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Identical small subunit ribosomal RNA gene nucleotide sequence of bovine *Theileria* isolates (Korea and Japan) and *Theileria buffeli* (Marula, Kenya)

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Abstract: Small subunit ribosomal RNA (SSU rRNA) gene nucleotide sequences of bovine *Theileria* isolates from Korea (KLS and KCB) and Japan (JHS) were determined. The genes from each isolate were amplified by the polymerase chain reaction and the approximately 1.8 kb product cloned and sequenced by a modified dideoxynucleotide method. Overlapping gene segments produced with a series of primers were sequenced, resulting in a complete DNA sequence for both forward and reverse strands of the SSU rRNA genes of each isolate. SSU rRNA gene sequences (termed Type A) were identical among the bovine *Theileria* isolates from Korea and the isolate from Japan. A GenBank data library homology search showed the sequence to be the same as that listed as *Theileria buffeli* isolated from cattle in Marula, Kenya.

Key words: *Theileria sergenti*, *Theileria buffeli*, small subunit ribosomal RNA gene, DNA sequence, PCR

INTRODUCTION

The phylum Apicomplexa includes numerous and diverse hemoprotozoan parasites of vertebrates (Levine, 1988) including the tick-borne piroplasms, *Theileria* and *Babesia*, which cause economic losses in domestic and wild animals throughout the world (Brown *et al.*, 1990). While classification techniques of microorganisms by life cycle observations and microscopic, ultrastructural, and/or antigenic analyses have allowed some piroplasms to be assigned binomens as species within the *Theileria* or *Babesia* genera, the taxonomic status of other isolates remains equivocal.

The taxonomic relationships among the collective members of the *Theileria orientalis/sergenti/buffeli* group are particularly unclear at present. *Theileria sergenti*, a moderately pathogenic organism, was originally isolated from cattle in eastern Siberia and was the first of the group to be described (Yakimoff and Dekhtereff, 1930). *Theileria orientalis* (Yakimoff and Soudatschenkoff, 1931) was also isolated from cattle in Siberia, and was thought to be

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different from *T. sergenti*. *Theileria buffeli* was first described from the Asiatic buffalo, *Bubalus bubalis* (Neveu-Lemaire, 1912). The name *T. buffeli* has since been applied to apparently non-pathogenic bovine *Theileria* isolates in Australia, Africa, Indonesia and China. Uilenberg *et al.* (1985) recommended the name *T. orientalis* for the three species known as *T. sergenti*, *T. orientalis* and *T. buffeli* based on morphological and serologic examination and experimental tick transmission.

Rather than relying on morphologic criteria alone, polypeptide or nucleotide sequence comparisons offer additional means of evaluating phylogenetic relationships among lower organisms (Willson *et al.*, 1977). A unique tool for phylogenetic comparisons is provided by the sequence structure of ribosomal RNA which encodes a comprehensive record of evolutionary history (Pace *et al.*, 1986; Gajadhar *et al.*, 1991). Phylogenetic relationships among a number of *Theileria* species based on the nucleotide sequence of the small subunit ribosomal RNA (SSU rRNA) gene have been reported (Ellis *et al.*, 1992; Allsopp *et al.*, 1993 & 1994) but the relatively benign bovine *Theileria* spp., presumptively identified as *T. sergenti* or *T. orientalis*, were not included.

Recently, we sequenced the V4 variable region fragment of the SSU rRNA genes of bovine *Theileria* isolates from Korea, Japan, and USA (Chae *et al.*, 1997). We found five sequence types, one of which (designated Type A) was identical to the SSU rRNA gene sequence reported for *T. buffeli* (Marula, Kenya, GenBank Accession No. Z15106). In the current study, we sequenced representative SSU rRNA genes in the previously reported Korean and Japanese bovine *Theileria* isolates to determine if the Type A V4 fragments represent genes identical in total sequence to the *T. buffeli* SSU rRNA gene.

