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Identification and sequence analysis of small subunit ribosomal RNA gene of bovine *Theileria* isolates from Korea and Japan

Joon-seok Chae[1], Jin-ho Park*, Oh-deog Kwon*, Suryakant D. Waghela, Patricia J. Holman, Gerald G. Wagner, Joo-mook Lee*

Department of Veterinary Pathobiology, College of Veterinary Medicine, Texas A&M University, College Station, Texas 77843, USA
*College of Veterinary Medicine, Chonbuk National University, Chonju, 561-756, Korea**
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한국과 일본 소에 감염된 *Theileria* 분리주의 small subunit ribosomal 유전자의 동정 및 분석

채준석[1] · 박진호* · 권오덕* · Suryakant D. Waghela, Patricia J. Holman, Gerald G. Wagner · 이주묵*

미국 텍사스 A&M 대학교 수의과대학
전북대학교 수의과대학*
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초 록 : 한국과 일본의 서로 다른 지역으로부터 소의 *Theileria* 분리주에 있어서 6가지 type(A부터 E 그리고 H)과 subtype(B1)의 small subunit ribosomal RNA(SSUrRNA) 유전자를 밝혔다. 이들 유전자 염기서열을 비교하여 본 바 염기서열의 위치 212~231, 261~270 그리고 632~690으로 3군데의 hypervariable region이 관찰되었다. SSUrRNA 유전자 염기서열 type A는 한국의 전북 분리주(KCB), 충남 분리주(KCN), 제주 분리주(KCJ) 그리고 실험실 보관주(KLS)와 일본의 Shintoku 분리주(JHS)인 5개의 분리주에서 나타났으며, 이 염기서열은 Kenya의 Marula 분리주인 *Theileria buffeli*의 SSUrRNA 유전자(GenBank accession number Z15106)와 일치하였다. 한국의 경북 분리주(KKB)에서는 type B만이 관찰되었으나 그 외의 분리주에서는 2 type 이상의 유전자 염기서열이 관찰되었다. KCB와 JHS 분리주에서는 type A와 B, 강원 분리주(KKW)에서는 type B와 H, KCN 분리주에서는 type A, C 및 D 그리고 KCJ 분리주에서는 type A, B, E 및 subtype B1이 관찰되었다. 한국과 일본 소의 *Theileria* 분리주에 있어서 여러 type의 SSUrRNA 유전자 염기서열이 나타나는 것으로 보

[1]Present address : Department of Medicine and Epidemiology, School of Veterinary Medicine, University of California, Davis, CA 95616, USA.

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Address reprint requests to Dr. Joo-mook Lee, College of Veterinary Medicine, Chonbuk National University, Chonju 561-756, Republic of Korea.

아 혼합감염이 되어 있는 것으로 판단된다.

Key words : *Theileria* spp., bovine, small subunit ribosomal RNA, PCR, sequencing.

Introduction

The current taxonomic relationships within the *Theileria sergenti/buffeli/orientalis* group[1] are not clear. *T sergenti*[2] was the first of the group to be described, from cattle in eastern Siberia. *T orientalis*[3] was isolated from cattle in the same area, and was described as being distinct from *T sergenti*, based on the ill-defined criterion that it had less variability in piroplasm morphology. However, the speciation of *Theileria* parasites by microscopic morphology is problematic; piroplasms are commonly highly variable, some are long and thin while others are round, and mixed infections are now known to be common, further confounding the identification. In addition, the schizonts of many species are rare, so their presence or absence among species has been a source of confusion. As a result of these uncertainties many workers since 1931 have suggested that *T orientalis* and *T sergenti* are one and the same species.

T buffeli[4] was first described from the Asiatic buffalo *Bubalus bubalis*. The name was little used until 1984, when it was applied in Australia to a *Theileria* species which had been isolated from cattle in 1976 and which was found to be transmissible to cattle from Asiatic buffalo[5,6]. Morel and Uilenberg[7] suggested that *T buffeli* was the same as *T orientalis* and *T sergenti*, and in 1985 the name *T orientalis* was recommended for all three species based on morphologic and serologic examination and vector tick transmission experiments[1].

