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Production of *Theileria sergenti* recombinant protein by *E coli* expression system

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Abstract : As an attempt to develop an effective control method against theileriosis, recombinant antigen protein was produced. Thirty-two kDa membrane protein(MP) gene of *T sergenti* was amplified through RT-PCR from extracted total RNA of *T sergenti* isolated in Chonbuk, Korea. The amplified 869 bp of Korean *T sergenti* membrane gene was cloned and the base sequences were analyzed. The amplified gene was cloned into *E coli* expression vector, pQE32 plasmid vector, and the vector was introduced into *E coli* strain M15 to produce the recombinant membrane protein. For the induction of *T sergenti* membrane protein(KTs-MP), the plasmid harboring *E coli* strain M15 were cultured in the presence of IPTG, and the recombinant protein were purified by Ni^{+}-NTA agarose. Then, to confirm the authenticity of the produced membrane protein, molecular weight of expressed recombinant KTs-MP was analyzed by SDS-PAGE and Western blotting. The molecular weight of expressed recombinant protein was 32 kDa as expected. The recombinant KTs-MP was successfully recognized by anti-His Tag antibody, antisera of *T sergenti* infected cattle and monoclonal antibody of *T sergenti* membrane protein. Therefore, we concluded that the authentic 32 kDa membrane protein of *T sergenti* was produced as immunologically recognizable form.

Key words : *Theileria sergenti*, recombinant protein, *E coli* expression vector.

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Introduction

Theileria sergenti, a protozoan hemoparasite of vertebrate, is a causative agent of theileriosis in cattle. Many studies mentioned that *T sergenti* is spreaded over east Asia including Korea, Japan, and China[1]. Lee *et al*[2] reported that most of the bovine in Korea were infected with *T sergenti*, and transmitted by the vector tick, Heamaphysalis longicornis[3-7].

For the diagnosis of *T sergenti*, many methods have been used. But, Giemsa' stain, which has a merit of facility that carried out at anywhere or anytime, is difficult to identify in carrier states or low parasitemia, and futhermore classify other similar hemoparasites like as *Babesia* spp. or *Anaplasma* spp. To overcome those of demerit, several methods such as electron microscopic method, indirect fluorescent antibody (IFA) method, immunoelectrophoresis method, Western blot analysis method, enzyme linked immunosorbent assay(ELISA) were reported[8-13]. In present, a new diagnostic method using the molecular biological tools were proposed. For example, southern hybridization using DNA probe, PCR amplification on the base sequenced target gene, and phylogenic assay using 18s rRNA were continually developed[14-16]. Those methods using molecular biological tools were admitted more sensitive than the conventional methods. Also, it provided the information about the characters of antigenic gene that could provide the key of control and prevention of theileriosis.

Consequently, the researches for control and prevention against babesiosis or theileriosis were moved on a recombinant major antigenic protein that capable of inducing immunity. For example, p67 antigen in *T parva* and p32 antigen in *T sergenti etc* [17,18] and many of major antigenic protein were considered to be used as a recombinant vaccine candidate. Actually, many researchers reported that vaccination with recombinant or synthetic immunodominant protein is effective for the control of babesiosis or theileriosis[19,20]. But, the effective prevention method against theileriosis in Korea is not established yet.

In this study, 32 kDa protein gene, one of the membrane protein genes, has been cloned from Korean *T sergenti* isolates by RT-PCR, and the sequence was analyzed. To confirm the authenticity of the gene, recombinant protein was produced through *E coli* expression system, and the recombinant 32 kDa protein was detected by the *T sergenti* infected cattle sera.

Materials and Methods

Isolation of total RNA : Total RNA was extracted from isolated *T sergenti* by the guanidinium isothiocyanate phenol method[21]. Briefly, 200μl of RNAzol(TEL-TEST inc., USA) was added to the precipitants of *T sergenti* and vortexed vigorously. After chloroform treatment, RNA was precipitated with isopropanol. RNA pellets were washed with ethanol, and dissolved in 20μl of 0.1 % diethyl pyrocarbonate(DEPC) water, and stored at -70℃.

First-strand cDNA synthesis : Prior to RT-PCR amplification, the target total RNA was converted to a cDNA by the use of AMV-RTase and oligo-dT primer(Promega Co., USA)[22]. First, the extracted RNA were denaturated 10 min at 65℃ and reaction solution was added. The RT reaction solution contained 4μl of 5 × RT reaction buffer, 2μl of 10mM dNTP, 2μl of AMV-RTase, 0.2μg of the oligo-dT primer. 20μl of total reaction volume was obtained by adding DEPC water. The mixture was incubated for 60 minute at 37℃, and stored at -20℃.

Reverse transcription PCR(RT-PCR) :

Oligonucleotide primers : The sense and anti-sense of oligonucleotide primers were chosen on the basis of the previously published sequence for the *T sergenti* mRNA for 32K protein[23](Table 1), GenBank accession No. D12689. The predicted size of amplification is 869 bp. Synthesis of oligonucleotide primers were carried out on DNA synthesizer(DNA International Inc, USA).