MATERIALS AND METHODS

Parasites and preparation of DNA

The bovine *Theileria* isolates used in this study have been previously described (Chae *et al.*, 1994 & 1996). Included were Korea laboratory stock (KLS) from Changsu-gun in Chonbuk, a field isolate from Kimje-gun in Chonbuk, Korea (KCB), and Japanese stock from Shintoku in Hokkaido (JHS). Each blood sample was collected from single bovine host. Purified parasites of KLS and JHS were collected by banding in 40-60% Percoll solution (Sugimoto *et al.*, 1991) and the DNA extracted from the purified parasites by standard procedures (Sambrook *et al.*, 1989). KCB piroplasm DNA was extracted from infected blood following the method of Allsopp *et al.* (1989).

PCR amplification of SSU rRNA genes

SSU rRNA genes were amplified from 5-50 ng of purified *Theileria* DNA using primers A and B (Sogin, 1990). PCR conditions were as previously described with an annealing temperature of 60°C (Allsopp *et al.*, 1989), except that the 72°C extension step was progressively increased by 30 sec with each cycle (autoextension) for a total of 30 cycles in a programmed heating block (MJ Research, Watertown, Massachusetts, USA). The PCR products were visualized by electrophoresis through a 1% agarose gel stained with ethidium bromide.

Cloning and sequence analysis

The amplicons were ligated into the plasmid vector pCR™ II, and INV α F' One Shot™ com-

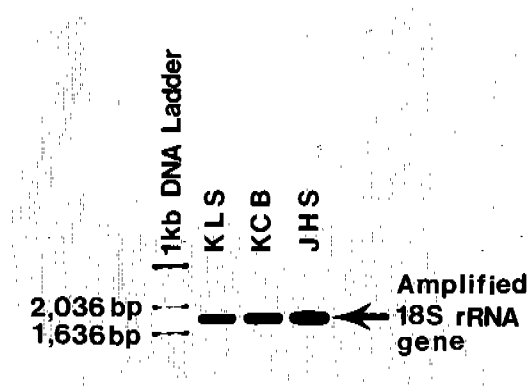

Fig. 1. Agarose gel stained with 2% ethidium bromide of amplified small subunit ribosomal RNA (18S rRNA) genes from a bovine *Theileria* laboratory stock from Changsu-gun Chonbuk, Korea (KLS), a bovine *Theileria* sp. from Kimje-gun Chonbuk, Korea (KCB), and a bovine *Theileria* sp. from Shintoku in Hokkaido, Japan (JHS). The first lane contains a 1 kb DNA ladder marker (Gibco-BRL).