Molecular analyses provide another means of characterizing related organisms. Phylogenetic relationships based on the small subunit ribosomal RNA(SSUrRNA) gene sequences have been recently reported for several *Theileria* species, but SSUrRNA gene sequences of bovine *Theileria* isolates presumptively identified as *T sergenti* or *T orientalis* were not included[8,9]. Previously we showed the presence of five sequence types(A through E) among bovine *Theileria* isolates from Korea and Japan based on the V4 variable region of the SSUrRNA gene[10]. In this study, sequence types A through E were confirmed among the whole SSUrRNA gene sequences of the Korean and Japanese *Theileria* isolates. Two new sequences, type H and a subtype B1, were also identified in the whole SSUrRNA genes of some Korean isolates.

Materials and Methods

Parasites and preparation of DNA : Each DNA sample used in this study for amplification of the SSUrRNA gene was derived from a blood sample collected from a single animal as previously described[10,11]. The field samples were collected from cattle showing no apparent clinical signs of the disease, and were from Kimje in Chonbuk(KCB), Kyoungbuk (KKB), Chungnam(KCN), Kangwon(KKW) and Cheju Island (KCJ) in Korea(Fig 1). The other isolates included were Korea laboratory stock (KLS) from Changsu in Chonbuk, and a bovine *Theileria* stock from Shintoku in Hokkaido, Japan(JHS)(Fig 1), that was kindly provided by Dr. M. Onuma, Faculty of Veterinary Medicine, Hokkaido University, Japan. DNA was extracted[12] from parasites purified by banding in 40~60% Percoll solution[13]. *Babesia bovis* DNA from cultured parasites served as a positive or negative control for PCR amplification based on the primers used.

PCR amplification of SSUrRNA genes : *Theileria* SSUrRNA genes were amplified from 5~50ng of purified DNA using primers A and B described by Sogin[14] for amplifying eukaryotic SSUrRNA genes. To confirm that presumptive *Theileria* SSUrRNA gene amplicons were in fact *Theileria* genes, the amplicons were subjected to amplification using internal primers 989 and 990[15]. The PCR conditions were as