RT-PCR : PCR reaction mixture contained 5μl of 10× PCR buffer{10mM Tris-HCl(pH 8.3), 50mM KCl, 2.5mM $MgCl_2$, 0.1% gelatin}, 3μl of 2.5mM dNTP, 1.5U of Taq DNA polymerase, 20 pmole of forward and reverse primer, 2μl of cDNA. 50μl of total reaction volume was obtained by adding nuclease free water. PCR amplification was carried out in a DNA thermal cycler(Perkin Elmer, USA) with

Table 1. RT-PCR primers used for amplification of *T sergenti* membrane protein gene

Primer	Length (mer)	Nucleotide sequence 5' to 3'	Expected amplicon size (bp)
Sense	21	5'-ACTAGATAATTTGCTATGTTG-3'	869 bp
Anti-sense	21	5'-TTAAACGTCGATAATATGTGA-3'	

the following cycling program : 94℃ denaturation for 1 minute, 55℃ annealing for 30 second, 72℃ polymerization for 1 minute. A total 30 cycles was performed. The final polymerization step was at 72℃ for 7 minute. Following PCR amplification, the PCR products was analyzed on 1% agarose gel in TAE buffer(0.04M Tris-acetate, 0.001M EDTA) and stained with ethidium bromide(EtBr).

Sequencing of PCR products :

Cloning of PCR products : PCR products were eluted using the Gene clean Kit(BIO 101) and directly ligated into pGEM-T vector system(Promega Co., USA). The recombinant plasmid DNA was transformed into competent *E coli* strain TOP10F' and selected by incubation on ampicillin(50mg/ml) added LB plate(10g Bacto-tryptone, 5g Bacto-yeast Extract, 5g NaCl, 15g agar adjust pH to 7.0). The plasmid DNA was isolated by alkaline lysis methods[24] and used as a sequencing template.

Sequencing of amplicon : DNA sequencing was performed by Sanger's dideoxynucleotide chain termination method[25] using the fmol DNA cycle sequencing system(Promega Co., USA). The primers used in the sequencing reaction were pUC/M13 forward and revers primers(Table 2). PCR was carried out in a DNA thermal cycler(Perkin Elmer, USA) with the following cycling program : A total 35 cycles was performed ; 95℃ for 1 minute, 42℃ for 1 minute, 72℃ for 1 minute.

Table 2. Sequence of pUC/M13 forward and reverse primers for sequencing

Primer	Length (mer)	Nucleotide sequence 5' to 3'
Forwad P.	17	5'-GTTTTCCCAGTCACGAC-3'
Reverse P.	17	5'-CAGGAAACAGCTATGAC-3'

Strategy for expression vector construction : pGEM-T vector plasmid DNA containing *T sergenti* membrane protein (KTs-MP) gene was digested with *Apa* I and *Nco* I enzymes. The digested DNA fragments were subcloned into a pRSET-C vector. In order to construct *E coli* expression vector, KTs-MP gene in a pRSET-C vector was removed to pQE32 vector(QIAGEN, USA) through *BamH* I and *Hind*Ⅲ digestion. The constructed recombinant expression vector was introduced in *E coli* strain M15 cells.

Harvest of recombinant protein :

Induction of recombinant protein : The transformants, *E coli* strain M15, harboring pQE32-KTs MP plasmid DNA were cultured in LB broth(10g Bacto-tryptone, 5g Bacto-yeast Extract, 5g Nacl, adjust pH to 7.0) added 20μl of ampicillin(50mg/ml) and 10μl of kanamycin(10mg/ml) at 37℃ for 5 hours(OD 600 = 0.5). The promotor activation was induced in the presence of 2mM isopropylthio-β-D galactoside(IPTG) and further incubation for 4 hours at 37℃.

Purification of recombinant protein : The transformant cells, *E coli* strain M15, in induced culture broth were harvested by centrifugation at 5,000rpm for 30 minute and dissolved in buffer B(8 M urea, 0.1 M Na-phosphate, 0.01 M Tris-Cl, adjust to pH 8.0). The supernatant were collected from the former dissolved solution by centrifugation at 6,000rpm for 30 minute at 4℃, and applied to 50% slurry of an Ni^{+}-NTA(QIAGEN) agarose resin column[26~28] of previously equilibrated in buffer A(6 M guanidine HCl, 0.1 M Na-phosphate, 0.01 M Tris-Cl, adjust to pH 8.0). The column was washed with ten column volumes of buffer A and five volumes of buffer C(buffer B, adjust pH 6.3). Purified proteins were eluted with buffer D(buffer B, adjust pH 4.5 supplemented with 200mM EDTA) and dialyzed against PBS.

