



Title	Development of ELISA to detect antibodies specific to <i>Mycobacterium avium</i> subsp. <i>paratuberculosis</i> with truncated 3.4 kDa proteins
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Development of ELISA to detect antibodies specific to *Mycobacterium avium* subsp. *paratuberculosis* with truncated 34 kDa proteins

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Abstract

To develop ELISA to detect antibodies specific to *Mycobacterium avium* subsp. *paratuberculosis* (*M. paratuberculosis*), the carboxyl termini of the 34 kDa proteins of *M. paratuberculosis* and *Mycobacterium avium* subsp. *avium* (*M. avium*) were expressed in *Escherichia coli* expression system. Antibodies specific to *M. paratuberculosis* were detected with the truncated 34 kDa protein of *M. paratuberculosis* in ELISA after pre-absorption of serum samples with the truncated 34 kDa protein of *M. avium*. All the serum samples from cattle confirmed to be infected with *M. paratuberculosis* were positive and those from healthy cattle were negative in the present ELISA system. These results indicate that the established ELISA detects antibodies specific to *M. paratuberculosis* with high specificity and sensitivity and is a useful tool for the screening of Johne's disease.

Key Words : cattle, diagnosis, ELISA, mycobacterium, paratuberculosis

Introduction

Bovine paratuberculosis (Johne's disease) is an important chronic granulomatous enteric disease of cattle caused by *Mycobacterium avium* subsp. *paratuberculosis* (*M. paratuberculosis*) [4]. The disease occurs in domestic and wildlife ruminants worldwide and causes great economic losses [3, 16, 20,

24]. Infection in non-ruminant wildlife has also been documented [1, 14]. Recent reports suggest that *M. paratuberculosis* is associated with Crohn's disease in humans [10, 22], although its causative role in the human disease remains controversial. The control of paratuberculosis relies on the identification of infected animals and culling in Japan. Fecal culture is the most specific and standard

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method for diagnosis of paratuberculosis [25, 28]. However, this method requires up to 12 weeks or more before the results are obtained. For higher sensitivity and rapid diagnosis, PCR and related methods, targeting *M. paratuberculosis* specific insertion sequence (IS 900), *HspX* gene or other species-specific genes, have been developed [2, 12]. Serological tests are currently adopted for rapid diagnosis in routine use [7, 26, 27]. Although most tests were neither sensitive enough, nor specific due to existence of antigenically related organisms, various ELISA systems have been developed and evaluated to reduce cross-reactions with related mycobacteria to improve the sensitivity and specificity [11, 15, 17, 29]. For the diagnosis at the early stage of the infection, detection methods to gamma interferon and its inducing antigens were also developed [18, 21].

The 34 kDa protein of *M. paratuberculosis* is immunodominant and contains epitopes specific for *M. paratuberculosis* [8, 9]. The carboxyl terminus of this protein was reported to contain specific epitopes for *M. paratuberculosis*, and thus was used as antigen for development of a diagnostic kit of paratuberculosis [9]. However, it has recently been reported that this protein also contains epitopes that cross-react with antibodies against *Mycobacterium avium* subsp. *avium* (*M. avium*) [23]. In the present study, an ELISA system has been established to detect antibodies specific to 34 kDa antigen of *M. paratuberculosis* after pre-absorption with a recombinant carboxyl terminus of the 34 kDa antigen derived from *M. avium*. The obtained results were compared to those of a commercial *M. phlei*-absorbed ELISA kit produced in Japan for the diagnosis of paratuberculosis in cattle.

Materials and Methods

Bacteria strains and culture : *M. paratu-*

erculosis strain ATCC19698, *M. avium* strain ATCC15769 and *Mycobacterium intracellulare* (*M. intracellulare*) strain NIAH-10746 were used in this study. *M. paratuberculosis* strain ATCC19698 was purchased from American Type Culture Collection (VA, USA). *M. avium* strain ATCC15769 was kindly provided by Dr. T. Shirahata (Obihiro University of Agriculture and Veterinary Medicine, Hokkaido, Japan). *M. intracellulare* strain NIAH-10746 was kindly provided by National Institute of Animal Health (Ibaraki, Japan). The bacteria were grown in Middlebrook 7H9 broth supplemented with Middlebrook ADC supplement (Becton Dickinson, NJ, USA) and 1 µg/ml Mycobactin (Kyoritsu Pharmaceutical Company, Tokyo, Japan). The bacteria were grown for 12 weeks at 37°C with shaking. The bacterial cells were pelleted by centrifugation, washed with phosphate buffered saline (PBS) and stored at -20°C until use.