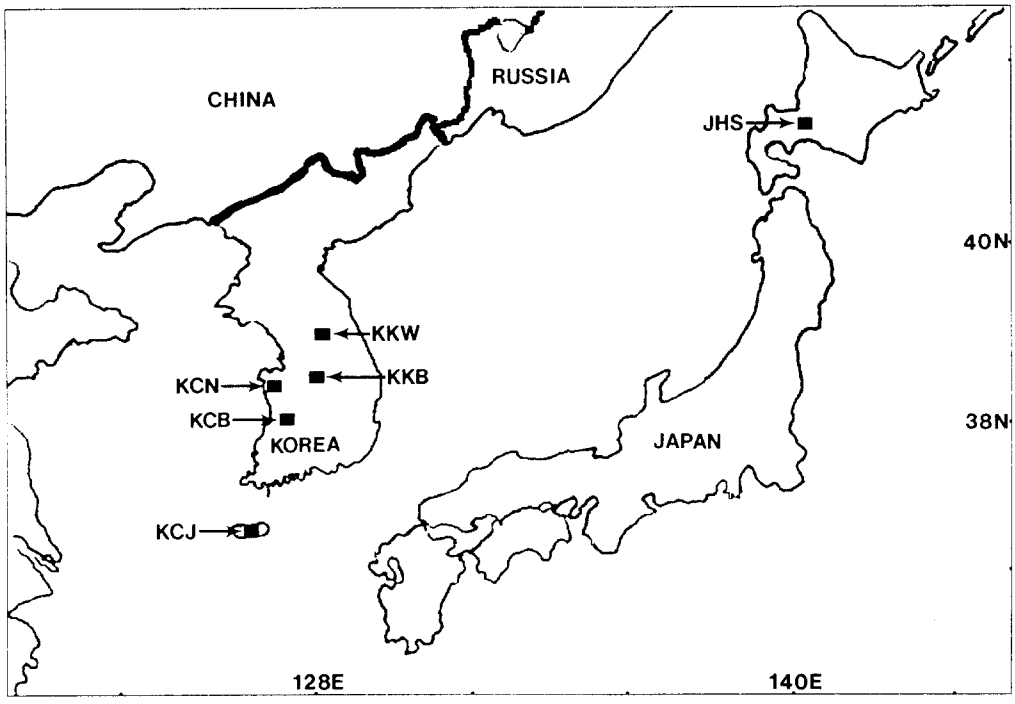


Fig 1. Map of Korea and Japan showing the general geographic locales of blood sample acquisitions for the bovine *Theileria* isolates. Isolates from Korea were from Kimje, Chonbuk(KCB), Kyoungbuk(KKB), Chungnam(KCN), Kangwon(KKW), and Cheju Island (KCJ). The isolate from Japan was from Shintoku, Hokkaido(JHS).

previously described with an annealing temperature of 60℃[16], except that the 72℃ extension step was progressively increased by 30 secs with each cycle in a PTC-200 Peltier Thermal cycler(MJ Research, Watertown, Massachusetts, USA). The PCR products were separated by electrophoresis through a 1% agarose gel and visualized by staining with ethidium bromide.

Cloning and sequencing : The amplified DNA products were directly ligated into the plasmid vector pCR™II and INVαF' One Shot™ competent cells transformed according to manufacturer recommendations(TA Cloning® Kit; In-Vitrogen, San Diego, California, USA). Transformed clones were color-selected and each colony subjected to amplification with primers A and B to ensure that the correct-sized DNA insert was present in the recombinant plasmid. For colony amplification, a portion of each selected colony was mixed in 5μl ddH_2O and amplified in a 25μl reaction volume as above but without auto-extension. Two clones of each type identified previously as type A, B, C, D or E[10] and the new type H and subtype B1 were arbitrarily chosen to sequence the forward and reverse strands. Small scale preparations of plasmid DNA for sequencing were made by a modified alkaline lysis protocol[17].

A primer complementary to the T7 promotor region of the plasmid vector(Stratagene, La Jolla, California, USA) and a series of previously described internal primers[18] were used in the sequencing reactions(Cyclist™ Exo-*Pfu* DNA Sequencing Kit; Stratagene, La Jolla, California, USA) to sequence the complete forward and reverse strands.

Sequence analysis : The SSUrRNA gene sequences obtained from the Korean and Japanese *Theileria* isolates were aligned and compared using CLUSTAL W (Version 1.60) multiple sequence alignment program[19] and MACAW multiple alignment construction and analysis workbench(Version 2.05 Win 32i)[20]. The obtained sequences were subjected to BLAST searches[21] in the GenBank database(National Center

for Biotechnology Information, National Institute of Health).

Results

The SSUrRNA genes amplified by primers A and B were visualized by agarose gel electrophoresis as a prominent single band of about 1.8kb for each of the 7 *Theileria* isolates from Korea(KLS, KCB, KKB, KCN, KKW and KCJ) and Japan(JHS)(Fig 2). *Babesia bovis* DNA amplified with primers A and B resulted in a product of approximately 1.7kb (Fig 2). Amplification of the SSUrRNA gene of *Theileria* spp. with primers 989 and 990 resulted in a band of 1.1 kb for each *Theileria* isolate, but *B bovis* DNA did not yield an amplicon with these primers(Fig 2). Two clones were selected for each type and subtype previously reported from the SSUrRNA gene variable(V4) region to sequence the complete forward and reverse strands of the SSUrRNA gene. The complete gene sequences were obtained by overlapping gene sequence segments obtained from the internal primers.