Analysis of recombinant protein :

SDS-PAGE : To confirm the authenticity of the produced

protein, molecular weight of purified recombinant KTs-MP was analyzed by sodium dodecyl-sulfate-polyacrylamide gel electrophoresis(SDS-PAGE). Briefly, the pellets were collected from induced former cultures by centrifugation at 12, 000rpm for 1 minute, and resuspended in 50μl of SDS gel-loading buffer(62.5mM Tris-Cl pH 6.8, 10% glycerol, 2% SDS, 1% β-mercaptoethanol, bromophenol blue and xylene cyanol 0.01mg/ml). The mixtures were boiled at 100℃ for 5 minute and separated on 12% SDS-PAGE. The gel was stained with Coomassie-blue(Gibco BRL, USA).

Western-blotting : The separated recombinant proteins on SDS-PAGE were transfer to nitrocellulose membrane and incubated in blocking solution(5% skim milk, 0.02% sodium azide and 0.02% Tween-20 in PBS) at room temperature. The membrane was washed with TBST(100mM Tris-Cl pH 7.5, 0.9% NaCl, 0.1% Tween-20) and was blotted using anti-His Tag antibody, antisera of bovine infected with *T sergenti* and monoclonal antibody against 32kDa membrane protein of *T sergenti*. Continually, anti-bovine IgG alkaline phosphatase(AP) conjugate(Promega Co., USA) diluted in TBST were added to allow the reaction. The used substrates for AP is 5-bromo-4-chloro-3-indolyl-phosphate(BCIP)/nitro-bluetetra zolium-chloride(NBT).

Results

Amplified 32kDa MP gene of *T sergenti* : Total RNA of *T sergenti* was extracted and its concentration was measured by UV/spectrophotometer at 260nm wavelength. The final concentration of the extracted total RNA was 45μg/μl. Using the cDNA as a template, MP gene of *T sergenti* isolated from Chonbuk area, Korea, was amplified by RT-PCR. The size of PCR products was a 869 bp as expected(Fig 1).

Sequencing alignment : The PCR product was eluted and directly cloned into pGEM-T vector. The size of recombinant plasmid DNA was 3.8Kb(Fig 2). Alignments of nucleotide and deduced amino acid sequences with Japan's stock are shown in Fig 3 and Fig 4., respectively. Also, the sequence of PCR products was examined for similarities to the GenBank using the BLASTN and BLASTX program. In

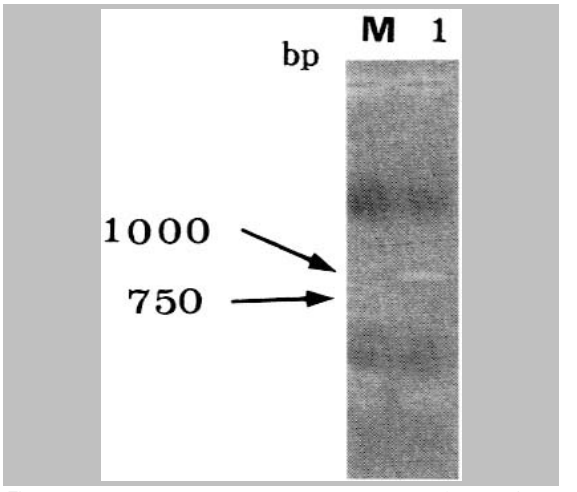

Fig 1. Amplification of *T sergenti* membrane protein gene by RT-PCR.
M : DNA size marker, 1 : Amplified KTs-MP gene.

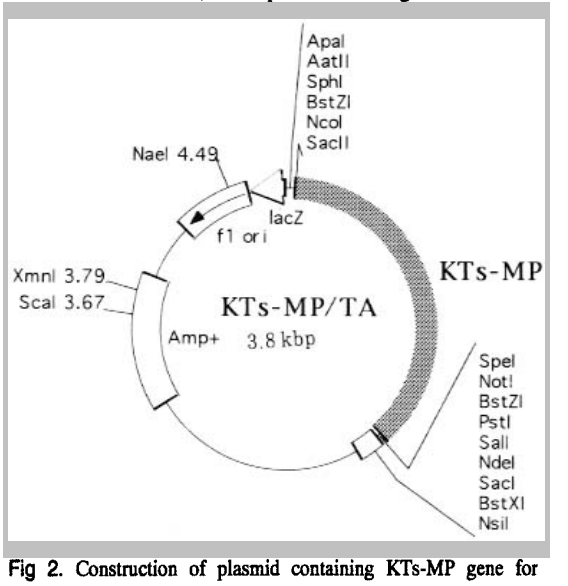

Fig 2. Construction of plasmid containing KTs-MP gene for sequencing.

a BLASTN search, the nucleotide sequence of PCR products from Chonbuk Korea isolates was showed 85% of homology with the mRNA for Ts-32K protein gene(GenBank accession No. D12689). Furthermore, high degree of homology was shown with the other *Theileria* spp. membrane protein genes such as *T buffeli* mRNA for major piroplasm surface protein gene, and *T orientalis* gene for piroplasm major immunodominant protein etc. Also, in a BLASTX search, the deduced amino acid sequence was showed 70% of homology