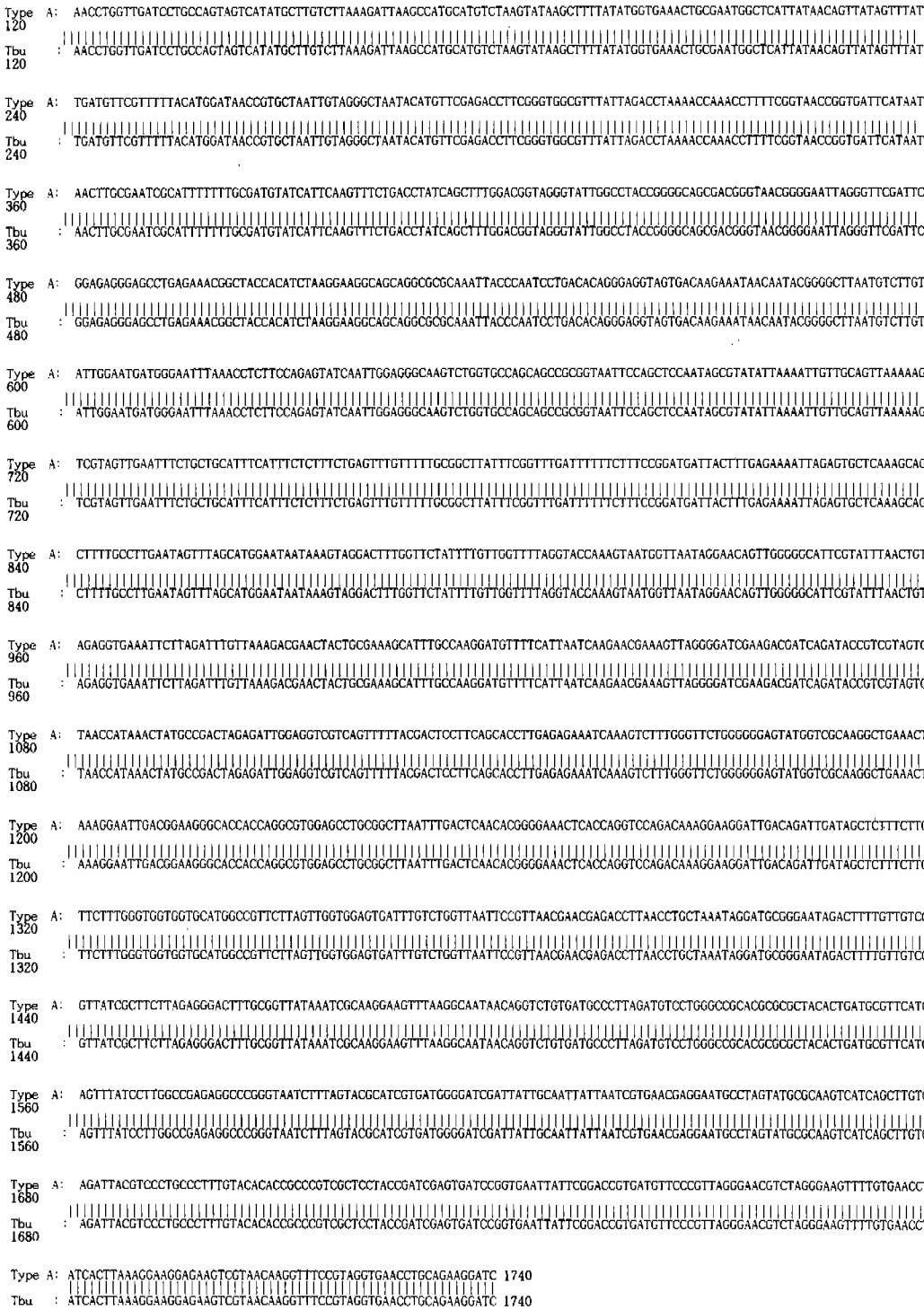

Fig. 2. Sequence alignment of the Type A small subunit ribosomal RNA gene sequence (GenBank Accession No. U97047) from the Korean (KLS and KCB) and Japanese (JHS) *Theileria* isolates with the corresponding gene sequence from *Theileria buffeli* from the GenBank database (GenBank Accession No. Z15106).

petent cells were then transformed following manufacturer's protocols (TA Cloning Kit; InVitrogen®, 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 insert was present as follows. A portion of each selected colony was mixed in 5 μl ddH_2O and amplified in a 25 μl reaction volume as above (without auto-extension). Two clones for each isolate, identified as KLS4, KLS6, KCB9, KCB11, JHS1 and JHS2, were chosen to sequence the forward and reverse strands. Small scale preparations of plasmid DNA for sequencing were made by a modified alkaline lysis protocol (Sambrook *et al.*, 1989).

Sequencing reactions (Cyclist™ Exo⁻*Pfu* DNA Sequencing Kit; Stratagene®, La Jolla, California, USA) with a T7 primer complementary to the promotor region of the plasmid vector (Stratagene®) and a series of previously described internal primers (Elwood *et al.*, 1985) were used to sequence the complete forward and reverse strands. A GenBank database BLAST search (National Center for Biotechnology Information) was conducted with the obtained sequence.