Six different sequence types, A through E and H, and a subtype, B1(Table 1 and Fig 3), of the SSUrRNA gene of bovine *Theileria* isolates from different geographic areas in Korea and Japan(Fig 1) were identified. The SSUrRNA gene types varied in size from 1,740 to 1,750 base pairs as follows: A(1,740), B(1,748), C(1,750), D(1,741), E(1,747) and H(1,746). The aligned sequences had a total of 1,753 positions including gaps generated by the CLUSTAL W multiple sequence alignment program and three hypervariable regions were located between nucleotide positions 212 and 231, 261 and 270, and 632 and 690. Microheterogeneity was observed in type B and subtype B1 which shared the same number of base pairs, 1,748, but differed in nucleotides at positions 648, 653, 750, 778 and 788.

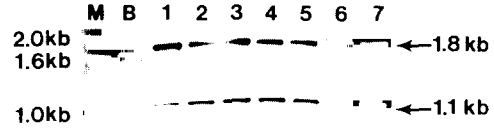
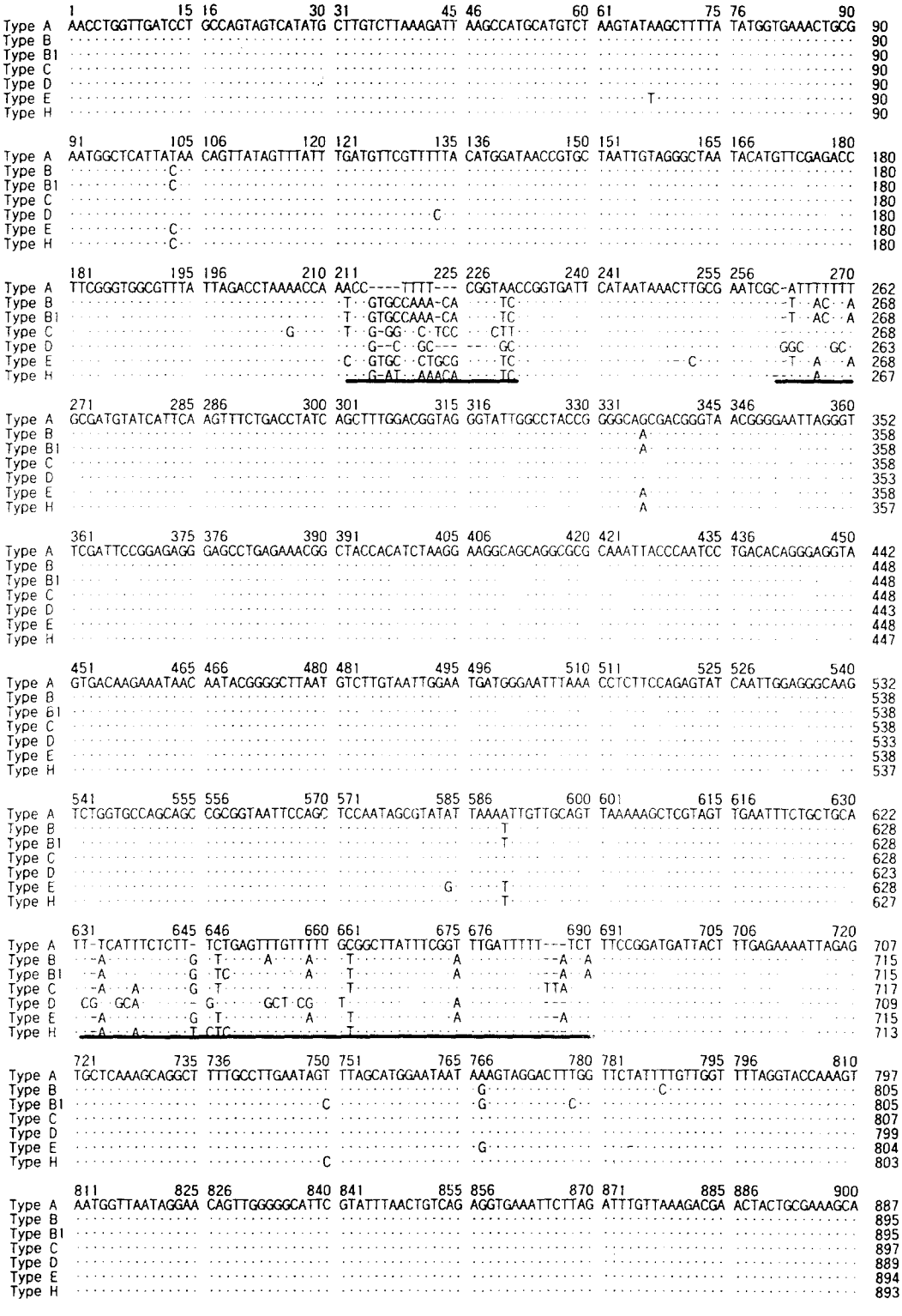