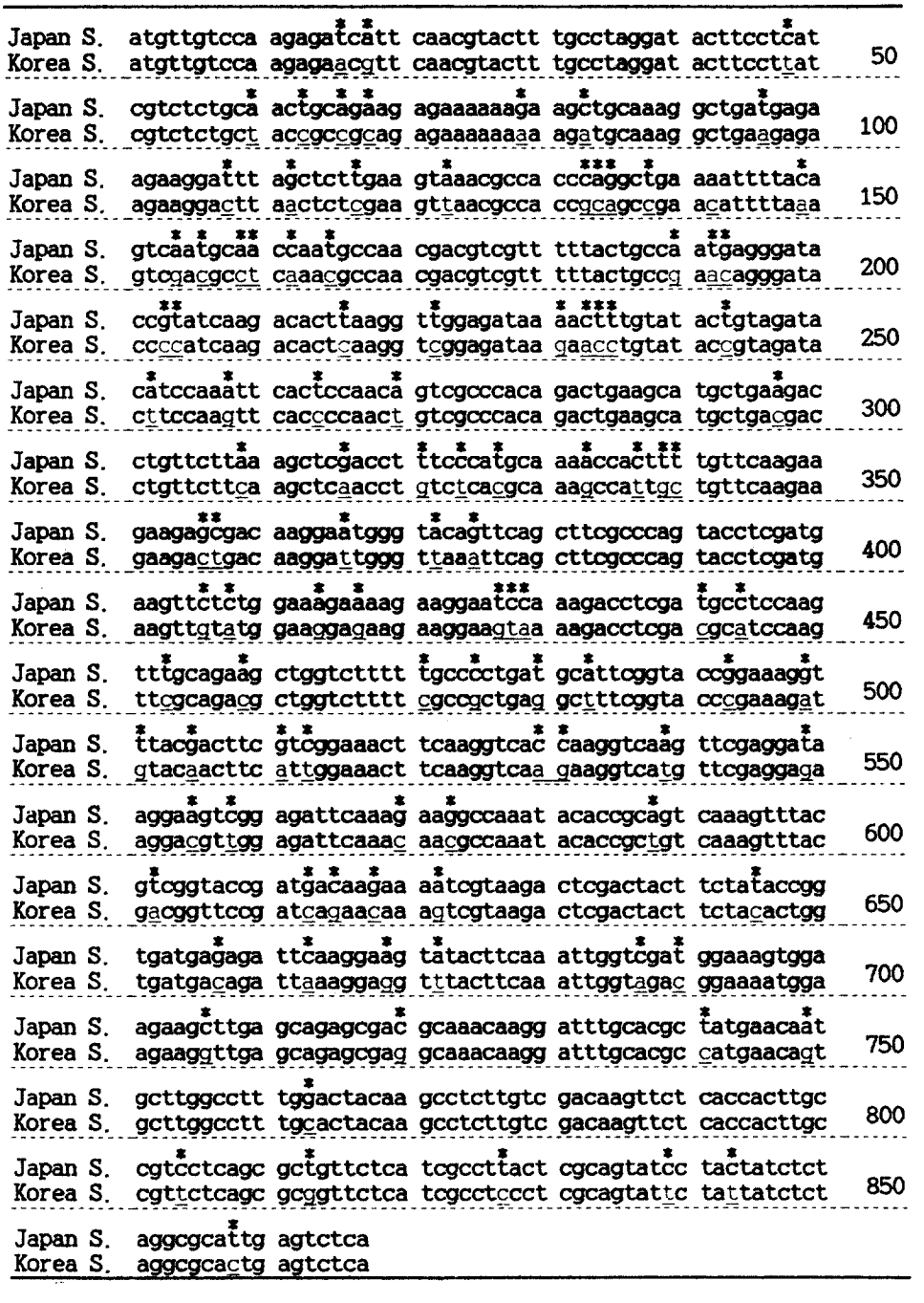

Fig 3. Alignment of nucleotide sequence of Ts-32K protein gene between Chonbuk Korea isolate and Japan's chitose strain(GeneBank Accession No. D12691).

with that of the predicted form.

Construction of expression vector : pRSET C-KTs MP plasmid construction : KTs-MP gene cloned into pGEM-T vector were digested with *Apa* I and