DNA extraction, polymerase chain reaction and gene cloning : The genomic DNAs of *M. paratuberculosis* strain ATCC19698 and *M. avium* strain ATCC15769 were extracted as described by Coetsier *et al.* [6]. Based on the nucleotide sequence of *M. paratuberculosis* 34 kDa protein gene [13], a set of oligonucleotide primers (5'-GAATTCCGGGTGGTCAGCAGCATTC-3' and 5'-AAGCTTAGGCGGGCGTCGGCCAGAC-3' containing restriction sites for *EcoRI* and *Hind III*, respectively) were designed to amplify the about 300 bp PCR products encoding nearly 100 amino acids of the carboxyl terminus of the whole 34 kDa protein. For DNA amplification, 1 µl of the extracted DNA was added to 37 µl of PCR mixture consisting of 10 mM Tris-HCl (pH8.8), 1.5 mM MgCl₂, 50 mM KCl, 4% dimethyl sulfoxide (DMSO), dNTP mixture (2.5 µM each), 2.5 U *Takara Ex Taq* (Takara Bio Inc., Tokyo, Japan) and 10 pM of each primer. The final reac-

tion mixture was made up to 50 µl with distilled water. After an initial denaturation step (5 min at 96°C), 35 cycles of amplification were performed as follows: denaturation at 96°C for 1 min, annealing at 53°C for 1 min, and extension at 72°C for 2 min, followed by a final extension at 72°C for 3 min. Amplification was carried out in a PTC-200 thermocycler (Bio-Rad Laboratories, Inc., CA, USA). Amplification of the *M. avium* gene was done using the same primers and conditions described above. After amplification, PCR products were cloned into pET30c(+) plasmid vector (Novagen, WI, USA).

Expression and purification of recombinant antigen: Escherichia coli (E. coli) BL 21 (DE 3) cells were transformed with pET30c(+) carrying the gene that encodes the truncated 34-kDa protein derived from M. paratuberculosis (MP-34kD-C) and M. avium (MA-34kD-C) carrying a histidine tag. Expression of the recombinant protein was performed according to the manufacturer's instructions (Novagen). The recombinant proteins were extracted and purified using the HisTrap purification kit (GE Healthcare Bio-Science Corp., NJ, USA). Expression and purity of the recombinant proteins were determined by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as previously described [19].

Serum samples: Hyperimmunized rabbit sera against M. paratuberculosis strain ATCC 19698, M. avium strain ATCC15769 and M. intracellulare strain NIAH-10746 were prepared for the specificity test. Japanese white rabbits (Japan SLC, Inc., Shizuoka, Japan) were immunized with formalin inactivated whole cells four times subcutaneously. Ten days after the final immunization, serum was collected and stored at -20°C until use. Bo-

vine serum samples (68 samples) were kindly provided from Dr. K. Yamaguchi (Hokkaido Institute of Public Health, Hokkaido, Japan). Eighteen serum samples were prepared from cattle that were diagnosed as paratuberculosis with Johne LISA II (Kyoritsu Seiyaku pharmaceuticals) or fecal culture and 50 serum samples were also prepared from healthy cattle as negative controls.