Fig 2. Ethidium bromide stained-agarose gel of small subunit ribosomal RNA (SSUrRNA) gene amplicons from bovine *Theileria* isolates from Korea and Japan. Amplicons at approximately 1.8kb resulted from amplification of parasite genomic DNA with primers A and B. Amplicons at approximately 1.1kb resulted from nested amplification of the SSUrRNA gene amplicons with primers 989 and 990(internal primers specific for *Theileria* SSUrRNA genes). Lane M; 1kb DNA ladder(Gibco), lane B; *Babesia bovis*, lane 1; Korea laboratory stock(KLS), lane 2; Kimje, Chonbuk, Korea(KCB), lane 3; Kyoungbuk, Korea(KKB), lane 4; Chungnam, Korea (KCN), lane 5; Kangwon, Korea(KKW), Lane 6; Cheju Island, Korea(KCJ), lane 7; Bovine *Theileria* isolate from Shintoku, Hokkaido, Japan(JHS).

Table 1. SSUrRNA gene sequence types in the bovine *Theileria* isolates from Korea and Japan. Korean isolates were from Kimje, Chonbuk(KCB), Kyoungbuk(KKB), Chungnam(KCN), Kangwon(KKW), and Cheju Island(KCJ). The isolate from Japan was from Shintoku, Hokkaido(JHS).

Country	Isolate	Sequence types					
		A	B	C	D	E	H
Korea	KLS	+	-	-	-	-	-
	KCB	+	+	-	-	-	-
	KKB	-	+	-	-	-	-
	KCN	+	-	+	+	-	-
	KKW	-	+	-	-	-	+
	KCJ[a]	+	+	-	-	+	-
Japan	JHS	+	+	-	-	-	-

[a] KCJ also had a subtype B1.