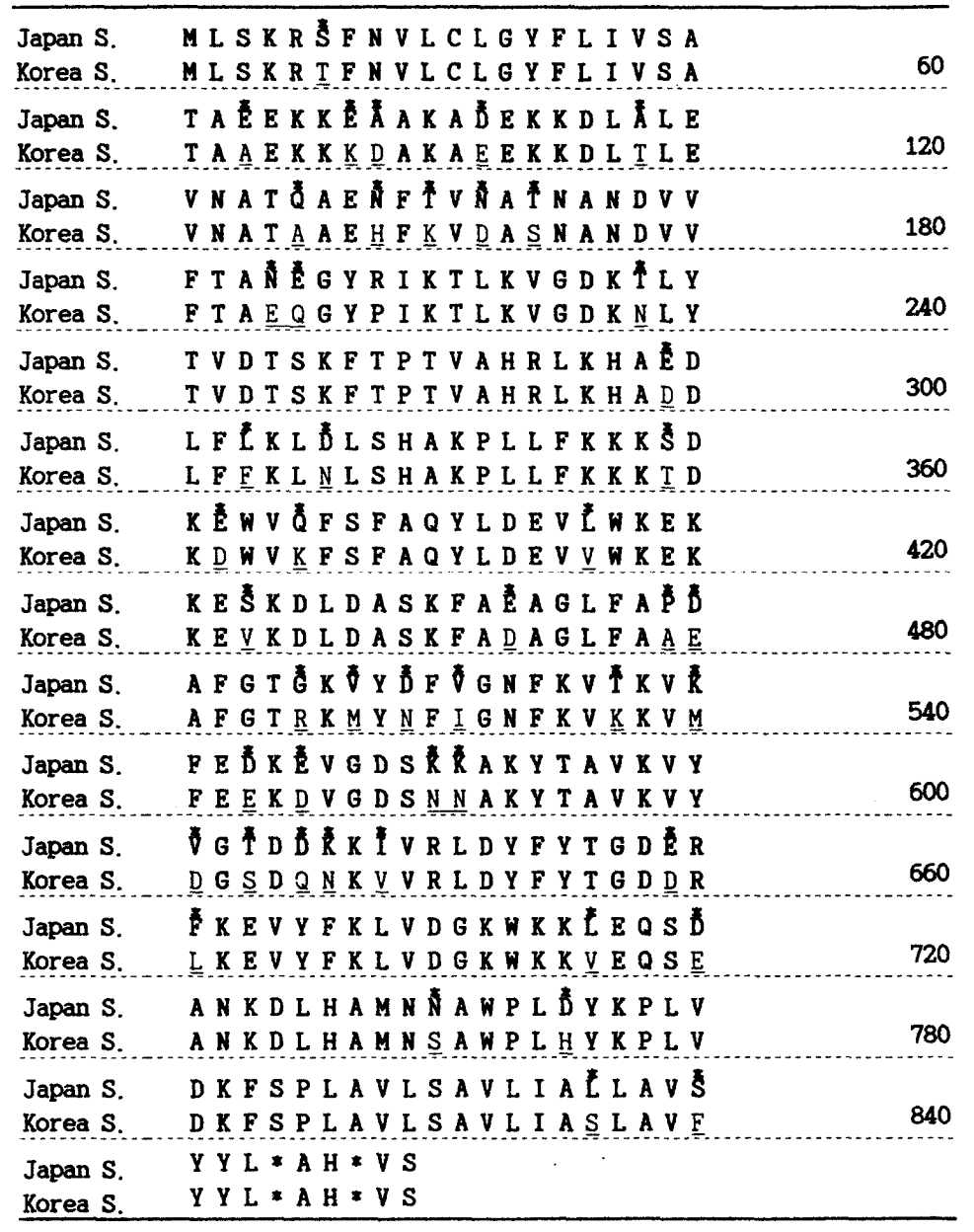

Fig 4. Alignment of deduced amino acid sequences of Ts-32K protein gene between Chonbuk Korea isolate and Japan's chitose strain (GeneBank Accession No. D12691).

Nco I enzyme. The truncated KTs-MP gene having a *Apa* I /*Nco* I site were eluted and subcloned into a pRSET-C vector(Fig 5).

pQE32-KTs MP plasmid construction : Recombinant pRset c-KTs MP plasmid DNA were digested with *Bam*H I and *Hind*Ⅲ enzyme. The truncated KTs-MP gene having a *Bam*H I /*Hind*Ⅲ site were eluted and subcloned into a *E coli* expression vector, pQE32 vector. The constructed recombinant plasmid DNA were named pQE32-KTs MP(Fig 6). When the recombinant pQE32-KTs MP plasmid DNA were digest