Enzyme-linked immunosorbent assay (ELISA): Plates were coated with 50 µl of 2.5 µg/ml MP-34kD-C protein, blocked with 200 µl of 1 % Bovine Serum Albumin Fraction (Roche Diagnostics GmbH, Mannheim, Germany). Fifty µl of 1 : 200 diluted test sera, which were incubated for 30min with 5 µg/ml of the MA-34kD-C antigen before being added to the plates, were added and incubated for 1 hr. After washing with PBST, 50 µl of horseradish peroxidase labeled anti-bovine IgG (Bethyl, Inc., TX, USA) or anti-rabbit IgG (Bio-Rad Laboratories, Inc.) were added and incubated for 1 hr. After washing with PBST, 100 µl of substrate solution containing 0.2 mM ABTS (Roche Diagnostics) and 0.004% H₂O₂ in citrate buffer (0.05M, pH4.0) were added and incubated for 30min. Optical densities with 405nm filter (OD₄₀₅) were read with plate reader Multiskan JX (Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan). Sample to positive (S/P) ratios were determined from OD₄₀₅ values according to the formula : $S/P = \frac{OD_{450}sample - OD_{450}MeanNeg}{OD_{450}MeanPos - OD_{450}MeanNeg}$,

where MeanNeg is the average of the OD₄₀₅ values of the negative control sera, and MeanPos is the average of the OD₄₀₅ values of the positive control sera. The cut-off point was determined as the Mean + 3 standard deviation (S. D.) of negative samples from healthy cattle.

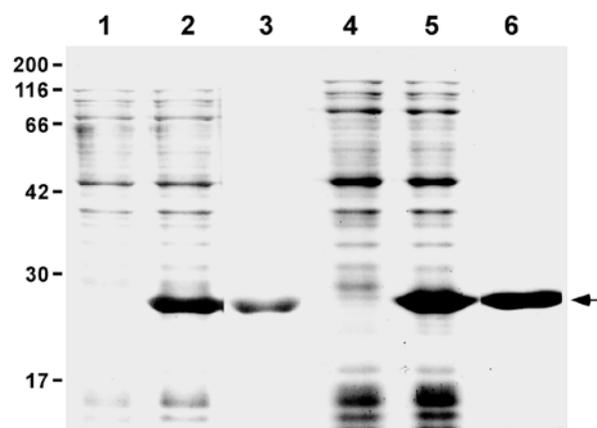


Fig. 1 SDS-PAGE of the carboxyl termini of 34kDa proteins of *M. paratuberculosis* and *M. avium*. The proteins expressed in *E. coli* system (Lanes 1 - 3 ; MP-34kD-C, Lanes 4 - 6 ; MA-34kD-C) were electrophoresed on a 10% sodium dodecyl sulfate polyacrylamide gel. Lanes 1 and 4 ; whole extract of the *E. coli* before the IPTG induction, Lanes 2 and 5 ; whole extract of the *E. coli* after the IPTG induction, Lanes 3 and 6 ; affinity purified recombinant MP-34kD-C and MA-34kD-C, respectively. The arrow on the right indicates the position of the expressed proteins. Molecular weight markers are shown on the left margin.

Results

Expression and purification of recombinant proteins : From the sequence analysis, the PCR products derived from *M. paratuberculosis* strain ATCC 19698 and *M. avium* strain ATCC 15769 were 297 bp and 309 bp, and estimated length of amino acids were 99 and 103, respectively. The amino acid sequences of these carboxyl termini of 34-kDa proteins were identical between these two strains except the insertion of 4 amino acids (QHGQ) for the *M. avium* strain ATCC15769 at the 4 th position from N terminus. These carboxyl termini of 34-kDa antigen of *M. paratuberculosis* and *M. avium* were expressed as recombinant proteins (MP-34kD-C and MA-34kD-C). After the induction with Iso-

propyl β -D-Thiogalactoside (IPTG), abundant expression of the recombinant proteins was observed at the estimated molecular weight (Fig. 1, lanes 2 and 5). The molecular weight of the recombinant proteins were estimated as 18-kDa containing the multiple tag regions (7 kDa) derived from the pET30c(+) expression vector. These recombinant fusion proteins with a histidine tag were purified with Ni^{2+} affinity chromatography and purified protein was observed at the appropriate position in the SDS-PAGE (Fig. 1, lanes 3 and 6).