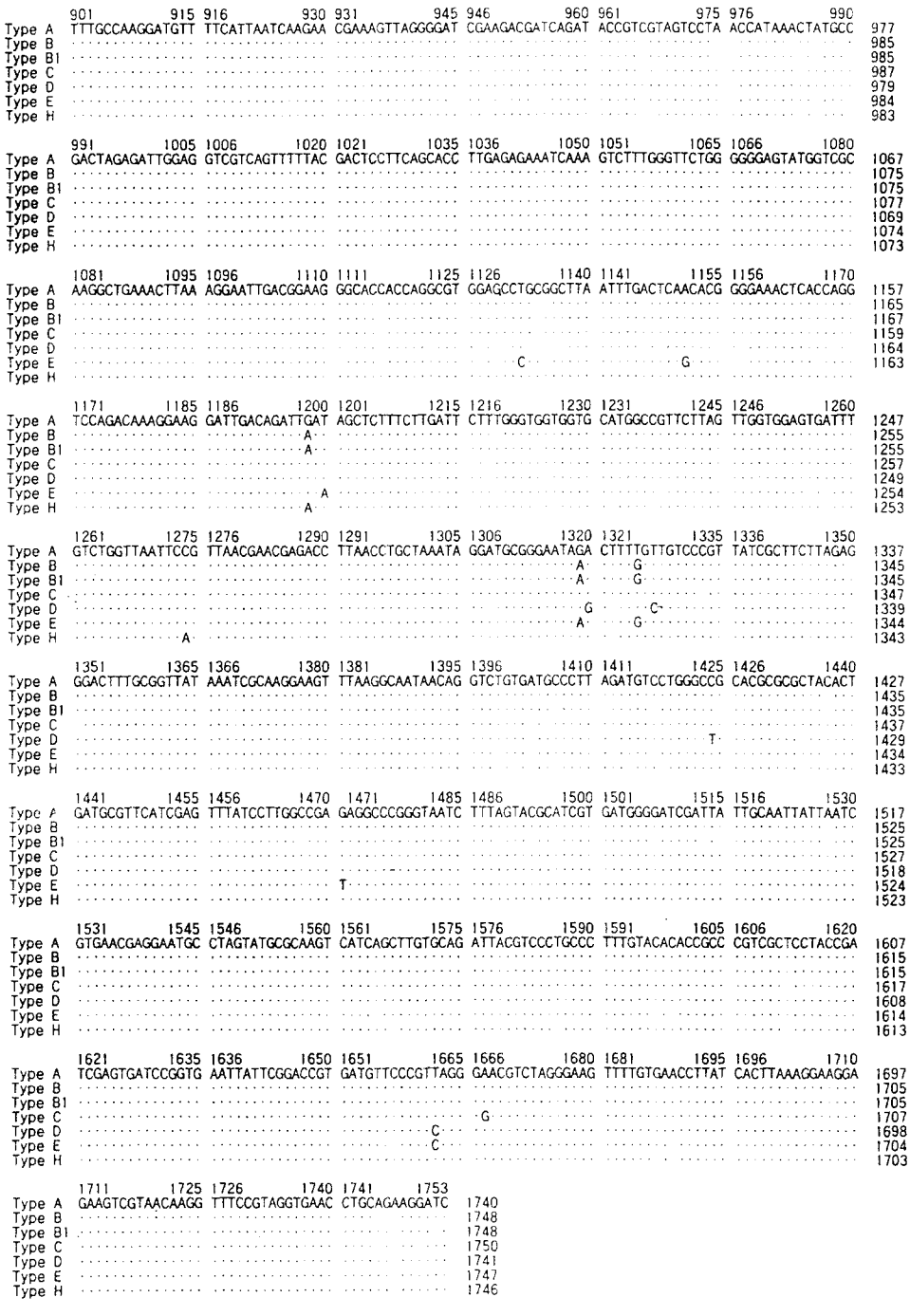


Fig 3. Sequence alignment of complete small subunit ribosomal RNA gene types A through E and H and a subtype B1(shown as type B1) from bovine *Theileria* isolates from Korea and Japan. Gaps(-) represent spaces introduced into the aligned sequences by the CLUSTAL W multiple sequence alignment program to maximize homology between the aligned sequences. Bold lines indicate hypervariable regions among the types located by the MACAW multiple alignment construction and analysis workbench program(Version 2.05 Win 32i). Print nucleotides in types B through E and H and B1 sequences vary from the type A sequence. GenBank Accession Nos.: type A; U97047, type B; U 97048, type B1; U97049, type C; U97051, type D; U97052, type E; U97053, type H; U97050.

Type A was found in five isolates, KLS, KCB, KCN and KCJ from Korea and JHS from Japan, and was identical to the sequence reported for *T buffeli* (GenBank accession No. Z15106[8]) isolated from cattle in Marula, Kenya.

The number of SSUrRNA gene sequence types or subtypes found within an isolate varied from one to four(Table 1). Only the type B SSUrRNA gene sequence was found in the KKB isolate. The KCB and JHS isolates had types A and B. The KKW isolate had types B and H. The KCN isolate had types A, C and D. The KCJ isolate had types A, B, E and subtype B1. Korean isolates had all types plus the B1 subtype of the SSUrRNA gene, but only types A and B were found in the Japanese isolate.