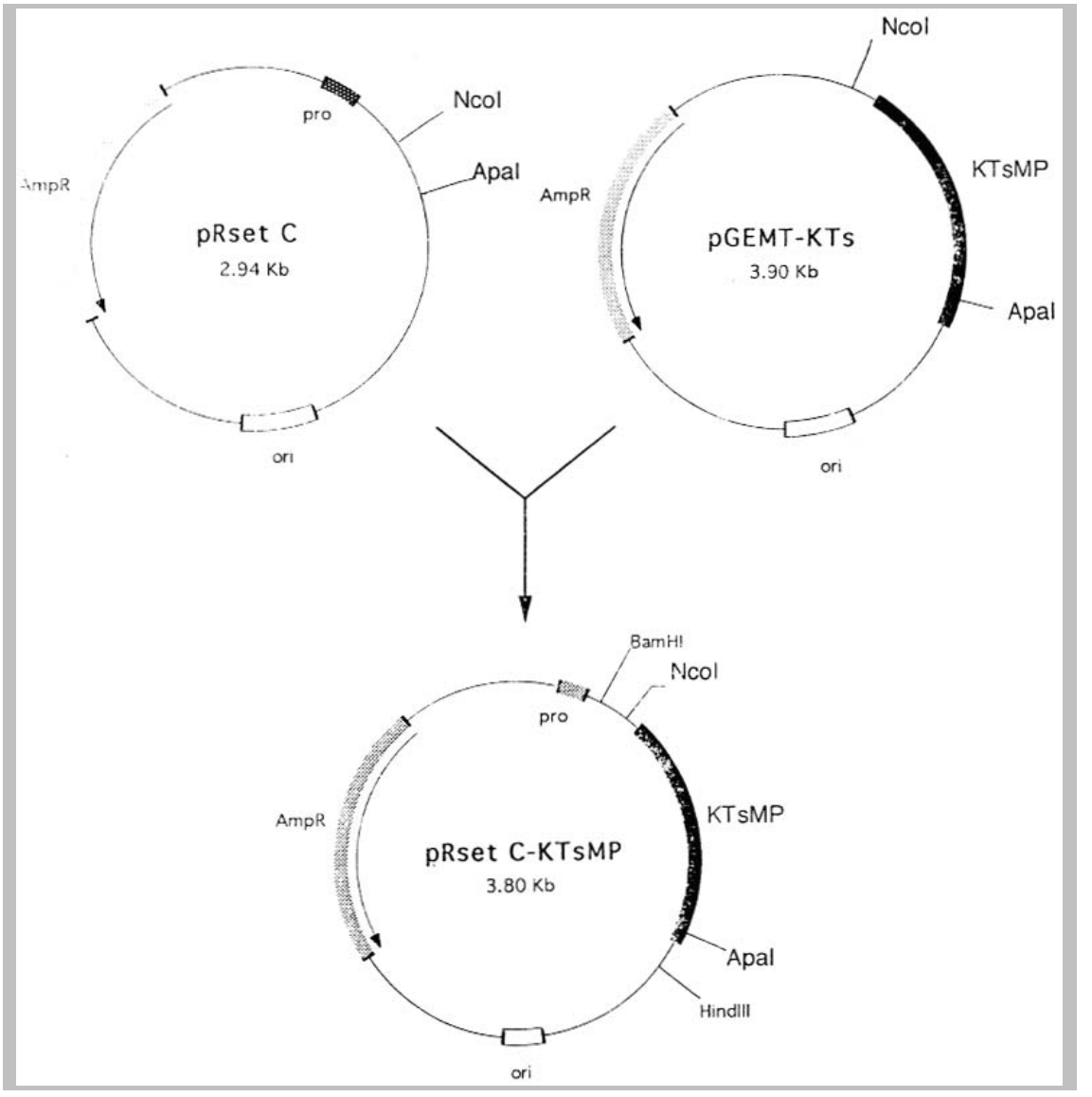

Fig 5. Construction of plasmid containing KTs-MP gene(pRSET C-KTs MP).

with *Bam*H Ⅰ and *Hind*Ⅲ, the predicted size of inserts was observed(Fig 7). Therefore, we could confirmed the construction of pQE32-KTs MP plasmid.

Recombinant KTs-MP protein : Recombinant KTs-MP was induced from *E coli* strain M15 cells harboring pQE32-KTs MP plasmid DNA in the presence of 2mM IPTG. The recombinant KTs-MP in induced grown cells was purified by Ni^{+}-NTA agarose resin column. When the induced cell type was compared to the genotype of M15 cell, the proper inducing time is about 4 hrs in IPTG.

Authenticity analysis of KTs-MP : Molecular weight of induced recombinant protein was about 32 kDa in SDS-PAGE as expected. A quantity of recombinant KTs-MP was successfully purified(Fig 8).

The recombinant KTs-MP was detected by anti-His Tag antibody, antisera of *T sergenti* infected cattle and monoclonal antibody of *T sergenti* membrane protein(Fig 9). All of those antibodies were strongly reacted with recombinant KTs-MP. Therefore, we concluded that the authentic 32 kDa membrane protein of *T sergenti* was produced as immunologically recognizable form.