RESULTS

The amplified SSU rRNA gene was visualized by agarose gel electrophoresis as a prominent single band of about 1.8 kb for each of the three *Theileria* isolates from Korea and Japan (KLS, KCB and JHS) (Fig. 1). An identical nucleotide sequence (termed Type A) was found in all three isolates. This sequence was identical to the SSU rRNA gene sequence reported for *T. buffeli* (GenBank Accession No. Z15106) (Allsopp *et al.*, 1994) isolated from cattle in Marula, Kenya (Fig. 2). The sequences were aligned by overlapping and determined to be 1,740 nucleotides in length. The nucleotide sequences obtained in this study have been submitted to the GenBank database (Accession No. U97047).

DISCUSSION

Complete nucleotide sequences of SSU rRNA genes from three geographic isolates of bovine *Theileria* from Korea and Japan were identical to that of the Marula, Kenya, isolate of *T. buffeli* (GenBank Accession No. Z15106) (Allsopp *et al.*, 1994). High homology (99.5% identity by the ALIGN program) was also found between this sequence (termed Type A in our study) and that of *T. buffeli* Warwick stock (GenBank Accession No. AB000272). However, lesser homology (98.3% identity) was seen when *T. buffeli* Marula sequence was compared with that of *T. sergenti* Ikeda (GenBank Accession No. AB000271). Other genetic analysis, together with some antigenic analysis, also suggests dissimilarities between *T. sergenti* Ikeda and *T. buffeli* Warwick (Kawazu *et al.*, 1992a & 1995).

T. buffeli, considered nonpathogenic, is prevalent in Australia, Indonesia, and Indochina (Uilenberg *et al.*, 1985). Stocks of benign *Theileria* isolates from cattle in Japan, Australia, Great Britain, Iran, and the United States, representing the *T. sergenti/buffeli/orientalis* group, are serologically and morphologically indistinguishable from a more pathogenic stock in Korea (Uilenberg *et al.*, 1985). Uilenberg and others have proposed the union of these isolates into one species, *T. orientalis* (1985). Our results based on SSU rRNA gene sequence analyses show that these Korean (KLS and KCB) and Japanese (JHS) *Theileria* parasites are also very closely related to *T. buffeli* Marula.

The identification of identical SSU rRNA gene sequences of the *Theileria* isolates from Korea (KLS and KCB) and Japan (JHS) appear in conflict with the genomic dissimilarities found among some isolates by Southern blot analysis (Chae *et al.*, 1996). Onuma (1996) reported that many cattle from Eastern Asia were infected with a mixed population of bovine *Theileria* parasites, and classified 6 types based on the p32/34 gene sequence. Our previous study indicated the probability of mixed infections since multiple SSU rRNA gene sequence types based on the V4 variable region were reported among Korean and Japanese bovine *Theileria* isolates (Chae *et al.*, 1997). Work is now underway to sequence the entire gene of each type identified. In this study, *Theileria* parasite DNA from KLS, KCB and JHS isolates was analysed based on the Type A SSU rRNA gene V4 region sequence,

and more sequence analysis is required to show the presence of multiple SSU rRNA gene sequence types in mixed infections.

Serologic methods and sequence analysis of genes encoding the immunodominant surface proteins showed distinction between *T. sergenti* Ikeda and *T. buffeli* Warwick (Kawazu *et al.*, 1992a, b & c). However, Ngumi *et al.* (1994) indicated that the Marula isolate could not be clearly identified as *T. buffeli* using serologic methods. These differences may be explained by isolate or strain variation, or, possibly, the presence of mixed genotypes in a parasite population (isolate). This is supported by the SSU rRNA gene variable region sequence differences found within isolates of bovine *Theileria* (Chae *et al.*, 1997). Also intraspecific sequence diversity in genomic DNA among isolates of *T. parva* has been documented (Allsopp *et al.*, 1988).