Discussion

A previous study[10] of *Theileria* isolates from cattle, deer and elk indicated sequence heterogeneity in the V4 region of the SSUrRNA gene fragment among and within the isolates, irrespective of geographic source. Here, the presence of types A through E in the bovine isolates from Korea and Japan were confirmed in the full SSUrRNA gene sequence. An additional type H and a subtype B1 were also found among these isolates. The type A sequence[22] was the most prevalent and was found in five of the seven bovine *Theileria* isolates examined in the present work.

The type A sequence is identical to the SSUrRNA gene sequence first reported for a bovine *Theileria* isolate from Marula, Kenya(GenBank Accession No. Z15106), which was not identified by a species name[15]. The GenBank identification of *T buffeli* for this sequence was based on the original designation of this isolate as *T buffeli* [23]. However, in a later study of this isolate[24], the authors suggest that it could be a species of low pathogenicity of the *T sergenti/buffeli/orientalis* group. The latter study did not include molecular characterization. We find that the type A SSUrRNA gene sequence occurs in the following isolates: Korean parasites KLS, KCB, KCN and KCJ, and Japanese isolate JHS.

The present work shows that bovine *Theileria* isolates from both Korea and Japan may consist of mixed populations since multiple SSUrRNA gene sequences were found in five of the six isolates. This finding supports an earlier suggestion that *T sergenti/buffeli* distributed in Japan and Australia is a mixture of parasites with various combinations of four different allelic types for the p33/32 and p 34 genes[25]. Bovine *Theileria* species from Korea, Japan, China and Australia have been classified into six types by aligning nucleotide sequences of the p32/34 genes[26].

Serologic cross reactions between stock *T sergenti* (Ikeda) from Japan and *Theileria* isolates from Korea have been reported[27]. The *Theileria* species found in Korea that is moderately pathogenic for cattle has also been designated *T sergenti*. Although *T mutans* has been reported in cattle in Korea[28], it was later argued to be *T sergenti* because the vector tick, *Haemaphysalis longicornis*, is highly prevalent in Korea[29].

It has been proposed that *T sergenti* (Japan), *T buffeli* (Australia) and *T orientalis* (Great Britain), be classified into two groups, *T sergenti* and *T buffeli/T orientalis*, respectively[30]. The proposal has been based on compiled data from tick transmission experiments[31,32], serologic dissimilarities[33], and sequence analysis of genes coding for immunodominant surface proteins[34]. On the other hand, the name *T orientalis* has been recommended for all three species based on morphologic and serologic examination and vector tick transmission experiments[1]. It is impossible, at this distance in time, to obtain the original material to which the names *T sergenti*, *T buffeli* and *T orientalis* were given. Any future characterization of the *Theileria* species in this group will therefore undoubtedly draw on the SSUrRNA sequence data, since these are the only data which unequivocally show differences between many of the isolates in the group.

Summary

Six different sequences types(A through E and H) and a subtype(B1) of the small subunit ribosomal RNA(SSUrRNA) gene were found in bovine *Theileria* isolates from different areas of Korea and Japan. The sequences were aligned and three hypervariable regions were observed in the nucleotide

position ranges 212~231, 261~270 and 632~690. Five of the *Theileria* isolates yielded sequence type A; these were the field isolates KCB, KCN, and KCJ, and the laboratory stock KLS, all from Korea, and a single isolate from Japan (JHS). This sequence type is identical to the SSUrRNA gene sequence listed for *Theileria buffeli* (GenBank Accession No. Z15106) from Marula, Kenya. The Korean field isolate KKB yielded only a single sequence type (B), but multiple sequence types were found in some isolates. For example, KCB and JHS isolates yielded both types A and B; isolate KKW showed types B and H; isolate KCN showed types A, C, and D; and isolate KCJ showed types A, B, E, and a subtype B1. Finding of the multiple sequences SSUrRNA gene sequences suggests that bovine *Theileria* isolates from both Korea and Japan may consist of mixed populations.

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