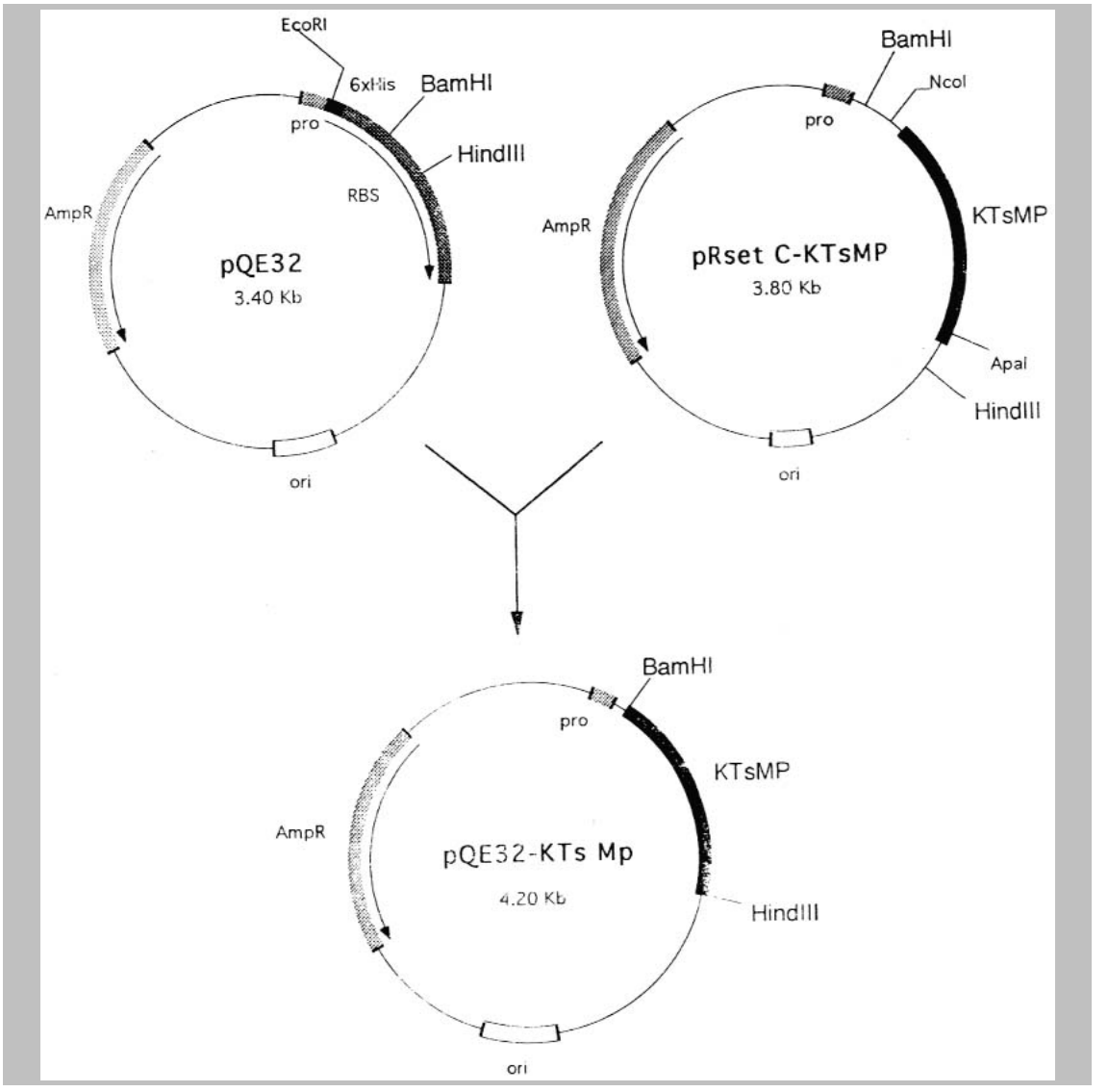

Fig 6. Construction of plasmid containing KTs-MP gene(pQE32-KTs MP).

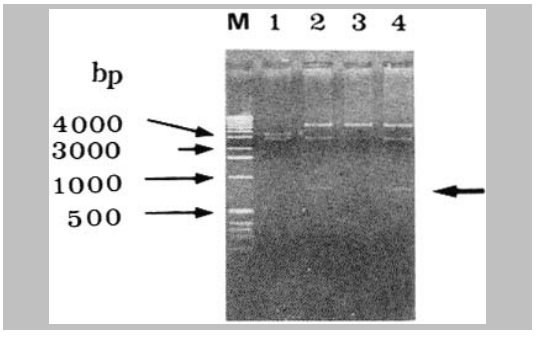

Fig 7. Restriction endonuclease analysis of pQE32-KTs MP plasmid. M : 1 kb DNA size marker, 1 : pQE32 plasmid(uncut). 2~4 : pQE32-KTsMP plasmid(*Bam*HI and *Hind*III treated).

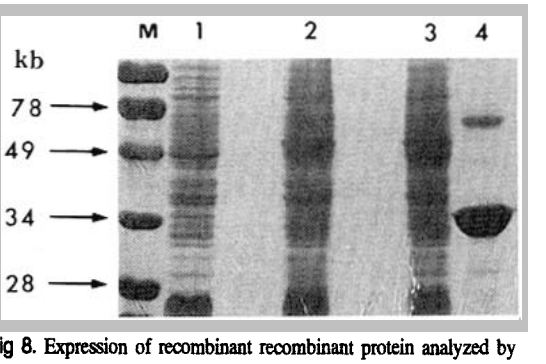

Fig 8. Expression of recombinant recombinant protein analyzed by SDS-PAGE. M : Molecular weight marker, 1 : Before induction with IPTG, 2 : 3hrs induction with IPTG, 3 : 4hrs induction with IPTG, 4 : Purified recombinant protein by Ni^{+}-NTA agarose.