Clearly, the taxonomy of the cosmopolitan benign *Theileria* spp. remains unresolved. Fundamental to understanding the relationships at the molecular level among these piroplasms will be the identification and characterization of multiple defined isolates from similar geographic locales as well as different stocks from diverse geographic locations. An understanding of the evolutionary relationships among different piroplasms within Apicomplexa will significantly aid the intensive studies currently aimed at the development of efficacious recombinant vaccines against economically important members of these genera.

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REFERENCES

Allsopp BA, Allsopp MTEP (1988) *Theileria parva*: genomic DNA studies reveal intra-specific sequence diversity. *Mol Biochem Parasitol* **28:** 77-84.

Allsopp BA, Baylis HA, Allsopp MTEP, *et al.* (1993) Discrimination between six species of *Theileria* using oligonucleotide probes which detect small subunit ribosomal RNA sequences. *Parasitology* **107:** 157-165.

Allsopp BA, Carrington M, Baylis HA, Sohal S, Dolan T, Iams K (1989) Improved characterization of *Theileria parva* isolates using the polymerase chain reaction and oligonucleotide probes. *Mol Biochem Parasitol* **35:** 137-148.

Allsopp MTEP, Cavalier-Smith T, De Waal DT, Allsopp BA (1994) Phylogeny and evolution of the piroplasms. *Parasitology* **108:** 147-152.

Brown CDG, Hunter AG, Luckins AG (1990) Disease caused by protozoa. *In* Handbook on Animal Diseases in the Tropics, Sewell MMH, Brocklesby DW (eds.). p161-226, Baillie re Tindall, London, UK.

Chae JS, Lee JM, Kwon OD, Chae KS (1994) Genomic DNA probe and purification of *Theileria sergenti* merozoites in Korean cattle. *Korean J Vet Res* **34:** 387-394.

Chae JS, Lee JM, Kwon OD, Lee SO, Chae KS, Onuma M (1996) Comparative analysis of *Theileria sergenti* isolated from Korea and Japan by Southern hybridization and polymerase chain reaction. *Korean J Vet Res* **36:** 187-193.

Chae JS, Lee JM, Kwon OD, Holman PJ, Waghela SD, Wagner GG (1997) 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*: (in press).

Ellis J, Hefford C, Baverstock PR, Dalrymple BP, Johnson AM (1992) Ribosomal DNA sequence comparison of *Babesia* and *Theileria*. *Mol Biochem Parasitol* **54:** 87-96.

Elwood HJ, Olsen GJ, Sogin ML (1985) The small subunit ribosomal RNA gene sequences from the hypotrichous ciliates *Oxytricha nova* and *Stylonychia pustulata*. *J Mol Biol Evol* **5:** 399-410.

Gajadhar AA, Marquardt WC, Hall R, Gunderson J, Ariztia-Carmona EV, Sogin ML (1991) Ribosomal RNA sequences of *Sarcocystis muris*, *Theileria annulata* and *Crypthecodiu cohnii* reveal evolutionary relationships among apicomplexans, dinoflagellates, and ciliates. *Mol Biochem Parasitol* **45:** 147-154.

Kawazu S, Kamio T, Sekizaki T, Fujisaki K (1995) *Theileria sergenti* and *Theileria buffeli*: polymerase chain reaction-based marker system for differentiating the parasite species from infected cattle blood and infected tick salivary

gland. *Exp Parasitol* **81:** 430-435.

Kawazu S, Sugimoto C, Kamio T, Fujisaki K (1992a) Antigenic differences between Japanese *Theileria sergenti* and other benign *Theileria* species of cattle from Australia (*T. buffeli*) and Britain (*T. orientalis*). *Parasitol Res* **78:** 130-135.

Kawazu S, Sugimoto C, Kamio T, Fujisaki K (1992b) Molecular cloning and immunological analysis of immunodominant piroplasm surface proteins of *Theileria sergenti* and *T. buffeli*. *J Vet Med Sci* **54:** 305-311.

