado-Fornelio, A., Martinez-Marcos, A., Buling-Sarana, A., Barba-Carretero, J.C., 2003a. Presence of *Mycoplasma haemofelis*, *Mycoplasma haemominutum* and Piroplasmids in cats from southern Europe: a molecular study. *Vet. Microbiol.* 93: 307-317.


제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Criado-Fornelio, A., Martinez-Marcos, A., Buling-Sarana, A., Barba-Carretero, J.C., 2003b. Molecular studies on *Babesia*, *Theileria* and *Hepatozoon* in southern Europe: Part Ⅰ. Epizootiological aspect. *Vet. Parasitol.* 113: 189-201.

Gubbles, M.J., Hong, Y., Weide, M., Qi, B., Nijman, I.J., Guangyuan, L., Jongejan, F., 2000. Molecular characterization of the *Theileria buffeli/orientalis* group. *Int. J. Parasitol.* 30: 943-952.

Inoue, M., Nguyen, D.V., Meas, S., Ohashi, K., Sen, S., Sugimoto, C., Onuma, M., 2001. Survey of *Theileria* Parasite Infection in Cattle in Cambodia and Vietnam Using Piroplasm Surface

Protein Gene-Specific Polymerase Chain Reaction. *J. Vet. Med. Sci.* 63(10): 1155-1157.

Iwasaki, T., Kakuda, T., Sako, Y., Sugimoto, C., Onuma, M., 1998. Differentiation and quantification of *Theileria sergenti* piroplasm type using type-specific monoclonal antibodies. *J. Vet. Med. Sci.* 60: 665-669.

Jeon, Y., 1978. A survey on *Babesiosis* and *Theileriasis* in Korean cattle. *Korean J. Vet. Res.* 18: 23-26(in Korean)

Jeong W., Kwen CH., Kang SW., Paik SG., 2003. Diagnosis and quantification on *Theileria sergenti* using TaqMan PCR. *Vet. Parasitol.* 111: 287-295.

Kakuda, T., Shiki, M., Kubota, S., Sugimoto, C., Brown, W.B., Kosum, C., Nopprn, S., Onuma, M., 1998a. Phylogeny of benign *Theileria* species from cattle in Thailand, Chaina, the U.S.A based on the major piroplasm surface protein and small subunit ribosomal RNA genes. *Int. J. Parasitol.* 28: 1261-1267.

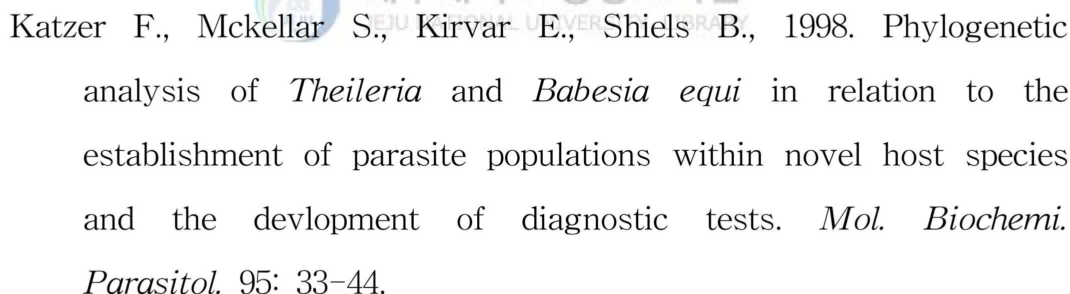
Kakuda, T., Kubota, S., Sugimoto, C., Baek, B. K., Yin, H., 1998b. Analysis of Immunodominant Piroplasm Surface Genes of Benign *Theileria* Parasites Distributed in China and Korea by

제주대학교 중앙도서관

Allele-Specific Polymerase Chain Reaction. *J. Vet. Med. Sci.* 60(2): 237-239.

Kang, S.W., Choi, E.J., Kweon, C.H., 1997. Cloning and sequencing of p33 in a Korean isolate of *Theileria sergenti. Korean J. Parasitol.* 35: 105-110.

Kang, S.W., Kweon, C.H., Choi, E.J., Yoon, Y. D., 1999. Expression of major piroplasm protein (p33) of *Theileria sergenti* (Korean isolate) and its immunogenicity in guinea pigs. *Korean J. Parasitol.* 37: 277-283.



Katzer F., Mckellar S., Kirvar E., Shiels B., 1998. Phylogenetic analysis of *Theileria* and *Babesia equi* in relation to the establishment of parasite populations within novel host species and the devlopment of diagnostic tests. *Mol. Biochemi. Parasitol.* 95: 33-44.

Kawazu, S.I., Kamio, T., Sekizaki, T., Fujisaki, K., 1995. *Theileria sergenti* and *T. buffeli*: Polymerase Chain Reaction-Based Marker System for Differentiating the Parasites Species from Infected Cattle Blood and Tick Salivary Gland. *Exp. Parasitol.* 81:430-435.

Kawazu, S.I., Okumura, T., Hirogari, Y., Miyhara, T., Terasaka, Y., Hida, M., Terada, Y., Kamio, T., Fujisaki, K., 1997. A polymorphism Observed in the Experimentally Successful Peptide Vaccine Sequence Derived from *Theileria sergenti* Piroplasm Major Surface Antigen (p33). *J. Vet. Med. Sci.* 59: 829-831.

Kawazu, S.I., Kamio, T., Kakuda, T., Terada, Y., Sugimoto, C., Fujisaki, K., 1999. Phylogenetic relationship of the benign *Theileria* species in cattle and Asian buffalo based in the major piroplasm surface protein (p33/34) gene sequences. *Int. J. Parasitol.* 29: 613-618.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Kim, G.H., Lee, K.K., Onuma, M., 1999. Susceptibility of *Theileria sergenti* Infection in Holstein Cattle Compared to Korea Native Cattle on Cheju Island. *J. Protozool. Res.* 9: 103-112.