Effect of the pre-absorption of serum sample with MA-34kD-C to improve specificity in the ELISA : To evaluate the efficacy of pre-absorption of serum sample with MA-34kD-C for the improvement of specificity of the ELISA, hyperimmunized rabbit sera against *M. avium*, *M. intracellulare* and *M. paratuberculosis* were pre-incubated with 5 $\mu\text{g}/\text{ml}$ of MA-34kD-C and used for the ELISA to detect antibodies specific to *M. paratuberculosis* (Fig. 2). All antisera were high S/P ratio in the ELISA when they were not pre-treated with the MA-34kD-C before the ELISA reaction. On the other hand, rabbit serum against *M. paratuberculosis* still showed high S/P ratio, even after the test samples were pre-treated with MA-34kD-C before the ELISA reaction. These results indicated that antibodies against common epitope between *M. avium*, *M. intracellulare* and *M. paratuberculosis* were pre-absorbed with MA-34kD-C and specific antibody against *M. paratuberculosis* was detected in this ELISA system.

Detection of antibody specific to *M. paratuberculosis* in the serum samples from cattle : To set the cut-off point between positive and negative as a result of ELISA, OD values of 50 serum samples from healthy cattle were measured in this ELISA. S/P value of each

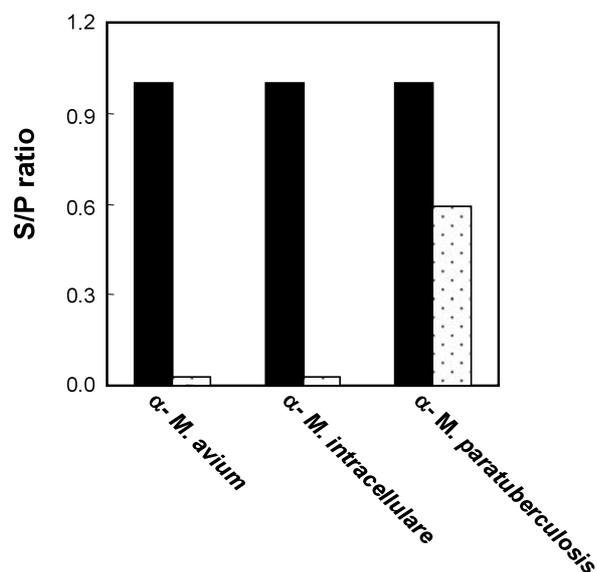


Fig. 2 Effect of the pre-absorption of serum with MA-34kD-C to improve specificity in the ELISA. Sera from rabbits hyper-immunized with *M. avium*, *M. intracellulare*, *M. paratuberculosis* were treated without pre-absorption (Black bars) or with pre-absorption (dotted bars) using MA-34kD-C protein. The samples were incubated on the ELISA plate coated with MP-34kD-C, followed by a goat anti-rabbit IgG conjugate. Sample to positive (S/P) ratios were determined from OD_{405} values according to the formula described in Materials and Methods.

serum was calculated described above and cut-off line was set at $S/P=0.37$ according to the "Mean + 3 S. D." of the S/P values from negative samples. Then 68 serum samples including 18 serum samples from cattle that were diagnosed as paratuberculosis were tested by this ELISA. To compare the specificity of our ELISA system, commercial ELISA kit to detect antibodies to *M. paratuberculosis* (Johne LISA II) was also used for the antibody detection (Fig. 3). Total 18 serum samples were diagnosed as positive in our ELISA and eleven of them were also positive by Johne LISA II. Seven of them, which were prepared from cattle confirmed to be positive with fecal culture, were negative by Johne LISA II. All 50 sam-