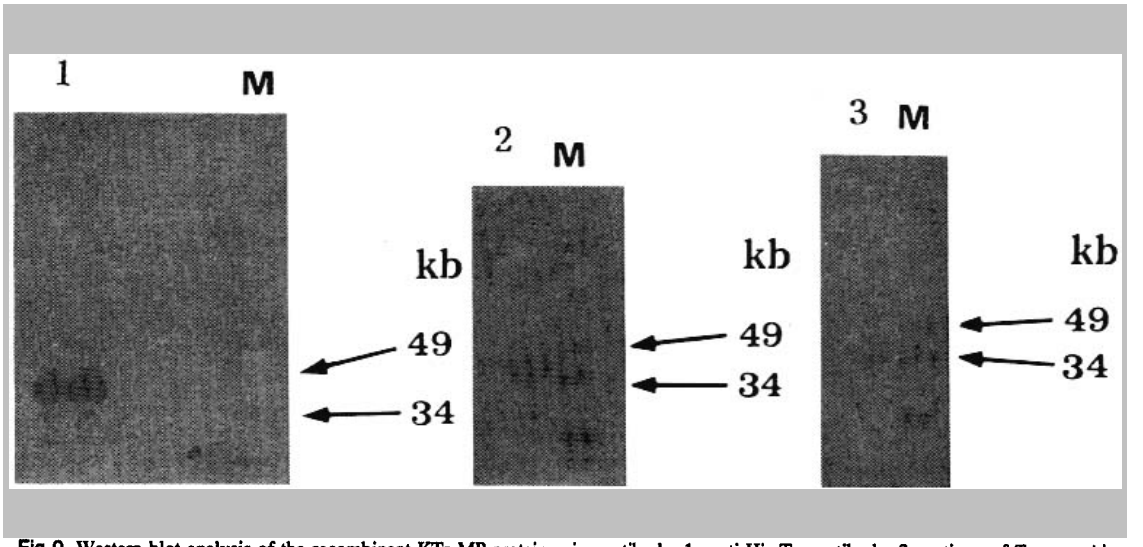

Fig 9. Western blot analysis of the recombinant KTs MP protein using antibody. 1 : anti-His Tag antibody, 2 : antisera of *T sergenti* infected cattle, 3 : monoclonal antibody against *T sergenti* membrane protein.

Discussion

Recently, a recombinant protein that capable of inducing immunity has been considered as a safe and effective new vaccine candidate. So, the researches were focused on collecting the information of the antigenic membrane protein genes and producing the recombinant proteins. For example, gene information of antigenic protein such as 42/44 kDa of membrane surface anetigen gene(MSA-1) and glutathione S tranferase(GST) fusion protein in *B bovis*, 60 kDa of merozoite rhoptry proteins in *B equi*, 67 kDa of sporozoite stage specific surface antigen and 104 kDa microneme rhoptry protein in *T parva*, and sporozoite surface antigen in *T annulata*, etc were elucidated and used as a recombinant protein vaccine candidate[17~20]. The antigenic protein gene of *T sergenti*, known as an agent of theileriosis in Korea including Japan and China, was reported, too[29]. The 32/34 kDa of immunodominant piroplasm surface protein gene of *T sergenti* was reported in Japan's stock type but not yet in Korea's and China's.

In this study, 32 kDa MP gene of *T sergenti* isolated from Chonbuk Korea was amplified through RT-PCR, and the amplicons have been sequenced. In the alignment of nucleotide and deduced amino acid sequence, the homology of Korea isolates nucleotide sequence was 85% compared to the Japan's stock(GenBank accession No. D12689). Furthermore, a 85-97% of high degree of homology was shown with the other membrane protein gene such as mRNA for *T sergenti* piroplasm major immunodominant protein gene (GenBank No. D11046), *T sergenti* (isolated from Fukushima) gene for piroplasm major immunodominant protein (GenBank No. AB016280), *T buffeli* mRNA for major piroplasm surface protein gene(GenBank No. D78015), and *T orientalis* gene for piroplasm major immunodominant protein (GenBank No. AB008369) etc. Also, in a BLASTX search, the deduced amino acid sequences showed 70% of homology with predicted form. To produce the recombinant membrane protein, *E coli* expression vector, pQE32 plasmid vector, was used. The induced recombinant KTs-MP were confirmed the authenticity by SDS-PAGE and Western blotting. The three of antibody(anti-His *Tag* antibody, antisera of *T seregnti* infected cattle and monoclonal antibody against 32 kDa MP of *T sergenti*) were reacted with induced recombinant protein in Western blotting. From these results, we confirmed that the recombinant KTs-32 MP were produced as immunologically recognizable form.

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