Kim, S.J., Tsuji, M., Kubota, S., Wei, Q., Lee, J.M., Ishihara, C., Onuma, M., 1998. Sequence analysis of the major piroplasm surface protein gene of benign bovine *Theileria* parasites in East Asia. *Int. J. Parasitol.* 28: 1219-1227.

Knowles D.P., Kappmeyer L.S., Perryman L.E., 1997. Genetic and biochemical analysis of erythrocyte-stage surface antigens

belonging to a family of highly conserved proteins of *Babesia equi* and *Theileria* species. *Mol. Biochemic. Parasitol.* 90: 69-79.

Kubota, S., Sugimoto, C., Kakuda, T., Onuma, M., 1996a. Analysis of Immunodominant Piroplasm Surface Antigen Allele in Mixed Populations of *Theileria sergenti* and *T. buffeli*. *Int. J. Parasitol*. 26: 741-747.

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

Lee, J.M., Kim, M.C., 1987. Studies for the Effective Diagnosis and Treatment of Bovine Piroplasmosis. *Korean J. Vet. Res*. 27: 321-330.

Onuma, M., Kakuda, T., Sugimoto, C., 1998. *Theileria* parasites infection in East Asia and control of the disease. *Comp. Immun. Microbiol. Infect. Dis.* 21: 165-177.

Sarataphan, N., Nilwarangkoon, S., Tananyutthawongese, C., Kakuda, T., Onuma, M., Chansiri, K., 1999. Genetic Diversity of Major Piroplasm Surface Protein Genes and Their Allelic Variants of

Theileria Parasites in thai Cattle. *J. Vet. Med. Sci.* 61: 991-994.

Sarataphan, N., Kakuda, T., Chansiri, K., Onuma, M., 2003. Survey of Benign *Theileria* Parasites of Cattle and Buffaloes in Thailand using Allele-Specific Polymerase Chain Reaction of Major Piroplasm Surface Protein Gene. *J. Vet. Med. Sci.* 65(1): 133-135.

Song, K.H., Sang B.C., 2003. Prevalence of *Theileria sergenti* infection in Korean native cattle by polymerase chain reaction. *Korean J. Parasitol.* 41(3): 141-145.

Stockham, S.L., Kjemtrup, A.M., Conrad, P.A., Schmidt, D.A., Scott, M. A., Robinson, T.W., Tyler, J.W., Johnson, G.C., Carson, C.a., Cuddihee, P., 2000. Theileriosis in a Missouri Beef Herd Caused by *Theileria buffeli*: Case Report, Herd Investigation, Ultrastructure, Phylogenetic Analysis, and Experimental Transmission. *Vet. Pathol.* 37: 11-21.

Tsuji, M., Arai, S., Nakamura, Y., Kim, S.J., Cho, S.H., He, F.Q., Ishihara, C., 1999. Preparation of Antibodies Directed to the *Babesia ovata*- or *Theileria sergenti*- prasitized Erythrocytes. *J. Vet. Med. Sci.* 61: 73-76.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Wang, C.T., Kubota, S., Kakuda, T., Kuo, C.C., Hsu, T.L., Onuma, M., 1997. Survey of *Theileria* Parasite Infection in Cattle in Taiwan. *J. Vet. Med. Sci.* 60: 253-255.

Zhuang WZ, Sugimoto C, Matsuba T, Niinuma S, Murata M, Onuma M. 1994. Analyses of antigenic and genetic diversities of *Theileria sergenti* piroplasm surface proteins. *J. Vet. Med. Sci.* 56: 469-473.

http://www.ebi.ac.uk/clustalw/

초　　록

제주 흑한우의 *Theileria* Major Piroplasm Surface Protein Gene 항원형의 다양성

(지도교수 : 이 경 갑)

고 명 순

제주대학교 일반대학원 수의학과

Piroplasm은 진드기가 매개하는 주혈원충으로, 허약, 발열, 빈혈 및 황달을 일으킨다. 한국과 일본에 분포하는 것은 *Theileria sergenti*와 *Babesia ovata*로, 방목중인 소의 증체률 저하로 인한 경제적 손실이 큰 것으로 알려져 있다.

본 연구는 제주농업시험장에서 생산, 방목 사육중인 제주 흑한우 35마리를 대상으로, RLB-F2/R2 pirmer set를 이용한 PCR과 *Kpn* I 를 이용한 PCR-RFLP을 실시하여 *Theileria* 와 *Babesia* sp의 혼합감염와 Allele-specific PCR를 하여 *Theileria*의 MPSP gene 항원형의 다양성에 대한 조사하였다.

35마리의 혈액학적 검사 결과는 정상범위에 속하였으며, Giemsa염

색한 혈액도말표본에서는 35마리 중 9마리에서 충체를 발견하였으나, 그 빈도는 매우 낮았다.

PCR결과 35마리 모두 *Theileria*에 감염되어있었으며, 그 중 20마리 (57.1%)가 *Theileria* 와 *Babesia* sp.의 혼합감염이었다.

*Theileria*의 MPSP gene 항원형에 대한 조사시, C (Chitose) type, I (Ikeda) type 과 B (Buffeli) type이 혼합감염되어 있었으며, 주된 type은 C와 B type이었다. B type에 대한 RFLP을 실시하여 변이형을 확인하였다.

주요어 : *Theileria, Babesia,* Major Piroplasm Surface Protein gene, 항원형의 다양성, 제주 흑한우