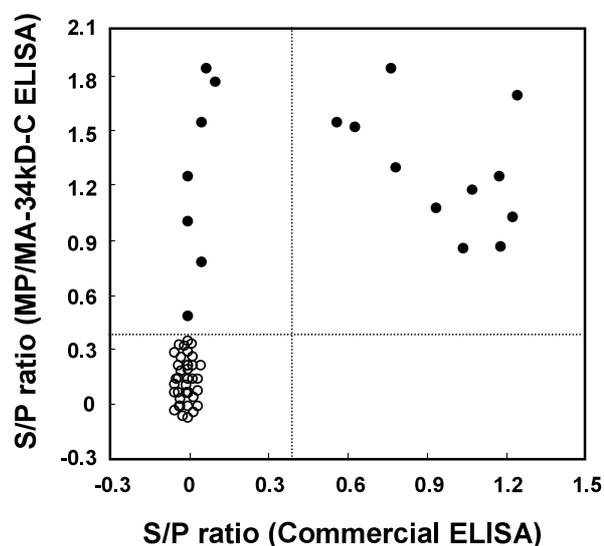


Fig. 3 Comparison of the reactivity in the developed ELISA (MP/MA-34kD-C ELISA) and commercial ELISA kit for 68 serum samples from cattle. Fifty sera prepared from healthy cattle (open circles) and 18 samples from those that were diagnosed as paratuberculosis (solid circles) were tested in the both ELISA tests. The cut-off lines (shown as broken lines) of the developed MP/MA-34kD-C ELISA and commercial ELISA kit were set at $S/P=0.35$ and 0.37 respectively.

ples from healthy cattle were negative in our ELISA and Johne LISA II.

Discussion

The control of paratuberculosis relies on the identification of infected cattle and culling. This, however, has been hampered by lack of sensitivity or specificity of most diagnostic tests in current use [7, 26, 27]. To resolve this defect, we developed an antibody detection method based on recombinant antigens for specific diagnosis of paratuberculosis in cattle. The 34kDa protein of *M. paratuberculosis* has been reported to be immunodominant [8]. The extracellular carboxyl domain of this protein was identified to have epitopes specific

for *M. paratuberculosis*, and was subsequently used as antigen for the development of an antibody detection ELISA [9]. Common epitopes were, however, found on the 34kDa proteins of both *M. paratuberculosis* and *M. avium* [23]. The difference of amino acids between MP-34kD-C and MA-34kD-C was 4 amino acids (QHGQ) insertion at the N terminus region in MA-34kD-C, where is the neighborhood of the B cell epitope reported by Ostrowski et al. [23]. In the present study, we cloned and expressed the MA-34kD-C for the use as pre-absorption antigen to remove cross-reacting antibodies before testing of the samples. We first tested the effect of pre-absorption with MA-34kD-C for the serum samples from rabbits immunized with *M. avium*, *M. intracellulare* and *M. paratuberculosis*. The results indicated that pre-absorption with MA-34kD-C was effective to reduce cross reactivity of the sera against *M. avium* and *M. intracellulare* to the MP-34kD-C antigen coated on the ELISA plate. The significant difference on the amino acid level in this region was the N terminus area, where is the neighborhood of the B cell epitope. Conformational difference of this area might be the critical for antigenicity to detect specific antibody against *M. paratuberculosis* in this ELISA system.

The assay correctly identified that all sera obtained from infected cattle (n=18) as positive, and gave negative results for all sera from non-infected cattle (n=50). From the comparative analysis of our established ELISA and commercial Johne LISA II, both ELISA have enough specificity and our ELISA system has higher sensitivity than Johne LISA II. Johne LISA II is composed of *M. paratuberculosis* strain P-18 whole cell extract as coating antigen and the whole cell extract of *M. phlei* strain ATCC354 as pre-absorption antigen [5, 29]. The present assay to

detect antibodies to MP-34kD-C after pre-absorption with MA-34kD-C was therefore considered more sensitive than the commercial kit. It is noted that none of the sera tested were obtained from cattle manifesting clinical signs of paratuberculosis. Therefore, the test was able to detect cattle in the subclinical stages of infection.

We therefore conclude that the developed ELISA in this study is specific and more sensitive than the commercial ELISA kit. Moreover, this method offers advantages over the commercial ELISA kit because antigens as recombinant proteins in *E. coli* system are able to be prepared quickly and safety for manufacturing. However, this method is not enough to diagnose all cattle infected with *M. paratuberculosis* because it is not able to detect specific antibody from the cattle at the early stage of the infection. Furthermore, fecal culture is still most reliable method and PCR and related methods are advanced rapidly for the diagnosis. The combination of antigen detection, gamma interferon detection and other methods with antibody detection ELISA developed in this study is still required for the reliable diagnosis of Paratuberculosis.

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