Kawazu S, Sugimoto C, Kamio T, Fujisaki K (1992c) 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:** 169-176.

Levine ND (1988) Blood parasite: The piroplasms. *In* The Protozoan Phylum Apicomplexa. FL p35-45, CRC Press, Boca Raton, Florida, USA.

Neveu-Lemaire M (1912) Parasitologie des animaux domestiques. Maladies parasitaires non bactériennes. p288-289, J. Lamarre et Cie, Paris, France.

Ngumi PN, Lesan AC, Williamson SM, *et al.* (1994) Isolation and preliminary characterization of a previously unidentified *Theileria* parasite of cattle in Kenya. *Res Vet Sci* **57:** 1-9.

Onuma M (1996) Workshop summary: Haemospordia. *Vet Parasitol* **64:** 167-169.

Pace NR, Olsen GJ, Woese CR (1986) Ribosomal RNA phylogeny and the primary line of evolutionary descent. *Cell* **45:** 325-326.

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning, a Laboratory Manual. 2nd ed. p9.14-9.23, Cold Spring Harbor Laboratory Press, New York, USA.

Sogin ML (1990) Amplification of ribosomal RNA genes for molecular evolution studies. *In* PCR Protocols, Innis MA, Gelfand DH, Suninsky JJ, White TH (ed.). p307-314, Academic Press, San Diego, USA.

Sugimoto C, Sato M, Kawazu S, Kamio T, Fujisaki K (1991) Purification of merozoites of *Theileria sergenti* from infected bovine erythrocytes. *Parasitol Res* **77:** 129-131.

Uilenberg G, Perie NM, Spanjer AAM, Franssen FFJ (1985) *Theileria orientalis*, a cosmopolitan blood parasite of cattle: demonstration of the schizont stage. *Res Vet Sci* **38:** 352-360.

Willson AC, Carlson SS, White TJ (1977) Biochemical evolution. *Ann Rev Biochem* **46:** 573-639.

Yakimoff WL, Dekhtereff NA (1930) Zur frage über die Theileriose in Ostsibirien. *Arch Protistenkd* **72:** 176-189.

Yakimoff WL, Soudatschenkoff WW (1931) Zur frage der piroplasmiden der rinder in Ost-Sibirien. *Arch Protistenkd* **75:** 179-190.

= 초록 =

한국과 일본의 소에서 분리한 *Theileria* 분리주와 *Theileria buffeli* (Marula, Kenya)의 small subunit ribosomal RNA 유전자 염기서열의 일치

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소의 주혈원충인 *Theileria* sp.의 small subunit ribosomal RNA (SSU rRNA) 유전자의 염기서열분석을 위해 한국의 전북 장수로부터 분리하여 계대보관 중인 실험실 보관주 (KLS)와 김제 분리주 (KCB), 그리고 일본의 Shintoku 분리주 (JHS)를 실험에 사용하였다. 이들 분리주로 부터 원충을 회수한 후 유전자를 추출하여 중합효소연쇄반응에 의해 1.8 kb의 SSU rRNA 유전자를 증폭시킬 수 있었으며, 증폭된 유전자를 이용하여 클론을 제작하고, 이들 클론으로 부터 플라스미드를 추출하여 유전자 염기서열분석을 실시하였다. 각 *Theileria* 분리주의 SSU rRNA 유전자의 염기서열분석은 forward와 reverse 양쪽 다 중복하여 실시하였으며 연속적인 primer를 이용하였다. 그 결과 한국의 소로부터 분리한 *Theileria* sp. (KLS, KCB)의 SSU rRNA 유전자 염기서열 (Type A로 명명하였슴)은 일본 분리주와 동일하였으며, 이 Type A를 GenBank로부터 유전자 검색을 해본 결과 Kenya의 Marula 분리주인 *Theileria buffeli*의 SSU rRNA 유전자 염기서열과 일치하였다.

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